CHAPTER-III

Material & Methods
3. MATERIAL AND METHODS:

3.1 Sample Collection:
Fermented food products were collected from all the six agro-climatic zones of Assam. The samples were mainly collected from the local haats of the district headquarters and the vicinity of the towns. The sample collected included:

Gundruk:

Gundruk is a fermented vegetable product indigenous to the Nepali people of the Himalayan region. It is commonly prepared during winter i.e., October to December, when perishable leafy vegetables are plenty. These vegetables are mainly leaves of mustard (Brasicca juncea), rayo-sag (Brasicca rapa), cauliflowers (Brasicca oleracea), radish (Raphanus sativus) and some other locally grown vegetables (Tamang and Tamang, 2009).

For its fermentation, fresh leaves of the selected vegetables are first wilted and shredded using a sickle or knife. These are then crushed mildly and pressed into an earthen pot. The container is then made air tight and left to ferment naturally at room temperature for about 7 to 10 days.

After the incubation period the leaves takes a mild acidic taste which indicates the completion of fermentation. The gundruk is then removed and sun dried for 3 to 4 days, which helps in storage. It is eaten as a soup...
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or pickle. The soup which is prepared after mixing gundruk with certain ingredients serves as a good appetizer (Tamang and Tamang, 2009).

Kharoli:

This is a kind of fermented mustard (Brassica juncea or B. nigra) seeds called chutney. The mustard seeds are washed properly. After proper drying they are grinded and sieved. The powder is then placed over the dorsal side of a banana (Musa sp.) leaf which heated over the fire in order to soften it. A little salt is added and the mass is kneaded for about 15 minutes by adding kolakhar (indigenous soda water) little at a time.

This is made into flattened balls and wrapped with the leaf which is again tied with a string. This parcel is kept in a warm place for about 3 days after which the kharoli gets ready. It is a sour favourite among the people of Assam and is eaten as chutney with rice (Hughes et al., 2001).

Fermented bamboo shoot:

This is another fermented product which is extensively used in the states of North-East India and bears resemblance to kardi in Orissa, India. It is mainly used as a taste enhancer and flavour provider. Many varieties of bamboo are used separately by the different tribes using their own traditional techniques.
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The outer inedible and hard casings of succulent bamboo sprouts are peeled off and the soft portions are chopped and pressed tightly into wooden or earthen pots and left to ferment for 6-12 months.

The noney/ kwatha type is the more preferred one which is batch type fermentation that results more acidic product with more acidic taste and is carried out in traditionally designed bamboo chamber which is covered with leaves of wild plants or polythene sheet. The product after completion of fermentation can be stored for more than a year. The product is whitish in colour with faint aroma and sour taste.

Hawaijar (Fermented Soya):

Basically produced in the state of Manipur, Hawaijar is a sticky fermented soybean product. Its name is derived from “hawai” meaning pulses and “jar” which is shortened form of achar, meaning pickle. In Manipur soybean is known as nung hawai (nung = stone, hawai= legumes/ pulses) and two varieties, viz. the local variety with small seeds and the bigger, round seeded variety are used for the preparation on Hawaijar. The produce is brown in colour and covered with a white slimy substance.

For its preparation, soybean seeds are soaked overnight after which they are washed thoroughly with water and boiled till the seeds become soft. They are then washed with hot water and packed tightly in a small bamboo basket (lubak) with a lid. The base of the basket is layered with the leaves of fig
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plant (*Ficus hispida*) or banana plant (Musa sp.) leaves. The basket is then wrapped with a jute cloth and kept in the sun or near to stove or buried in paddy. This helps in maintaining the optimal temperature (> 40 °C) required for fermentation to take place.

The fermented product becomes ready for consumption after 3-5 days. The final product is brown in colour, has a sticky texture and emits ammonia like odour. The fermented product is then wrapped with banana leaves for storage. Hawaijar has a very short shelf-life of 3-4 days. Hence, the product is sometimes dried in the sun for long-term storage.

