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The gut microbiome is a collection of microbes that exist in the digestive tract, which includes bacteria, viruses and archaea. These microbes are symbiotic to their host and are essential to the proper development and ongoing health of digestive and immune systems. Any fluctuations in the environment can dramatically affect this relationship, resulting in changes of gut microbiome and human health, such as diabetes, asthma, bowel diseases, cancers and obesity. To explore the roles of the gut microbiome in the development and/or prevention of diseases, the profiling of gut microbiome and their activities must be determined.

Messenger RNA (mRNA) is a large group of RNA molecules that would be translated into functional proteins molecules, which then perform all sorts of biological activities and structures. In the past, mRNA have been used as biomarkers for monitoring cellular activities, but the use of metatranscriptome to study the gut microbiome activities is limited. For this reason, we set out to explore the use of mRNA as biomarker for monitoring the gut microbiome activities.

In this study, we build a simple gut microbe model aiming to demonstrate the relationship between the level of mRNA and gut microbe activities. *L. reuteri,* a probiotic Gram-positive bacteria was chosen as our model. In order to carry out the initial studies with the selected model, there are a number of technical issues that ought to be addressed. In the first case, due to the lack of 3' poly-A tail in the bacterial mRNA and the limited number of reports on the extraction of RNA from Gram positive bacterial cells, we had compared and evaluated various methods for extracting total RNA from bacterial cells. The goal was to establish a reproducible method that would provide sufficient purity and yield of total RNA.

We used Triton X-100 Boiling method for the extraction of total RNA from *L. reuteri* cells after comparing various methods. The total RNA extracted was usually less than 900 ng/µL in quantity which reduces our choice of using the spectrometer to measure the concentration and purity of our sample. The concentration and purity was measured by the use of NanoDrop 1000 spectrophotometer, which uses about 1-2 µL of sample.

The technique used for studying mRNA was gel electrophoresis. Sequencing is another method available to identify presence of a particular mRNA, but there is yet some information required to build a library that will

allow us to match a single mRNA to its parent bacterial strain. As this being our first step for the feasibility study for mRNA being used as biomarkers, it's important to look for a cheaper, easier and quicker method. When it comes to cheap and easy, agarose gel electrophoresis is a well-established method available in the science world.

Total RNA were extracted from the bacterial cells and gel electrophoresis was performed on it to explore the changes in mRNA due to a given growth conditions. Since there wasn't an effective gel electrophoresis method available to inspect all the mRNA from the cell, re-extraction of mRNA smear from an agarose gel was done. With this developed method to monitor mRNA changes in *L. reuteri* cells, we can get insightful information about the functionality of gut microbe.

# METHOD DEVELOPMENT FOR STUDYING BACTERIAL MESSENGER RNA AS BIOMARKERS

by

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# CHAPTER I

# INTRODUCTION

## 1.1 Gut Microbes

In the field of medical science a new era has dawned with the realization of the critical role of the gut microbiota in the health and disease. The gut microbiota is a diverse collection of microbes in and on the human body, which includes bacteria, unicellular eukaryotes, viruses and archaea. Gut microbes and humans have evolved together for over thousands of years, which must mean that these microbes are essential to human life and function. Central to this beneficial interaction between the microbiota and the host, is the manner in which bacteria and most likely other microorganisms contained within the gut communicate with the host's immune system and participate in a variety of metabolic processes of mutual benefit to the host and the microbe.

The advancement of high throughput methodologies and sophisticated analytical systems have allowed the detailed description of the composition of the microbial constituents of the human gut, as never before, and are now enabling comparison to be made between health and various disease states. The Human Microbiome Project, has completed the profiling of human gut microbiome. The microbiome consists of >100 different types of microorganisms[1]. With this advancement we now understand the interaction between the host and gut microbiome better.

The gut microbiome may positively or negatively influence the human health and a dysbiosis of the microbiome can lead to a variety of health issues. There have been studies that show certain microbes are present in a diseased condition and in different quantities. For example, studies have shown that carcinogenic processes could be mediated by changes in metabolic activity and composition of the colonic microflora. This tells us that there is some connection with the physical state of our body and the gut microbiome. Thus, to explore the roles of the gut microbiome in the development or prevention of disease, the gut microbiome profile and their activities must be determined[2].

### 1.1.1 Host-Microbe Interaction on Immune System

After characterizing microbial community membership and dynamics, it is critical to understand the functional activities that ultimately affect the host physiology. Gut microbiota are integral to host digestion and nutrition, and they can generate nutrients from substrates that are otherwise indigestible by the host. For instance, xyloglucans are commonly found in dietary vegetables such as lettuce and onions, and the capacity for microbial digestion of xyloglucans was recently mapped to a single locus in a certain species of *Bacteroides*[3]. The ability to digest xyloglucans was shown to be a relatively rare trait in members of the phylum Bacteroidetes, and the importance of this capability to the human host was demonstrated by analysis of a public metagenome database showing that 92% of individuals contained at least one of these rare *Bacteroides* species capable of digesting xyloglucans.

Most studies of the microbiota target one specific disease or state, but comparison of the microbiota across diseases reveal common changes in the gut environment. For example disturbed mucous layers lining the intestinal cell wall and concomitant inflammation are observed in individuals with IBD, celiac diseases, HIV enteropathy, acute diarrhea, diverticulosis,

carcinoma and IBS[4]. Given these similarities, one might expect similar microbes to increase or decrease in abundance across these different disturbances, although elucidating these differences may require a proper analytical tool that allow studies along the length of the gut.

Further, competition in the densely populated gut environment is also expected to be important. Microbes can compete to use the same resources, or inhibit each other directly using antimicrobial products. We might expect phylogenetically related bacteria to compete because of overlapping functional roles or habitats. Therefore, understanding how to manipulate positive and negative feedback at the level of the host, the individual microbes and of the entire gut ecosystem will be critical for understanding how to maintain healthy stable states, and how to switch from an unhealthy to a healthy state. Hence for this understanding, we need an analytical tool that will allow the study of the whole gut ecosystem.

### 1.1.2 Activity of Microbes

In order to look at microbial activity, it is logical to first find out the biological molecules that is involved in the cause of their activity. The three most potential indicators for their activity are the mRNA, proteins and metabolites. Each represents a step in the activity of microbes from gene expression, to protein production and to what the proteins produce.

Each detection target has their pros and cons when studying microbiomes. For instance, metabolites are typically smaller molecules which can be easily detected by using modern techniques like mass spectrometry and they provide an accurate image of the activity profile of the microbes. At the same time, although extracting the metabolites does not need to maintain biological conditions, isolating all the classes of molecules can be time consuming and difficult. In the case for metabolites, the identity of the microbes producing the metabolites cannot be traced back and therefore only the activity of the microbiome, not the individual microbes can be detected[5].

Proteins are the workhorses of the cell and would show us a true and complete depiction of the activity in the microbiome. Moreover, proteins can be sequenced and identified by their structural components and therefore it is

possible to trace the identity of the microbes to the activity. However, proteins are difficult to work with and since they are larger molecules, they are not as easy to measure as metabolites. Further, protein sequencing is complicated in a mixed culture and therefore proteomics is the most labor intensive of the activity measurements[6].

Messenger RNA (mRNA) are a large group of RNA molecules that represents the conversion step of genetic information carried in a cell's DNA into functional proteins. Using mRNA for activity detection is the easiest for tracing back to microbes since nucleic acid sequencing is well established and high-throughput. Therefore, metatranscriptomics[7] could provide both activity information as well as identity of microbes. However RNA is highly unstable and easily degradable. On the downside, mRNA provide only a snapshot of activity at the time of collection and multiple proteins can be made from a single mRNA, so relative levels of activity are not as clear[8]. Since the goal is to determine activity in gut microbes in relation to human health and disease development, mRNA can be used to throw light on to the physical state at that particular point. Based on this understanding of how mRNA work, the gut microbe activity can be directly represented by the mRNA. Here we use this fact of nature to follow the gut microbe activity.

## 1.2 A Survey of mRNA Extraction Methods

There are no methods available for direct extraction mRNA from Gram-positive bacteria. The different methods that are available to obtain high yield of mRNA are first, to carry out total RNA extraction followed by various methods to isolate mRNA form the rest of the RNA community. Therefore, the first step would be to identify a suitable method for total RNA extraction and later a method to concentrate mRNA for detecting microbial activity.