Hawaijar is known for its unique organoleptic properties. A special delicacy of the Manipuris called chagempomba is prepared using hawaijar, rice and other vegetables. It is also eaten as a paste with chilli and salt and is known as ametpa. A fermented fish product called ngari is sometimes added to ametpa to enhance the flavour. Hawaijar is also added while cooking other vegetables (Das and Deka, 2012).

**Fermented Fish:**

Following two methods are practiced.

1. Fish is washed with water using porous bamboo based baskets and allowed to drain. Next day morning, the head and bones are removed and pressed using gunny bags to remove excess water. Oil released from head during pressing is believed to cause initiating fermentation. In the
meantime, inner surface of earthen pot (capacity of 45-50 kg) is coated with mustard oil. For new pots, 8-10 times oil coating are required with interval of 7-10 days. In old pots, only one coat is sufficient for fermentation. Oil coating might be creating anaerobic environment inside the chamber. The pressed dry fish is packed tightly inside the pot. After packing, pots are sealed with polythene sheet, fish scales, oil slurry, mud and cow dung slurry. These packed pots are kept in dark up to 6-12 months at room temperature.

2. The fish (*Puntius sophore*) is rubbed with salt, dried in the sun for 3-4 days, press tightly in an earthen pot, sealed airtight and then stored at room temperature for 4-6 months.

Fermented Pork:

Some of the methods used for the preparation of different varieties of fermented pork.

- Boiled rice smeared over the boiled pork pieces filled tightly in bamboo cans and sealed-fermentation to obtain the dahi or curd occurs within few days and acts as preservative.
- Pieces of pork fat-half boiled and kept inside the sathu (Water gourd) container which is placed near the fire for 4-5 days. It has long shelf life and is used in curry.
- Boiled fats of pork is kept in air tight bottle and used in curry preparation.
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→ Small pork pieces mixed with ginger, garlic and salt paste stuffed into bamboo hollow container- burnt in fire- cool & consume in slice form.

Dahi (Curd):

Dahi is generally prepared by adding a starter culture in raw or boiled, kept for a day in warm place. Tribals also prepare it by keeping it in bamboo shoot and covering it airtight with banana leaves and sometimes burying in mud and leaving it for a few days to obtain the curdling effect in milk. There is also a tradition of keeping milk undisturbed for three to four days.
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Gundruk

Hawaijar (Fermented Soya)

Fermented bamboo shoot

Fermented Fish

Fermented Pork

Figure 3.1: Fermented food products collected for study from Assam
3.2 Isolation of lactic acid bacteria from samples:

Dahi was thoroughly mixed and one ml of this was inoculated into the MRS broth. The fermented bamboo shoot, kharoli, fermented beans, fermented fish and fermented pork were weighed one gram each and these were then crushed in one ml of sterile phosphate buffered saline pH 7.2 with mortar and pestle that were also autoclaved. The cells would have theoretically burst if the normality is not maintained during isolation of all the diluting buffers. Hence normal saline solution or phosphate buffered saline was used while isolation from the food products.

The crushed samples were then inoculated into the sterile Man de Rogosa and Sharpe (MRS) [Hi Media] broth medium. All the samples were left for overnight growth at 37 °Celsius. The next day a loop of the overnight grown cultures was used to observe gram staining of the mixed population. One ml of sample from each of the overnight cultures was inoculated into fresh 10 ml tubes of MRS broth at 37 °Celsius. Again a loop of the overnight cultures was used to observe gram staining of the mixed population.

These cultures were then pour plated at very low concentration as well as streak plated on the MRS agar in duplicates and incubated at 37 °Celsius for overnight growth to obtain individual colonies. Individual colonies from these plates were then picked up with loops, inoculated in 3 ml of MRS broth and incubated overnight at 37 °Celsius. These pure cultures were then used to determine the gram staining of the bacteria. All the Gram positive pure cultures were kept for further analysis and the remaining were discarded. An overnight lawn at 37° Celsius of the Gram positive cultures was prepared on the MRS agar plates and drops of 0.3% H₂O₂ was used to
determine the catalase test. Appearance of bubbles on application of H₂O₂ gave a positive test. These strains were then observed for sporulation.