### 1.2.1 Investigation on Total RNA Extraction Methods

Several methods are used in molecular biology to isolate RNA from samples, the most common of these is guanidium thiocyanate-phenol-chloroform extraction. For my thesis, the sample type used is Gram positive *L. reuteri* bacterial cells. Therefore a survey was done from the literature upon the best suitable method of RNA extraction on Gram positive bacteria.

A number of methods have been reported for the isolation of RNA from Gram positive bacteria[9]. These methods involve enzymatic lysis[10], sonication, bead beating and cesium chloride precipitation. These methods are all time-consuming, laborious and costly. Moreover, the RNA preparations are loaded with >70% rRNA and 10-15% tRNA. Only a minor

population of the RNA obtained represents the mRNA. Hence, three methods were compared here for yields of good quality of RNA which also has good quantity of mRNA and does not require costly and additional purification steps.

RNA isolation by the Qiagen method was one of our three methods of investigation. The Qiagen's RNeasy Plus Mini Kit is one of the widely used kits for the extraction of RNA and therefore was chosen to represent most commercial kits available. The other two methods selected for this investigation were the hot phenol extraction and extraction by the Triton-X 100 boiling method.

Table 1. Comparison of RNA Yield by Different Methods

| Bacteria | Qiagen method | | Hot phenol method | | Triton X-100 Boiling method | |
|---|---|---|---|---|---|---|
| | $A_{260}/A_{280}$ | Yield (mg/ml) | $A_{260}/A_{280}$ | Yield (mg/ml) | $A_{260}/A_{280}$ | Yield (mg/ml) |
| Gram positive | | | | | | |
| *E. faecium* | 2.03 | 1.02 | 1.99 | 0.95 | 2.01 | 1.01 |
| *S. aureus* | 2.05 | 0.92 | 1.98 | 1.02 | 2.03 | 0.95 |
| *L. lactis* | 1.99 | 0.65 | 2.01 | 0.69 | 2.05 | 0.72 |
| *L. reuteri* | 1.97 | 0.83 | 2.03 | 0.92 | 1.98 | 0.88 |
| Gram negative | | | | | | |
| *E. Coli* | 2.03 | 2.40 | 1.99 | 2.32 | 1.99 | 2.10 |
| *S.typhimurium* | 1.92 | 0.20 | 1.96 | 0.22 | 1.97 | 0.22 |
| *A veroni* | 2.03 | 2.40 | 2.01 | 2.12 | 2.02 | 1.99 |
| *C. jejuni* | 2.01 | 0.23 | 2.01 | 0.22 | 1.99 | 0.26 |

It is reported in the literature that the Qiagen and hot phenol extraction methods yields RNA with a very high yield of 16S and 23S rRNA compared to the Triton X-100 boiling method which provided good quality RNA with lesser rRNA. The quantity of RNA obtained by the boiling method ranged from 0.2 to 2 mg $ml^{-1}$ and had an *A*260/*A*280 ratio between 1.9 and 2.0, which indicated a good quality of RNA. Therefore, the Triton-X 100 boiling

method is quantitatively superior to Qiagen and hot phenol extraction methods.

### 1.2.2 Enrichment of mRNA

Messenger RNA usually comprises only 1-3% of total RNA, while rRNA constitutes approximately 90%. Next-generation sequencing technology has greatly advanced metagenomics study by allowing to assess the taxonomic composition and genetic functionality of microbial assemblage[11]. A major obstacle to microbial RNA-sequencing is the difficulty in enriching mRNA, as the prokaryotic mRNA species are not stably polyadenylated. Therefore, oligo d(T) mediated messenger enrichment is not applicable.

Removal of abundant rRNA is an alternative approach to enrich mRNA. Commercial rRNA depletion kits, such as Microexpress Bacterial mRNA Enrichment kit (Ambion), RiboMinus Transcriptome Isolation Kit (Bacteria) (Life Technologies), and mRNA-ONLY Prokaryotic mRNA Isolation kit (Epicentre) that preferentially degrades rRNA with an exonuclease, have been used for removing rRNA[12]. In order to make our method less costly and be even more efficient compared to the kits, we used

the XRN-1 exonuclease enzyme (New England) for the depletion of rRNA in our extracted total RNA.

### 1.2.3 Gel Electrophoresis - Comparison of mRNA

Gel electrophoresis is a common technique that is used to visualize proteins, DNA and RNA. Gel electrophoresis has allowed for the visualization of DNA and RNA with use of markers. This process of separation is achieved by sorting of a sample by size and charge. A gel slab is prepared with either a specific concentration of agarose a polyacrylamide. A matrix is formed by the gel through which the sample travels. The matrix is a cross linked polymer which contains and separates the sample. The higher the concentration of gel, the more hindered the matrixes mesh. The different concentrations of the gel determines the length in kilobase pairs (KB) which are visualized through the slab. The lower percentage of agarose will visualize a higher KB, while a higher percentage agarose will visualize a lower KB. The use of polyacrylamide is done for visualization of smaller KB pairs rather than agarose at a higher percentage.

Depending on the type of sample, a staining reagent is either added to the sample, or the entire gel is stained after electrophoresis to visualize the

bands under the ultra violet light. Sybr green stain is used for staining gels with RNA samples after electrophoresis.

### 1.3 Fluoroscent Microscope

EVOS FL Imaging System is a fluorescent microscope that allows images to be taken with and without fluorescence. It uses a LED light source which gives high intensity output over a short light path for the most efficient fluorophore excitation. It is also used to distinguish dead and live cells on a glass slide or plate. Various fluorescence staining reagents can be used to stain cells depending on the conditions. For instance propidium iodide dye is used to stain dead cells.

The advantage of this technology in this project is to signifying the treatments given to gut microbes and their conditions at which the cells would be able to show a significant change in mRNA. Cell viability curve was developed using the fluorescent microscope to shade light on when and how the cell tries to adjust to the new environment by fluctuating their functionality.

## 1.4 Specific Aims

1) Establish a Method for Extraction and Quantitative Comparison of Cellular mRNA

In order to compare the activity of microbial cells by gel electrophoresis of mRNA, a study model needs to be built. Therefore the first aim is to build a simple model of the gut microbes and optimize the conditions for detecting specific changes in mRNA in the model system.

2) Determine Effectivity of a Treatment Using Fluorescent Microscope.

To simulate atypical conditions (disease) in the gut, the *L. reuteri* cells were exposed to different growth environments and mRNA changes were studied by gel electrophoresis. The entire process before we are able to see a change in mRNA by gel electrophoresis, is a very long process. Therefore, in order to verify if a given treatment is causing any changes to the bacterial cells before the mRNA extraction, a fluorescent microscope will be used to verify the effectiveness and optimize conditions of that treatment.

3) Establish mRNA as a Biomarker for Monitoring Gut Microbial Activity.

Different environmental conditions causes changes in the expression of mRNA. Based on this fact, the goal of this specific aim is to demonstrate that variation of mRNA expression correlates with different conditions under

which the gut microbes are being cultured, thus demonstrating mRNA as a biomarker for monitoring gut microbial activity.

# CHAPTER II

# EXTRACTION OF MESSENGER RNA

## 2.1 Introduction

### 2.1.1 Gut Microbe Activity

Identification of the activity the gut microbes in the gut environment including how they interact with food and medicine as well as how they live in symbiosis with the host, is important in order to understand how the gut microbes affect human health and diseases. If the fashion of gut microbial behavior is detected, then may be how the gut microbiome influences the development or prevention of diseases can be unraveled. Hence, to study the pattern of activity, models of the gut microbes need to be established and tested to simplify the development and optimization of new methods of detection.

### 2.1.2 Simulation of Gut Microbiome

Most of the gut microbes cannot be cultured on a laboratory scale due to the conditions required for the growth. Therefore, the easiest option for a model system is probiotic bacteria. Probiotics are good microbes that produce health benefits to humans. They can be easily cultured in a lab and are found naturally in your body and in many types of food such as yoghurt. It is because of these properties that probiotics would make an ideal choice for developing a model for testing the gut microbes. While the probiotics are easy to culture, a large proportion of them are Gram-positive. This means they have a thick peptidoglycan layer around their cell wall. Because of this, it may be difficult to break open the thick cell wall for total RNA extraction.

Therefore starting with a simple model would help solve this difficulty. To begin with, the simplest model is a single pure culture of probiotic bacteria. The strain chosen was *L. reuteri.* It is a Gram-positive bacteria that is commonly found in certain cheese cultures and also has been detected in the gut[13]. It is from the *L.* genus, which is made up of many other probiotics and it is one of the better studied genuses of bacteria[14]. Therefore studying *L. reuteri* will provide a good foundation for developing methods of microbial activity detection.

### 2.1.3 Messenger RNA for Microbial Activity

The collection of proteins within a cell determines its health and function. Proteins are responsible for nearly every task of cellular life, including cell shape and inner organization, product manufacture and waste cleanup, and routine maintenance. Proteins also receive signals from outside the cell and mobilize intracellular response. They are the workhorse macromolecules of the cell and are as diverse as the functions they serve.

The genes in DNA encode proteins molecules. In the simplest sense, expressing a gene means manufacturing its corresponding protein, and this multilayered process has two major steps. In the first step, the information in the DNA is transferred to the mRNA molecules by a process called transcription. During transcription the DNA of a gene serves as a template for complementary base-pairing and RNA polymerase II catalyzes the formation of a pre-mRNA molecule, which is then processed to form mature mRNA. The resulting mRNA is a single stranded copy of the gene, which is translated into a protein molecule. Therefore following the changes in mRNA would allow us to study the gut microbe activity.

Any changes in the growth environment of the bacteria will induce direct changes in mRNA. May overexpress, underexpress or even form new

proteins to cope up with the changed environment. The changes in the environment here reflect the changes in the gut due to a disease or any other abnormal activity in the gut. To induce such a change in our model system, we grew the *L. reuteri* cells with slight changes in their growth conditions. The treatment given to the cells was, a lower pH 4 growth media.

### 2.1.3.a Low pH Treatment

In order for a bacterium to function as probiotic, it must survive passage through the gastrointestinal tract, where it is exposed to variety of environmental changes that might normally disturb bacterial growth or survival. Since *L. reuteri* is a probiotic, it likely adapts to the increasing acidity via changes in the expression levels of various stress response proteins. This also means that the expression mRNA would also be changing due to the low pH conditions. Therefore treatment of the cells at low pH 4 instead of the normal pH 7 is able to show various changes in terms of overexpression or underexpression of certain mRNA that enables us to prove mRNA as a biomarker for studying the gut microbial activity.

## 2.2 Method

### 2.2.1 Bacteria Stock

*L.* reuteri was purchased from the American Type Culture Collection (ATCC). The culture was started in 5 mL of deMan, Rogosa and Sharpe (MRS) media using the provided protocol. Growth was at 37°C with 5% $CO_2$ for 24 hours. After this initial growth, 10µL of *L. reuteri* was inoculated into fresh 5mL tube of MRS media and grown until the late log phase based on optical density (OD) at 600nm. Then the culture was decanted into 15mL centrifuge tube and centrifuged at 3000 x g for 15 minutes at 4°C. The supernatant was removed and the cells were then resuspended in an equal volume of fresh MRS media as begun with, containing 20% (w/v) sterile glycerol. The stock was aliquoted into cryogenic tubes and stored at -80°C.

### 2.2.2 Growth Curve

The cells are likely to produce a high amount of mRNA at their most active state. In order for us carry out the total RNA extraction from the most active cells, a growth curve was determined to identify the mid log phase of the *L. reuteri* growth cycle. *L.reuteri* was grown in MRS media for about 14 hours or till their death phase, with measurements of OD at 600nm approximately every 2 hours. 10 µL of cells were inoculated in a 5mL

volume of MRS media in duplicate and OD was tested directly in the culture tube in a Genesis 10 UV/Vis Scanning Spectrometer (ThermoSpectronic). The average OD for each time point was calculated and plotted against the time in hours.

### 2.2.3 Triton X-100 Boiling Method

After the comparison of various methods of extraction of total RNA from Gram positive bacteria for a high yield of mRNA and purity of extracted RNA, the Triton X-100 boiling method[15] was chosen as a suitable method for the analysis of extracted mRNA. Firstly, the *L. reuteri* was grown in 5 mL MRS media at 37°C with 5% $CO_2$ to the mid log phase.

Then 3 mL of cells at approximately 1.2 OD (mid log phase) were centrifuged at 3000 g force for 10 minutes to collect the cells. The pellet was then washed with 5mL of Tris-EDTA (TE) buffer. The cells were then suspended in 1mL TE buffer containing 0.2% Triton X-100 (Sigma). The suspension was then boiled at 100°C for 10 minutes and then transferred on an ice bath. An equal volume of chloroform was added. The tubes were inverted 10-15 times and then centrifuged at 12000 g at 4°C for 10 minutes. The top aqueous phase was collected and once more liquid-liquid extraction was carried out with an equal volume of chloroform.

After centrifugation, the RNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 5.2, 2 volumes of prechilled absolute ethanol, and incubated at -20°C for 2 hrs. The precipitate was then collected by centrifugation at 12000 g for 10 minutes at 4°C. The total RNA pellet was then washed twice with 1 mL of 70% ethanol and once with absolute ethanol. After centrifugation, the pellet was air-dried for 5 minutes and dissolved in 80-100 µL of nanopurified and autoclaved water.

### 2.2.4 Determination of RNA Concentration

The concertation of the extracted total RNA was determined using a Thermo Scientific NanoDrop 1000 spectrophotometer. The RNA sample concentrations are based on the absorbance at 260 nm, the selected analysis constant and baseline correction. For RNA quantification, a modification of the Beer-Lambert equation is used to calculate sample concentration using the Nanodrop instrument.

$C = (A*\varepsilon)/b$

- C is the RNA concentration in ng/µL
- A is the absorbance in AU
- ε is the wavelength-dependent extinction coefficient in ng-cm/µL

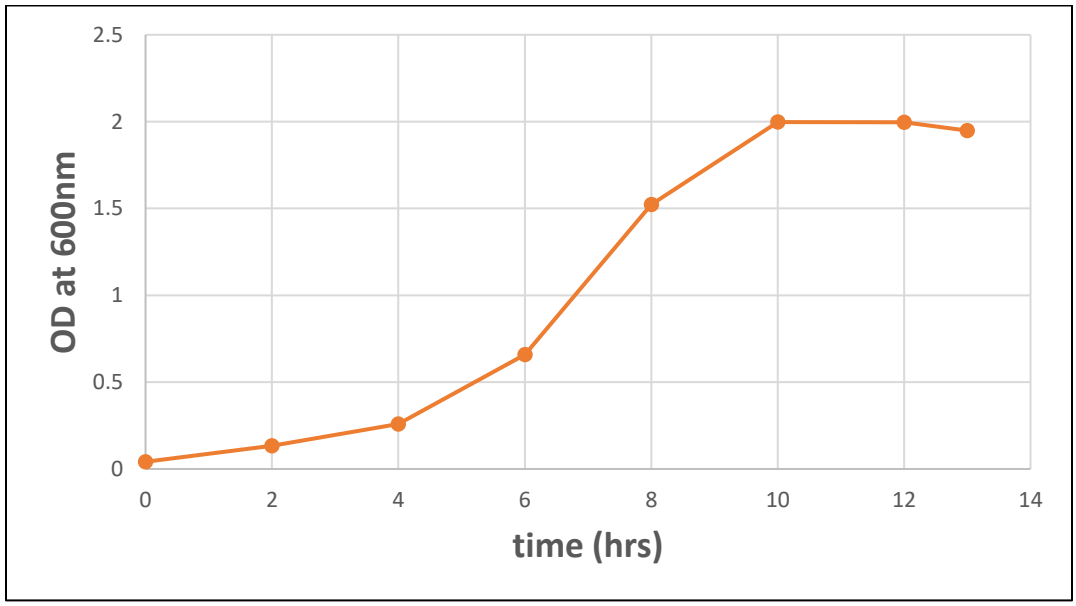
- b is the pathlength in cm

The Nanodrop also gives a purity ratio in terms of $A_{260}/A_{280}$ ratio. The ratio of absorbance at 260 nm vs 280 nm is used to assess contamination of proteins solutions, since proteins absorb light at 280nm. An $A_{260}/A_{280}$ ratio of ~2 is generally accepted as "pure" for RNA.