All such samples which were found to be sporulating and positive for catalase test were discarded. Since the LAB group is a very big family of both motile and non-motile bacteria, we have considered gram positive, catalase negative and non-sporulating strains as probable lactic acid bacteria as literature review and Bergey's manual of determinative bacteriology. Various biochemical tests were performed to further characterize the bacterial isolates.
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Crushed Sample → MRS Broth → MRS Agar Overlaid with Soft MRS Agar

- Enlarged View

MRS Agar Overlaid with Soft MRS Agar

Cocci shaped LAB → Rods shaped LAB

Figure 3.2: Isolation of Lactic Acid Bacteria
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3.3 Growth of *Lactobacilli* in varying pH:

The *Lactobacilli* strains were grown in MRS broth, pH adjusted to 7.2, and then the overnight culture (approximately 16 hours) was centrifuged at 3000 X g to obtain a pellet of cells. This pellet was resuspended in 1 ml PBS buffer, pH adjusted to 7.2, and again centrifuged at 3000 X g to wash the cells. The washing step was repeated again. This pellet of cells was then resuspended in PBS, pH adjusted to 7.2, with the concentration of cells adjusted to obtain approximately $10^9$ CFU per ml by plating the cells.

Man de Rogosa and Sharpe (MRS) agar [Hi Media] plates were then prepared of varying pH from 7.2 to 2.0. These plates were kept overnight in the incubator to check for any contamination and then stored at 4 °C for further Use.

These plates with pH 7.2 were then inoculated with 0.1 ml of the stock prepared as mentioned above. The plates were incubated overnight at 37 °C and the colonies counted. This was taken as a base point. Again 0.1 ml of the stock was used to inoculate 0.9 ml of MRS broth of varying pH and incubated for varying time periods followed by 0.1 ml from each being spread plated on MRS agar plates with respective pH values. The plates were incubated overnight at 37 °C and the colonies counted.

The general ranges in common acceptance for countable numbers of colonies on a plate are 30 – 300 and 25 – 250. This was taken as a reference point for the consideration of plates bearing the colonies. The probability of countable colonies decreased with reducing pH, so at least two dilutions were used. The colonies from these were counted and a mean from them was considered. The plates with very low
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pH and increased time intervals which were either having no colonies or very low colony numbers inconsistent with varying dilutions and hence were discarded.

3.4 Growth of Lactobacilli in varying bile salt concentration:

After ingestion, probiotics must be able to survive the detergent properties of bile acids in the small intestine. The concerted efforts of the potential probiotic candidate are to efflux out the bile as much as possible before it is overcome and the cell gets damaged beyond repair.

The Lactobacilli strains were grown in MRS broth, pH adjusted to 7.2, and then the overnight culture (approximately 16 hours) was centrifuged at 3000 X g to obtain a pellet of cells. This pellet was resuspended in 1 ml PBS buffer, pH adjusted to 7.2, and again centrifuged at 3000 X g to wash the cells. The washing step was repeated again. This pellet of cells was then resuspended in PBS, pH adjusted to 7.2, with the concentration of cells adjusted to obtain approximately 0.5 McFarland.

MRS broth of pH 7.2 was prepared with varying concentrations of Ox Bile (Hi Media Laboratories -- CR010) ranging from 0.05% to 1% and aliquots of 5 ml were transferred in culture tubes. 0.1 ml of the above prepared 0.5 McFarland Lactobacilli strains were inoculated into the 5 ml aliquots of the MRS broth and growth observed as a measure of turbidity. The growth was observed overnight as well as until three days of inoculation.
3.5 Growth of *Lactobacilli* under antibiotic stress:

Different techniques have been used to assay the antibiotic susceptibility of *Lactobacilli*. But a method to study antimicrobial susceptibility of genus *Lactobacillus* has not been standardized yet. For all the isolates, antibiotic susceptibility testing was performed by using Kirby-Bauer disc diffusion method following CLSI guidelines (CLSI, 2007) with slight modification of using MRS agar media.