## 2.3 Results/Discussion

### 2.3.1 Growth Curve

Figure 1. *L. reuteri* Growth Curve



The growth curve shown in figure 1 was created for *L. reuteri* by growing in MRS media. *L. reuteri* confirmed to grow well in the MRS media. It reached the beginning of the log phase at around two hours and ended at around 12 hours with 10 µL inoculation volume in 5 mL media. Further, it can be noticed that the bacteria reached its midlog phase at approximately seven hours and at an OD of ~ 1.200. This indicates that *L. reuteri* cells are most active at an OD of 1.200. Hence, extraction of total RNA at this point in their growth curve would result in a maximum amount of mRNA extraction.

### 2.3.2 Results of Total RNA Extraction

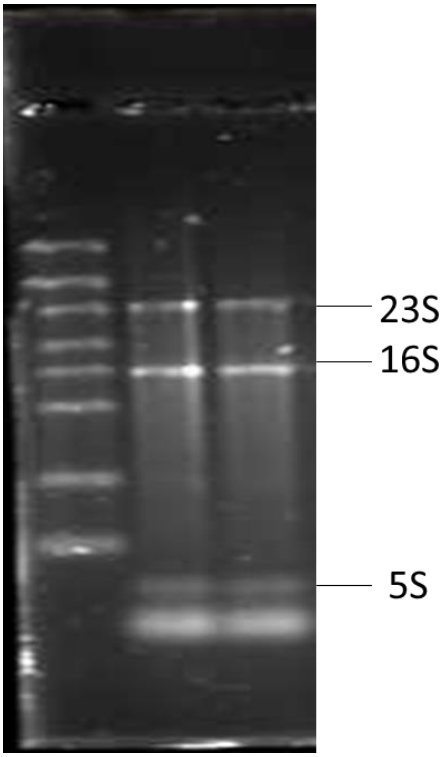
Starting the inoculation of *L. reuteri* with 10 µL in 5 mL of MRS media, the cells were grown to their mid log phase, approximately to around 1.2 OD. Triton X-100 boiling method was carried out on 3 mL of *L. reuteri* cells at 1.2 OD for total RNA extraction. The extracted RNA were then precipitated by the addition of 1/10 of 3M sodium acetate and 2 volumes of prechilled absolute ethanol and incubated at -20ºC for 2 hours. After centrifugation, the total RNA pellet was diluted in nanopurified and autoclaved water and stored at -80ºC for future use.

Table 2. Average RNA Yield by Triton X-100 Boiling Method

| Trials | Yield ng/µL | $A_{260}/A_{280}$ |
|---|---|---|
| 1 | 893.05 | 1.92 |
| 2 | 796.25 | 2.02 |
| 3 | 648.75 | 1.91 |

Triton X-100 boiling method provided good-quality RNA (and lesser 16S and 23S rRNA) and did not require any additional purification. The decreased rRNA in this preparation is probably due to heat degradation Using Thermo Scientific NanoDrop 1000 spectrophotometer, the quantity of RNA obtained from 3ml of cells grown to mid-exponential phase, by the boiling method ranged from 600-1000 ng/µL and had an $A_{260}/A_{280}$ ratio between 1.8 and 2.0, which indicated a good quality of RNA as shown in the table 2 above.

Figure 2. 1.2% Agarose Gel Showing Total RNA




A smear is obtained between 23S and 5S RNA when total RNA extraction is done by either Qiagen method, hot phenol methods or Triton X-100 boiling method. It represents different sizes of mRNA and some degraded RNA as shown in figure 2 above.

# CHAPTER III

# VISUALIZATION OF MESSENGER RNA CHANGES

## 3.1 Introduction

### 3.1.1 Gel Electrophoresis

Perhaps the most important and certainly the most often used technique in RNA analysis is gel electrophoresis. RNA have negatively charged phosphate backbones and therefore they migrate towards the anode in the presence of electric current. The gel acts as a sieve to selectively impede the migration of the RNA in proportion to its mass, given that mass is generally proportional to its charge. Because mass is approximately related to chain length, the length of RNA is more generally determined by its migration. Further, topology (i.e. circularity) can affect migration. Making RNA's appear longer on the gel than they are.

There are two common types of gels: polyacrylamide and agarose. Agarose gel electrophoresis is the most common method of analyzing RNA species, which has a relatively low resolution power but a large range of

separation from 50 bp to 500 kb in concentrations from 3% to 0.1% respectively. By using different concentrations of agarose, one can resolve different sizes of RNA fragments. Higher concentrations of agarose can facilitate separation of small RNAs, whereas low agarose concentration allows resolution of larger RNAs. Agarose gels are nontoxic and really easy to prepare. By completely melting agarose in the appropriate buffer and then pouring into the mold, the gels are formed.

For most applications involving RNAs of 600 nucleotides or less, acrylamide gels[16] are most appropriate. Acrylamide and *N,N'*-methylenebisacrylamide (bis) are mixed, then copolymerized by means of a vinyl addition reaction initiated by free radicals. Gels are formed as acrylamide monomers polymerizes into long chains that are linked together by bis molecules. The resultant structure has both solid and liquid components. It can be thought of as a mass of relatively rigid fibers that create a network of spaces, all immersed in liquid. The liquid (buffer) in the gel maintains the gel's three dimensional shape. Without the liquid, the gel will dry to a thin film. At the same time, the polymer fibers prevent the liquid from flowing away. During electrophoresis, the RNA molecules move

through the pores of gel. The migrating molecules segregate into discrete regions, corresponding to their individual gel mediated mobilities and sizes.

### 3.1.2 Ribosomal RNA Interference

The cell's total RNA pool is mainly composed of 82-90% of rRNA. Most studies that involve mRNA or other small RNA types, find these rRNA molecules interfering their study, especially with gel electrophoresis. The bigger rRNA molecules tend to block the pathway for the flow of smaller RNA through the gel pores. Further, conducting a total RNA gel shows that the background noise from rRNA on the gel is quite high, such that it interferes the visibility of other bands. Therefore, when other RNAs are to be analyzed, it is very advantageous to remove rRNA to eliminate background noise. The methods for removal of rRNA from total RNA extracted from bacteria are very limited, due to the fact that mRNA do not have polyadenylated (Poly-A) tails. Therefore mRNA cannot be selectively separated from the total RNA. Hence, the total RNA was conducted on both agarose and PAGE gels to confirm the difficulty in visualization of mRNA.

Formaldehyde de-ionized formamide denaturing PAGE[17] (FDF-PAGE) gel is used to denature small RNAs. Full denaturation of mRNA allows the real size to be exposed which increases the efficiency of gel electrophoresis. Once the RNA molecules are in their denatured state (straight and not circular), may ease flow of smaller RNA even with the interference from rRNA.

A method available in the market for removal of rRNA is by enzymatic digestion. XRN-1 exonuclease is an enzyme used for the digestion of rRNA. This procedure relies on the properties of certain exonucleases that digest RNA in a 5′ to 3′ direction and require a 5′ phosphate[18] for activity. Because rRNA, with the exception of 5S rRNA, contain a 5′-phosphate terminus, they are substrates for these nucleases. Due to the fact that 5′ ends of mRNA are protected by caps, they are resistant to degradation by these exonucleases. Therefore this method can be used to enrich RNAs without a 5′-phosphate groups (mRNA).

Another lengthy procedure available for separating mRNA is by extracting of mRNA bands from the electrophoresis gel. One of the simplest method of purifying mRNA from gels is by diffusion of mRNA. This procedure can be applied to both agarose and PAGE gels. Compared to

PAGE gel, agarose gel easy to handle and make. Also, very few ingredients are involved in making the agarose gel and therefore there is less chance of contamination or degradation of RNA and hence increases recovery of mRNA. Therefore diffusion of mRNA smear from agarose gel was carried out.

## 3.2 Method

### 3.2.1 Agarose Gel Electrophoresis

An agarose gel was made by simply melting agarose in appropriate amount of 1x trisborate EDTA buffer (TBE). A 1% agarose gel was used in order to achieve successful separation of both big and small RNAs. Approximately 0.5 g of agarose was melted in 50 mL of 1x TBE buffer. The melting was carried out by microwaving the mixture in intervals of 10 seconds with shaking of mixture, for 40 seconds or until a clear solution was formed.

Once the flask containing gel mixture was warm enough to be touched, it was poured into the casting tray and allowed to solidify and mold into a gel with appropriate wells for holding the samples for electrophoresis. Ribo Ruler high range RNA ladder was purchased from Thermo Scientific and the loading dye that came with it was used to prepare the sample for

electrophoresis. The RNA sample was made by mixing the total RNA with an equal volume of 2X RNA Loading Dye from Thermo Scientific. The total RNA mixture was then heated for 10 minutes at 70°C and snapped cooled on an ice bath for approximately two minutes before loading into the gel.

The gel was conducted at 70 V for about two hours in 1x TBE buffer. The gel was then stained using 1x Sybr green II RNA stain for 30 minutes, before visualization of the gel using spectrophotometer.

### 3.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

A 5% polyacrylamide with 7M urea gel was prepared by mixing the ingredients given in Table 3 below. Once all the ingredients were mixed and the apparatus set up to pour the gel 10 µL of TEMED, a source of free radicals and a stabilizer was added to initiate polymerization. Immediately after mixing in the TEMED, the mixture was poured into the casting apparatus and polymerization to occur for about an hour before loading in the RNA sample.

The gel was conducted without the samples at 50 V for half an hour. This was done to remove any solution in the well and also to attain an equal temperature in the electrophoresis chamber. The RNA samples were prepared exactly same way as they were for the agarose gel, heated for 10

minutes at 70°C and snapped cooled for about two minutes before loading into the gel. The gel was then ran at 70 V for about an hour. Then staining of the gel was also done the same way as for the agarose gel before its visualization under the same spectrophotometer.

Table 3. 5% Polyacrylamide Gel

| **Ingredients** | **Amounts in mL** |
|---|---|
| **47.5% acrylamide 2.5% bis-acylamide (19:1)** | **2** |
| **10 M Urea** | **14** |
| **10X TBE Buffer** | **2** |
| **10% fresh ammonium persulphate** | **0.2** |
| **DI Water** | **1.8** |

Ingredients for making 5% polyacrylamide with 7M Urea PAGE gel. This solution is enough for two mini gels.

### 3.2.3 FDF-PAGE Gel

Denaturation of the RNA in formamide prior to electrophoresis through a 7M urea polyacrylamide gel (F-PAGE) is commonly used to carry out denaturation of RNA samples. This is thought to allow RNA remain in

its denatured state for long enough to conduct the gel electrophoresis. Fully-denaturing formaldehyde polyacrylamide gel electrophoresis (FDF-PAGE) was recently proven to maintain the denatured state of RNA more efficiently than the F-PAGE[17]. For FDF treatment, RNA in 4 µl volume was mixed with 11 µL of FDF buffer (2.75 µL of formaldehyde (40%), 7.5 µL of de-ionized formamide, 0.75 µL of 10×MOPS buffer [200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0]) and incubated at 55˚C for 15 min. 2 µL of dye was added at room temperature before loading. The gel was conducted in 0.5 X MOPS buffer at 70 V for about 45 minutes.

### 3.2.4 XRN-1 Exonuclease Enzyme

XRN-1 exonuclease, M0338S was purchased from New England BioLabs. According to the manufacturer's description it is highly processive for 5′ to 3′ exoribonuclease, which requires 5′ monophosphate. The mRNA of *L. reuteri* do not have a 5′ monophosphate unlike its rRNA and therefore it qualified to be used for the process of rRNA removal when the above gel tricks failed. For the very first attempt XRN-1 enzyme was used exactly as per the manufacturer's instructions, which says that one unit (=1 µL) of enzyme digests 1 µg of monophosphorylated RNA in 60 minutes at 37°C.

After the first failed attempt, various conditions were tried for the complete and specific digestion of rRNA. The concentration of the enzyme was serially increased to try and achieve complete digestion, until we achieved unspecific digestion of mRNA as well. The time of RNA incubation with the enzyme was also increased to try and achieve complete digestion of rRNA. The likelihood of rRNA not being in the denatured phase and therefore the XRN-1 enzyme's unable to identify the 5′ monophosphate was also eliminated by carrying out XRN-1 digestion on denatured RNA and still achieved incomplete digestion of rRNA. The resulted RNA from the digestion were observed using both the PAGE and agarose gel to determine the best method for visualization of mRNA

### 3.2.4.a Increased Enzyme Concentration

Approximately 600 ng of total RNA was incubated with 0.75 µL of the XRN-1 enzyme along with appropriate amount of enzyme buffer and water for one hour at 37°C. In order to achieve complete digestion of rRNA, the concentration was increased by 2X (1.5 µL) and 3X (3.6 µL) under the same conditions.

### 3.2.4.b Increased Enzyme Incubation Time

After failing to achieve complete digestion with increased concentration, we suspected that the enzyme was not getting enough time to complete the digestion. Therefore, to verify our suspicion we increased the incubation time from 1 hour to 2, 4 and overnight incubation, with the rest of the conditions remaining the same.

### 3.2.4.c XRN-1 on Denatured RNA

When disappointing results with increased incubation time was obtained, we speculated that the enzyme was not able to recognize the 5′ position under natured (circular) condition of RNA. Hence, the total RNA were heated for 10 minutes at 70°C to denature it before carrying out the enzymatic digestion. The conditions for digestion used here were as per the manufacturer's instructions as well.

As temperature increases, so does the rate of reaction. But very high temperatures may denature the enzyme. Therefore a slight increase in the temperature for enzyme activity was carried out. The temperature was increased from 37°C to 42°C to know the effect in activity of the enzyme due to higher temperature.

### 3.2.5 Purification of mRNA from Agarose Gel

In the past recovery or purification of a particular band or RNA have been re-extracted from the gels by the process of diffusion. A band of interest is cut out from the gel and submerged into the electrophoresis buffer allowing for diffusion of the RNA in that particular band out into the buffer. The RNA is then recovered by ethanol precipitation.

The smear between 18S and 5S rRNA bands are the mRNA, which are seen as smear due to their multiple sizes. Therefore after conducting an agarose gel of total RNA, that smear of mRNA was cut and collected into the Eppendorf microcentrifuge tubes. Enough TBE buffer was added such that piece of gel is submerged. The plastic pipette tube used to dispense buffer into the microcentrifuge tube, was also used to crush the gel against the wall of the tube into tiny pieces to achieve complete diffusion of RNA from the gel. The mixture of the gel pieces and buffer were incubated on ice and on a rotor overnight to allow diffusion of mRNA.

The next morning, the tubes were centrifuged at 12000g for 2 minutes at 4°C to collect the supernatant fluid that contains the mRNA. Ethanol precipitation was carried out by adding of 1/10 of 3M sodium acetate and 2 volumes of prechilled absolute ethanol to the supernatant and incubated at -

20ºC for 2 hours. The mRNA pellet was collected by centrifugation at 12000g for 10 minutes at 4°C. The mRNA was then ready to be reloaded into a gel for the visualization of mRNA only. This achieved the isolation of mRNA for the study of gut microbe activity.

## 3.3 Results/Discussion

### 3.3.1 Messenger RNA on Agarose and PAGE Gel

Total RNA extracted from *L. reuteri* cells by Triton X-100 boiling method, were denatured by heating at 70°C for 10 minutes after mixing it with loading buffer that came with Ribo Ruler RNA Ladder. The gel photo obtained from conductedning RNA on 1% agarose gel and 5% PAGE is as shown in Figure 3. The 23S and 18S rRNA are seen very clearly, but a smear of RNA follows it all the way between 5S rRNA. Since rRNA makes up most of the constituents of total RNA as seen in the Figure 3 below, it is necessary to remove high background noise created by rRNA prior to the analysis of our target mRNA by using gel electrophoresis.