The *Lactobacilli* were grown overnight at 37°C in MRS broth. Then the broth was centrifuged to obtain a pellet of cells which was washed with PBS having pH adjusted to 7.2. The cellular pellet thus obtained was then diluted to 0.5 McFarland in Phosphate buffered saline, pH 2, before they were spread on MRS agar plates using sterile cotton swab to obtain an even lawn of the *Lactobacilli* test strain. Tests were initially done on nutrient media but the faint growth could not be gauged clearly. Hence nutritionally rich MRS agar plates were used to obtain a luxuriant growth where the effect of antibiotics could be easily deciphered.

Antibiotic discs were placed on the surface of the agar and the plates were incubated for 24 to 48 hours at 37°C. After the incubation, the diameter of the halos was measured.

Commercially produced antibiotics discs from HiMedia were used in the study. The antibiotics that were used in the study were:

i) Penicillin G (10 U/disc) – Mode of action involves inhibition of the cell wall synthesis
ii) Tetracycline (30 mcg/disc) -- Mode of action involves inhibition of protein synthesis

iii) Gentamycin (10 mcg/disc) -- Mode of action involves inhibition of protein synthesis

iv) Chloramphenicol (30 mcg/disc) -- Mode of action involves inhibition of protein synthesis

v) Ciprofloxacin (5 mcg/disc) -- Mode of action involves inhibition of nucleic acid synthesis

Any discreet data with consensus giving exact size of halo created by antibiotic disc for *Lactobacilli* could not be found. So we considered the Hi media chart of antibiogram (Sesitive=S, Intermediate=I, Resistant=R) for disc diffusion method for *Streptococci* other than *S. pneumoniae* (Chloramphenico-30 mcg, Penicillin G-10Units), *Streptococci* (Tetracycline-30mcg) and the general direction (Gentamycin-10mcg, Ciprofloxacin-5mcg) when data for the former was not given. We considered the following data for our analysis; Chlorampenicol -- R=17, I=18-20, S=21mm or more, Ciprofloxacin -- R=15, I=16-20, S=21mm or more, Gentamycin -- R=12, I=13-14, S=15mm or more, Penicillin G -- R=19, 20-27, S=28mm or more, and Tetracycline -- R=18, I=19-22, S=23mm or more.

3.6 Antipathogenic activity of *Lactobacilli* against *Salmonella enterica* and *E. coli*:

Clinically pathogenic strains of *E.coli* and *Salmonella enterica* were used to detect the antipathogenic activity of the *Lactobacillus* strains against them. The pathogens,
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*E. coli* and *Salmonella*, were grown overnight in MacConkey Broth (HiMedia: MH083) and Salmonella Enrichment Broth (HiMedia: MH1491) to 0.5 McFarland before they were spread on nutrient agar plates using sterile cotton swab to obtain an even lawn of the pathogenic bacteria. Then either a well (approximately 3 mm) was made where the overnight grown *Lactobacilli* were inoculated or sterile filter paper discs (approximately 3 mm) dipped in the *Lactobacilli* culture was overlaid on the lawn. These were then examined after being left overnight at 37°C and after 2nd and 3rd days of incubation.

The other methods employed initially to observe the antipathogenic activity against the clinically pathogenic strains of *E. coli* and *Salmonella* included the streaking of the pathogenic stain in a line with a sterile cotton swab and then putting the test *Lactobacilli* strain as another line against it. Another method employed was making a lawn of pathogenic strains of *E. coli* and *Salmonella* on nutrient agar plates and putting lines of the test strains of *Lactobacilli*.

The size of the zones of inhibitions has been calculated by subtracting the disc size or the size of the punched out wells (3 mm). A table of data has been recorded for the inhibition produced for all *Lactobacilli* that showed growth in pH 4.5. The table for the size of zones of inhibition have been made for the seven potential probiotic strains selected for study.
3.7 Molecular characterization of \textit{Lactobacilli}:

Seven strains of \textit{Lactobacilli} showed comparatively good growth in low pH of 2 thereby making them potential probiotic candidates of study that can withstand the gastric environment. Aliquots of 10 ml pure overnight culture (approximately 16 hrs) of the seven strains in MRS broth (\textit{Lactobacillus} MRS broth – M369, Hi-Media Laboratories, India) were centrifuged at 3000 X g and the pellet obtained was washed twice in PBS and then sonicated (Omni Sonic Ruptor Ultrasonic Homogenizer, 250 watts, Omni International, GA, US) at low intensity to just lyse the cells in 1ml of sterile ultra-pure water (obtained from TKA water pure systems).

The lysate was used to isolate the DNA by the standard phenol chloroform method as described by Maniatis \textit{et al.} This DNA was checked for integrity on 0.8% agarose gel.

This DNA was checked for integrity on 0.8% agarose gel. Then primers specific for the variable region of 16S ribosomal RNA gene V1 were used for the amplification of the 5' end (approximately 300 bases) of the 16S rRNA gene. These were

5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GTCTCAGTCCCAATGTGGCC-3'.

The PCR program consisted of 5 min at 94°C, followed by 30 cycles of 1min at 94°C, 2 min at 61°C, and 2 min at 72°C and then 5 min at 72°C.

The intergenic transcribed spacer (ITS) region between the 16S and 23S rRNA genes were amplified using 5'-GAAGTCGTAACAAGG-3' and 5'-GGTTTCCCCATTCGGA-3'.

Both these sets of primers were published by Plengvidhya \textit{et al.} in Applied and Environmental Microbiology, Dec. 2007, p. 7697–7702.
This ITS-PCR product was then digested with 5 units of Rsal enzyme at 37°C and the pattern obtained was correlated with RFLP data provided in the above mentioned publication. The variable region was also sequenced and BLAST analysed at PUBMED. These showed that three of the samples were *L. plantarum* and four of them were *L. fermentum*. A multiple alignment data was obtained using MultAlin software available free on the internet and the data was used to generate a phylogenetic tree using the Clustal-W software, also freely available on the net. The phylogenetic tree shows the formation of two specific groups, one of the *L. plantarum* and the other of the *L. fermentum* with slight variations among the strains of each type.

### 3.8 Immune Modulation by *Lactobacilli* in Mice:

#### 3.8.1 Animals used in study:

Animals selected for study was of the average group of 4-6 weeks old and of 16-20 gm of weight. The animals were of the Swiss albino group of mice. They were fed standard diet and kept in clean and dry cages without overcrowding with 12 hrs of light and dark period. All experiments related to animals were done after due permission from the animal ethical committee of the institution (Gauhati University) and by following the guidelines laid down thereupon.

#### 3.8.2 *Lactobacilli* administration to mice and sample collection:

The mice were injected intraperitoneally with lysate samples of *E. coli, L. plantarum* and *L. fermentum* diluted to 0.5 Mc Farland in normal saline individually and with normal saline acting as control.
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Each group composed of 18 animals with 3 animals from each group being sacrificed on each day of study, viz., 5th, 10th, 15th, 20th, 30th, and 40th day. Blood was drawn by puncturing the heart and transferred to Trizol reagent immediately for processing. Spleen and liver were kept in RNA later and then stored in -20°C.

A part of Liver and spleen tissue were kept for histopatopatology by fixing the tissue in 4% formalin solution. Blocks were made from these tissue in paraffin and sections were cut using the microtome and slides were prepared from these for H & E (Hematoxylin and Eosin) staining.
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**Drawing of Blood from Mice**

**Figure 3.3:** Sacrificing of animal and sample collection

*Mice under the effect of chloroform*  
*Dissection of Mice*  

*Drawing of Blood from Mice*
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Animals were first put under the effect of chloroform so that they become unconscious and then the procedure to obtain samples was done.