Figure 3. Results from Different Gel Electrophoresis

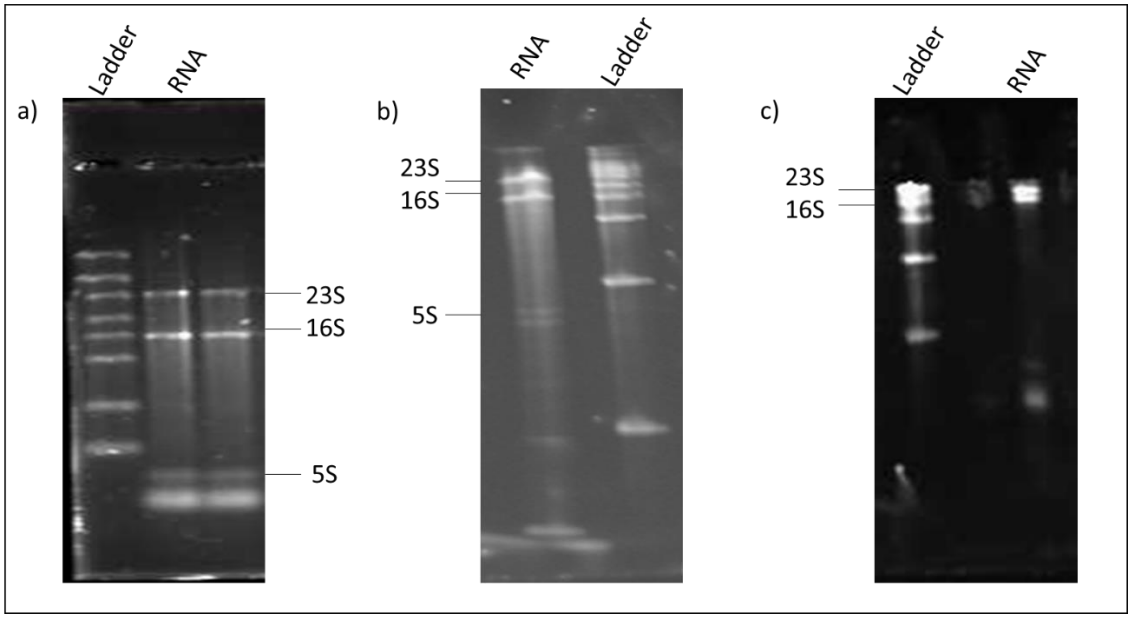



Image a) and b) shows total RNA samples after denaturing for 10 minutes at 70˚C. Image c) is total RNA after FDF treatment.

### 3.3.2 FDF-PAGE Results

Our assumption of RNA not being in fully denatured state and thus not achieving clearance with the mRNA smear, was not solved by the FDF-PAGE gel. The gel picture obtained by treating total RNA with FDF, showed full degradation of all types of RNA except rRNA. The mRNA smear was no longer observed as seen in the Figure 2 above. The rRNA were clearly seen and very small RNA were also seen on the gel except for mRNA smear between 18S rRNA and 5S rRNA. Our interpretion of these

results was that the treatment with FDF caused degradation of mRNA and therefore another option was required to observe mRNA on a gel.

### 3.3.3 Incomplete Activity of XRN-1 Enzyme

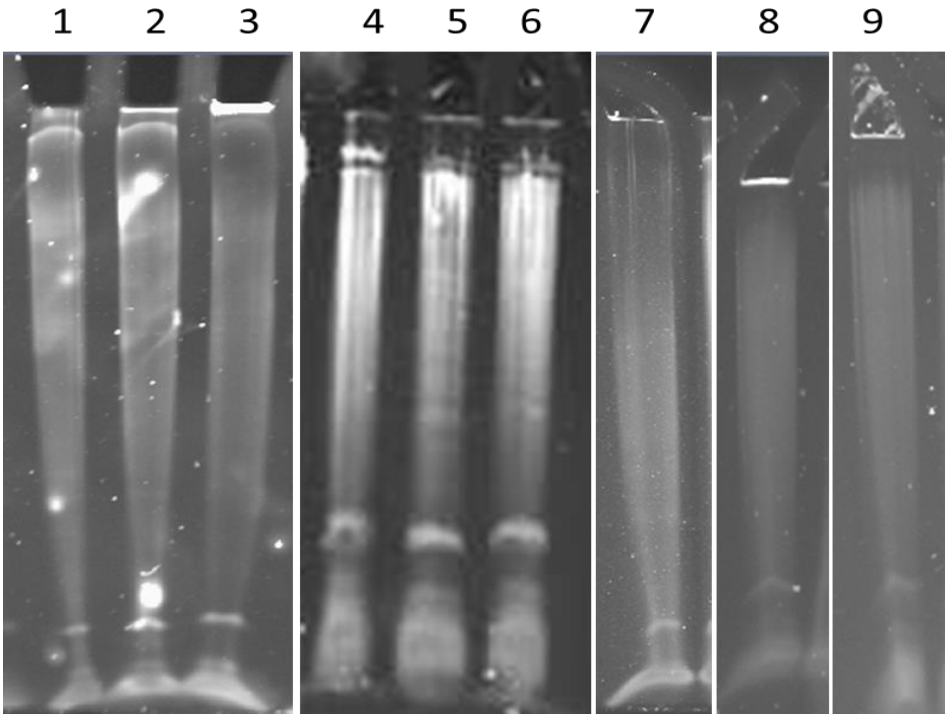
The results from XRN-1 enzyme activity as per the manufacturer's instructions confirmed that the enzymes were able to cause the digestion of the rRNA (2016-03-09), because the 23S and 18 S rRNA were no longer producing sharp body. Instead there was a whole smear conductedning all the way down form the 18S rRNA band till the 5S rRNA. The smear at the mRNA position also got sharper, which may indicate that the rRNA were broken down into various sizes that increased the sharpness of the smear of mRNA. If there was complete digestion of rRNA, there would be a very high increase in the small RNA that were below 5S rRNA, which we did not see. Therefore the observation indicates an incomplete digestion of the 23S and 18S rRNA, hence to resolve the interference from the incomplete digestion rRNA, higher concentration of the XRN-1 enzyme was used.

The Figure 4 shown is for the XRN-1 enzyme used at higher concentrations, and all the other conditions remain the same, (i.e. temperature at 37°C and incubated for an hour). About 600ng of total RNA were treated with 0.75 µL, 1.5 µL and 3.6 µL. There is no significant

difference in any of the smears obtained and all resulted in incomplete digestion of rRNA. A stronger smear was still obtained for mRNA showing the interference from the incomplete digestion of rRNA instead of the clear cut smear/band for mRNA. When even higher concentrations were used (increased by 5X) there was non-specific digestion of all RNA, for which results are not shown below. The enzyme ended up digesting mRNA along with other RNA types and therefore no further increase in enzyme was tried.

We then speculated that the incomplete digestion may have resulted from lack of enough time to complete the digestion. So, instead of increasing the concentration, we simply tried increasing the incubation time from 1 hour, to 2 hours, 4 hours and overnight. It can be observed from figure 4, that there was incomplete digestion of rRNA at all the three incubation times. This may be due to the inactivation of enzyme at 37°C after one hour. The enzymes may be denatured after one hour under these conditions and therefore there is incomplete digestion even when incubated overnight.

Figure 4. Total RNA Treated with XRN-1 Enzyme




Lane 1, 2 and 3 shows RNA treated with 0.75, 1.50 and 3.60 µL of XRN-1 enzymes respectively. Lane 4, 5, 6 and 7 shows RNA treated with 1.50 µL of XRN-1 enzyme for 1, 2, 4 hours and overnight respectively. Lane 8 shows RNA that was denatured before XRN-1 enzyme activity. Lane 9 shows RNA treated with XRN-1 enzyme with increased temperature of 42˚C.

There are chances of XRN-1 enzyme to miss out on recognizing the monophosphorilated 5′ position on rRNA due to its nondenatured state (circular in shape). To resolve this issue, the total RNA were heated at 70°C for 10 minutes to denature them before incubation with XRN-1 enzyme. The gel in Figure 4 shows that there no effect of carrying our XRN-1 digestion of

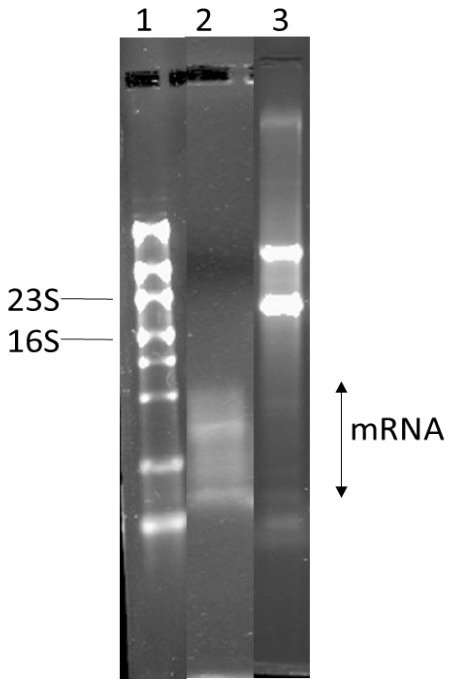
denatured RNA. The same smear was obtained showing the incomplete digestion of rRNA.