3.8.3 Haemotoxylin and eosin staining:
The histopathological study was performed for the mice of each group by obtaining the liver from the sacrificed mice. The liver from the mice were removed and then the tissue fixation was done by keeping in 10% formalin solution. Then this was used for making the paraffin blocks for saving the tissue and for further processing. The following protocol was employed for haemotoxylin and eosin staining.

1. DEPARAFFINIZE THE SECTION:
Flame the slide on a burner and then place in xylene for 5 min (2 changes)

2. HYDRATION:
Absolute alcohol: 2 min
90% alcohol: 2 min
70% alcohol: 2 min
50% alcohol: 2 min
Running water: 2 min
Dry the section before staining

3. STAINING:
Hematoxylin: 10 min
Running water: 10 min
1% acid alcohol: 1-2 dips
Running water: 5-10 min
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Eosin: 2 min

4. DEHYDRATION:

70% alcohol: 1 min
90% alcohol: 1 min
Absolute alcohol: 1 min

5. CLEARING:

Xylene 2 min (2 changes)

6. MOUNTING: with Dpx

3.8.4 Primers for Real Time PCR:

Primers were selected from published literature and verified online through freely available bioinformatics tools on the internet. The gene accession numbers against the primers have noted for verification. (Yang et al., 2006)

IL-2: primers -- (accession no. X01772);
forward --5' - CCC AAG CAG GCC ACA GAA TTG AAA-3' and
reverse --5' - AGT CAA ATC CAG AAC ATG CCG CAG-3'

IFN-γ: primers -- (accession no. M28381);
forward 5' - AGA GGA TGG TTT GCA TCT GGG TCA-3' and
reverse 5' - ACA ACG CTA TGC AGC TTG TTC GTG-3'

IL-4: primers -- (accession no. NM_021283);
Forward - 5' - AGA TGG ATG TGC CAA ACG TCC TCA-3' and
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Reverse - 5'- AAT ATG CGA AGC ACC TTG GAA GCC-3'

IL-10: primers — (accession no. M37897)
forward-5'- GGA CAA CAT ACT GCT AAC CGA C-3' and
reverse - 5'- TGG ATC ATT TCC GAT AAG GCT TG-3'

Endogenous control -- β-actin
Forward - 5'-' AAG TGT GAC GTT GAC ATC CGT AA- 3' and
Reverse - 5'- TGC CTG GGT ACA TGG TGG TA-3'

3.8.5 RNA extraction from the tissue and blood:
RNA was extracted from liver tissue and blood using trizol method. This RNA was stored at -20° c and further used to prepare cDNA. RNA from was also done in almost same manner with the exception that it does not need to be crushed severely as the cells (WBCs) are already separate individuals.

Principle: The trizol method involves use of trizol reagent which maintains intricity of the RNA while disrupting cell and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution in to aqueous phase and organic phase. RNA remains extensively in aqueous phase. After transfer of the aqueous phase RNA is recovered by precipitation with isopropanol.

Chemicals used:
1. Trizol reagent
2. Chloroform
3. Isopropanol
4. Ethanol
5. Liquid nitrogen
6. DEPC treated water or Nuclease free water.

Procedure for RNA isolation

A small bit of tissue was taken in a mortar, pestled with a little amount of liquid nitrogen

The tissue was crushed

1 ml Trizol was added (waited for a minute)

Crushed again

Waited and retained in room temperature for 5 minutes

280µl of chloroform was added, incubated for 15 minutes at room temperature

Centrifuged at 13,000 rpm for 15 minutes at 4°C

Upper aqueous phase was taken in fresh eppendorf tube
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600μl of ice cold isopropanol was added

\[ \downarrow \]

Incubated at -20°C for 20 minutes

\[ \downarrow \]

Centrifuged at 13,000 rpm for 20 minutes

\[ \downarrow \]

Washed with 500μl 70% ethanol

\[ \downarrow \]

Centrifuged at 13,000 rpm for 10 minutes at 4°C

\[ \downarrow \]

Alcohol was discarded

\[ \downarrow \]

Pellet was air dried

\[ \downarrow \]

DEPC treated H₂O was added
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**Agarose gel electrophoresis of the extracted RNA**

To check the quality of the RNA extracted from tissue sample it was run on the agarose gel with 1.5 % concentration.