As seen in Figure 4 the increase in incubation temperature also did not have any effect on the digestion of rRNA. The total RNA were incubated at 42°C instead of 37°C for one hour. It showed the same incomplete digestion results. After discussion about the project to the committee, we were suggested about re-extraction of the mRNA band back from the agarose gel. Hence, our next step was to carry out re-extraction of mRNA from the gel, to obtain mRNA for the feasibility study of mRNA being a biomarker for gut microbe activity assessment.

### 3.3.4 Successive Re-extraction of mRNA

Diffusion of mRNA from the agarose gel was carried out to separate mRNA from other RNA types. After obtaining the mRNA back from the gel by diffusion, an agarose gel of the mRNA was repeated to confirm our re-extraction process was successful. The gel picture shown in Figure 5 is of the mRNA repeated on the agarose gel after its re-extraction from the gel. All there smears at different sizes were seen again onto the gel.

Figure 5. mRNA Extracted from Agarose Gel




Lane 1 shows RNA ladder, lane 2 shows mRNA smear from agarose gel extraction and lane 3 shows total RNA.

As mentioned earlier, at least only about 3-5% of total RNA is mRNA, this method had to be multiplied atlist three times, to obtain enough mRNA in order to observe mRNA on an agarose gel. There is some loss of mRNA in the process of re-extraction form the gel and good chances of RNase contamination, there was need for great care and sterilization of the environment. Further due to small size of the agarose gel apparatus available in the department, re-extraction from atlist 3 gel conducted were needed in

order to obtain enough mRNA for the study. Each gel has 8 wells and each well can carry about 600 ng of total RNA for efficient separation. This added on to the stress of little quantity of mRNA sample to work with at a time. Overall, successive extraction of mRNA was done in expense of time and labor for further studies to be carried out on mRNA.

# CHAPTER IV

# STUDY OF LOW pH ON *L. REUTERI*

## 4.1 Introduction

### 4.1.1 Laboursome Extraction of Messenger RNA

When the time taken for the entire process of extracting messenger RNA from *L. reuteri* starting from the cells' incubation all the way to re-extraction of mRNA is considered, it takes approximately 10 days. *L. reuteri* cells are inoculated in MRS media the night before, to allow it to reach the mid-log phase for extraction to be carried out on matured and most active cells. Two or more hours add up when we considered treating the microbe cells with the lower pH before extraction of total RNA. It takes approximately one and half day to conducted one agarose gel and re-extract the mRNA by diffusion. Since only about 3-5 % of total RNA is mRNA, more than one total RNA gel electrophoresis is needed to be done to extract enough mRNA than can be visually studied for microbial activity.

Further, more than one gel is also required because of the availability of only the mini agarose gel casting plates, which limits the amount of total RNA that can be conducted in a single gel. More total RNA also need to be conducted in order to compensate the loss during the re-extraction process. Altogether, this work very laborious.

This means that, when the cells are treated with a lower pH, the end results will be available after about 10 days in order for to us conclude whether the treatment was effective enough to do the feasibility study of mRNA as biomarkers for gut microbes. In order to save time, money and effort put it, before carrying out a particular treatment on *L. reuteri* cells, it is productive to develop a method to identify the conditions under which a certain treatment is effective on mRNA. Fluorescent microscopy can be used here to identify the conditions for an effective treatment.

### 4.1.2 Cell Viability Study

The EVOS FL Cell Imaging System from ThermoFisher scientific was used to carry out this study. It allows capture of multiple images of a large area of a slide or plate to create high resolution images of the larger field of view. This fluorescent microscope can also be used for analyzing samples in the well of a 96-well plate. This fluorescent microscope allows the visualization of *L. reuteri* cells 20X magnification power.

Once the cells are treated with the low pH, there would be a change in the cell viability. This cell viability can be monitored using this EVOS FL imaging system. Further, the viability curve obtained from any treatments may allow rough estimate during which major changes are taking place in the cells. This is a region on a cell viability graph where the cells are trying to stabilize with a low death rate. This time interval informs us about the functional proteins change occurring in order for the cells to survive in a different environment. Therefore it gives information about exposure time treatments that would cause changes in mRNA, and hence saves our time on trial and failure for right conditions for the treatments.

Whereas, the gel electrophoresis would provide information about effectivity about a treatment in approximately 10 days, using EVOS FL

microscope would do this in a day. Therefore, EVOS FL Imaging is used here to study the right conditions under which the pH treatments are effective, hence the cells can be treated under those validated conditions and extraction of mRNA can be done for further studies.

### 4.1.3 Dead Cells Detection - PI Dye

Propidium Iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain. It is commonly used to detect dead cells in a population. PI dye reaches the nucleus by passing through disordered areas of dead cell membranes, and intercalates with DNA double helix of the cell. After the exposure of low pH treatment, the cells were treated with the PI dye and culture observed under the EVOS FL microscope to obtain viability curve for further studies.

### 4.1.4 pH Treatment on *L.REUTERI*

The reported proteomic profile for *L. reuteri*[19] under acid stress conditions, shows differential expressions in response. It also shows that the acid stress response of *L. reuteri* is a complex process. Some of the differentially expressed proteins are general stress response proteins, while others are key metabolic components involved in the glycolytic and pentose-phosphate pathways. Hence low pH condition is an excellent choice for

showing changes in mRNA for feasibility study of mRNA being a biomarker for gut microbe activity.

## 4.2 Method

### 4.2.1 pH 4 Treatment

The required pH of the media in which *L. reuteri* grow is pH 6.8. The proteomic profile for these bacteria show maximum change in protein fold to occur when the cells are exposed to low pH 4, hence the pH of the media in which *L. reuteri* cells grow was changed from 6.8 to 4. First, the *L. reuteri* cells were grown to mid exponential phase of 1.2 OD in the normal MRS media of pH 6.8. After the cells had grown to 1.2 OD, they were centrifuged down to a pellet at 3000 rpm for 10 minutes. The supernatant media was carefully poured out. The pellet of *L. reuteri* cells was then resuspended into the MRS media at pH 4 at 37°C in the presence of 5% $CO_2$. Alongside these treated cells a control was also carried out. The cells' pellet of the control were resuspended into the normal media at pH 6.8, while all the other conditions reaming the same.

At an hourly interval 100 µL of the cells were taken out into a 1.7 mL microcentrifuge tube from both the treated and control cells. The cells culture was returned into the incubator immediately after extracting out 100

µL of cells from it, to continue with the treatment. The 100 µL of cells were then treated with the propidium iodide (PI) as per the manufacturer's instructions. Firstly, PI dye was prepared in the ratio of 1 mg/ mL i.e. 1 mg of solid PI dye was dissolved into 1 mL of autoclaved DI water. The dye was then stored at -20°C for further use. This prepared PI dye was then used to stain the bacterial cells. 100 µL of cells were mixed with 2.7 µL of the PI dye and stored at room temperature for 5 minutes for the dye to diffuse through the damaged cell wall and into the DNA of the dead cells.

After the 5 minutes at room temperature, the cells were stored on ice for further analysis under EVOS FL Imaging system. 100 µL of cells were extracted every hour for first 4 hours of the treatment, and dyed with PI. Further the live Vs dead cells were counted using the fluorescent microscope for the analysis of effectiveness of the low pH treatment.

### 4.2.2 Treatment Evaluation

After the cells were treated with the pH 4 media, the effectivity of each treatment was analyzed using the EVOS FL Imaging system. In order for cells to fluorescent under the red fluorescent light, they were treated with propidium iodide dye for 5 minutes at room temperature. Once the cells

were dyed, 1.6 µL of these cells were put on a glass slide and covered with a round cover slip.

The cells were then observed under the EVOS FL microscope at 20X magnification. The images at five different positions on the slide were taken. These five positions were consistent with each trial (i.e. image was taken at the top, bottom, left, right and center of the slide). The cell images were taken with and without the red incident light. The PI dye would only stain the dead cells and therefore only the dead cells would be visible under the red incident light. While without the fluorescent light, all the live plus the dead cells were visible at that particular spot on the glass slide.

Image of one single spot on the slide was taken with and without the red fluorescent light in order to obtain the percent cell viability at that point of the treatment. After taking the images of dead (under red fluorescent light) and mixed population (without fluorescent) of cells at the same position of a slide, % cell viability was obtained by equation 1. The average of the 5 images was used to obtain the percent cell viability.

$$\%\ Cell\ Viability = \frac{Alive\ Cells}{No.\ of\ mixed\ population} * 100 \qquad \text{(Equation 1)}$$

Figure 6. *L.reuteri* Cell Images Using EVOS FL Imaging System

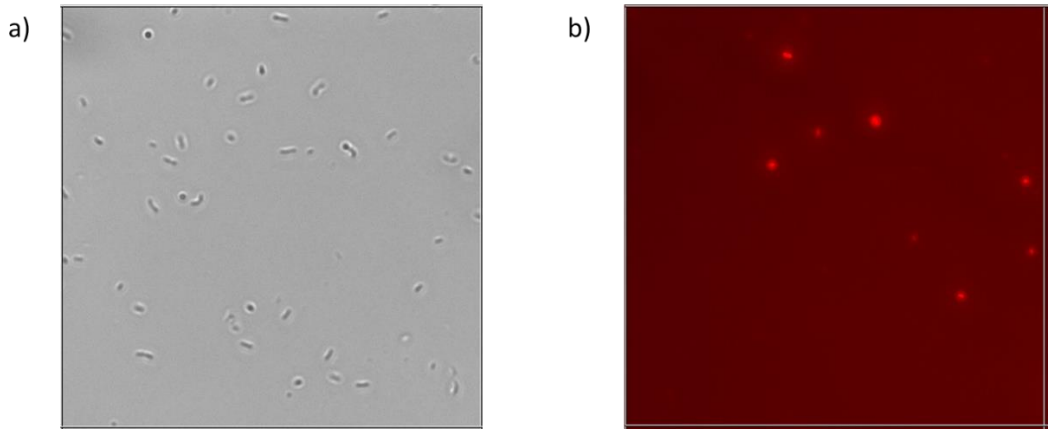



Image a) shows the total cells without fluorescent light while b) shows the fluorescent dead cells of *L. reuteri* bacteria

## 4.3 Results/Discussion

### 4.3.1 pH Treatment

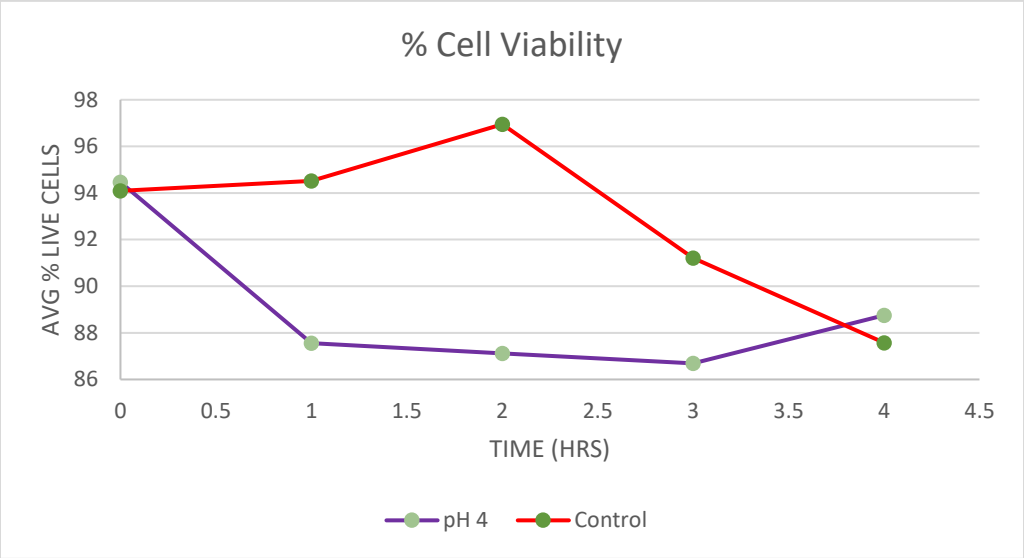
After the cells were treated at low pH 4 for four hours, 100 µL sample of cells was withdrawn at 0, 1, 2, 3 and 4 hours' time of interval and treated with PI dye. The viability of cells was then obtained by counting the number of cells of dead and mixed population (live and dead) of the cells using the fluorescent microscope. The equation 1 was then applied to calculate the % cell viability.

As observed, the percent viable cells decrease drastically in the first one hour than compared to the $2^{nd}$, $3^{rd}$ and $4^{th}$ hour for the pH 4 treated cells.

Between the first and the 3rd hour, the cells seem adapt the new environment. This may be supported by the fact that there is no change in cell viability during that time. The cells seem to slow down with their activity for adapting to their new environment. This also means that the major change in their proteins takes place during this time that enable them to adjust to the new environment and later thrive.

During the last 4th hour, it seems to have adapted or the change in their proteins has taken place and hence able to increase in number and therefore survive. Overall the graph for pH treated cells informs us that mRNA change can be visible after treating the cells between 1- 3 hours. If mRNA extraction is done within the first one hour, we may not observe any changes in mRNA as the cells are mostly dying, and very little change occurs in their proteins cell viability. The data was then plotted on a % cell viability graph as shown in 6.

Figure 7. % Cell Viability Graph for pH 4 Treated *L.reuteri* Cells




As also seen from the same graph that the cell viability for the control increases for the firsts 2 hours as they receive fresh media with all the nutrients required to grow. Later, the cells start to die as they conducted out of nutrients and space to grow further. The viability of the cells seems to not stabilize at any point, which also proves they do not need time to make any changes to adjust to the environment. Their proteins are stable enough to carry out normal functions required for them to survive in those conditions.

# CHAPTER V

# CONCLUSION

## 5.1 Extraction of Total RNA from Gram Positive Bacteria

Total RNA extraction was completed in *L. reuteri* bacteria. It provided high yield of total RNA for the study of mRNA for gut microbe activity. The extraction was done more than twice to confirm the preciseness of the method used. A simple step by step procedure was assembled from the growth of the bacterial cells for the extraction of total RNA.

Even though the Triton X-100 boiling method is expected to yield total RNA with less rRNA extraction due to the heating involved, the amount of rRNA is still high enough to block the visualization of mRNA on a gel. As rRNA make up about 90% of total RNA, it occupies most of the pores in a gel, blocking the smaller mRNA to flow down efficiently by electrophoresis and thus may interfere with our study of mRNA changes to follow the gut microbe activity. Therefore removal of rRNA must be done.

## 5.2 Successful Re-extraction mRNA from Agarose Gel

Though a very long procedure is involved, a step by step method was developed for separating mRNA from total RNA which was necessary for the feasibility study of mRNA being the biomarker for the study of gut microbes. It takes two whole days for the extraction of mRNA from an agarose gel and re-conducting it on fresh gel to observe the mRNA changes. Due to low concentration of mRNA in total RNA as well as the possibility for loss of mRNA in the extraction process, almost three whole agarose gel extraction need to be done in order to obtain band/smear of mRNA that can be analyzed. Limitation of the small agarose gel casting plates, also adds the pressure for extracting more than one agarose gel.

XRN-1 exonuclease enzyme proved to be an unselective enzyme for digestion of rRNA. It also continuously showed incomplete digestion under all concentration and higher temperature conditions. Higher incubation time also did not have any effect of completing the rRNA digestion. Therefore it can be concluded that XRN-1 enzyme is not suitable for complete digestion and elimination of rRNA from non-poly-A mRNA containing samples.

## 5.3 Study of Treatment Effect under Short Time Duration

Use of fluorescent microscope showed to be a perfect method to evaluate the treatment conditions for a change mRNA to occur for *L. reuteri* cells. If gel electrophoresis were to be used from the start, it would have taken almost ten days to verify if the mRNA changes is caused by a certain treatment under given conditions. For example, treatment of *L. reuteri* cells for 30 minutes at pH 4 may have showed any significant change in mRNA, and it would have taken almost 10 days to reach this conclusion. On the other hand, it took one day to collect data of pH 4 treated cells and another day to analyze the day to give the percent viability of cells.

It is easily concluded from percent viability graph the time needed to expose to pH 4 that would cause a change in cell functionality which is directly represented on the mRNA. Study of these changes on the mRNA of *L. reuteri* cells would build a feasibility study for following the gut microbe activity by using mRNA as a biomarker.

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