**Chemicals used:**

1. Agarose gel
2. Staining solution
3. 6X gel loading buffer
4. Electrophoresis buffer (TAE)

**Procedure:**

The edges of a of a clean dry open ends of a plastic tray was sealed with tape to form a mold and an appropriate comb was inserted for forming a sample slot in the gel.

A solution of agarose in electrophoresis buffer is prepared at a concentration appropriate for separating the particular size of the fragments.

Agarose gel was cast by melting the agarose in the presence of the desired buffer until a clear transparent solution was obtained.

To the agarose 0.5µl of EtBr was added allowed to cool.
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The warm agarose solution was poured into the mold

Carefully the comb and tape was removed from the mold

The gel was mounted in electrophoresis tank

The sample of RNA was mixed with 0.20 volumes of desired 6X gel loading buffer

Slowly the sample was loaded into the slots of the submerged gel using the micropipette

**3.8.6 cDNA preparation:**

Intact, pure poly (A+) RNA is essential for the synthesis of high-quality cDNA. The cDNA is then used further in mRNA expression analysis. We performed the cDNA synthesis using following procedure/protocol. Table -3.5.3 shows master mix1 and master mix2 for cDNA preparation.
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Mastermix1:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>6 µl</td>
</tr>
<tr>
<td>MQ H2O</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Random hexamer</td>
<td>1 µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNAse inhibitor</td>
<td>1 µl</td>
</tr>
</tbody>
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Blank

Mastermix2:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>dTT</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

Master mix1

↓

PCR condition 70°C for 10 minutes (incubate)

↓

Snap freeze

↓

Master mix2

↓

PCR condition 25°C for 15 minutes
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Snap freeze

↓

2μl RT (20unit/μl) was added

↓

PCR condition (42°C-1hr/72-10minutes/4°C-hold)

↓

cDNA (stored at 20°C)

3.8.7 Real-time PCR based gene amplification:

SYBR Green-based real time PCR was conducted for target gene mRNA. Briefly, SYBR Green Master mix (Applied Biosystems, Foster City, CA), primers, and template were mixed in 10μl reaction. An Applied Biosystems Step up-1 Real Time PCR Systems machine was used with the following amplification conditions. Samples were run in duplicate. Levels of mRNA were calculated using the $2^{ΔΔCT}$ method by expressing mRNA in case of fibrosis relative to control after normalizing to β-actin. Primers were used in a final concentration of 10 pico-Moles.

B- actin:

PCR conditions: Real Time PCR Systems machine was used with the following amplification conditions: 95°C for 5min; 95°C for 30s followed by 40cycles 59°C for 30sec; and a final dissociation step at 72 for 45sec. Samples were run in duplicate. Melt curve stage was for 95°C for 15 sec; 60°C for 1 min and 95°C for 15 sec.
**Material & Methods**

Table 3.1: *Reaction mixture for real-time PCR analysis for β-actin gene amplification:*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>volume for 10µl mix</th>
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</thead>
<tbody>
<tr>
<td>2X SYBR green master mix</td>
<td>5µl</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3.8 µl</td>
</tr>
<tr>
<td>Template(cDNA)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Table 3.2: *Reaction mixture for real-time PCR analysis for gene amplification:*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>volume for 10µl mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SYBR green master mix</td>
<td>5µl</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3.8 µl</td>
</tr>
<tr>
<td>Template(cDNA)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10µl</td>
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</table>