MATERIALS AND METHODS

Enumeration of bacteria

Air Sampling

Two sampling spots at four different slaughter houses (Russel market, Tannery market, K.R market, Johnson market in Bangalore city were selected for air sampling. The air sampling were done from procurement area and degutting area at a height of around 1 to 1.5 meter above the floor.

Two plates containing 25 ml of sterilized non selective media Plate Count Agar (PCA) were exposed in the sampling spots for a period of 10 minutes and transferred to the laboratory aseptically. Exposed plates were incubated at 37°C for 24-48 hours. Sterile conditions were maintained during all microbiological analysis in order to prevent the contamination of samples and the isolates obtained from the air samples.

Water Sampling

Four different slaughter houses (Russel market, Tannery market, K.R market, Johnson market) in Bangalore city were selected for water sampling analysis. Water samples were collected in sterile glass bottles from the above mentioned locations before slaughtering and after slaughtering and transported to laboratory in sterile conditions. Serial dilutions was carried out by transferring 1ml of the $10^{-1}$ dilution to the $2^{nd}$ tube which contained 9ml of sterile water to get $10^{-2}$ dilution.
Dilutions were made from $10^{-1}$ to $10^{-5}$. These dilutions were used for the isolation of microorganisms.

**Sample collection**

A total of 84 (seven from each market) raw meat samples (in triplicates) were collected from market places, Russel, Tannery, K.R. and Johnson markets in Bangalore. Samples were collected within 12 hours post-slaughter and during late morning, in order to minimize the microbial changes due to environmental temperatures and post-slaughter timings. Samples were collected throughout the year 2009-10 (once in three month), in sterile plastic bags and kept at 4 °C until further analysis. Whereas, 84 (seven from each markets) finished meat products (in triplicates) were collected from markets places, Russel, Tannery, K.R. and Johnson markets in Bangalore. Samples were collected at the end of the day i.e., during late hours of evening, in order to access the microbial changes due to storage temperatures and hygiene conditions. Samples were collected throughout the year 2010-11 (once in three months), in plastic bags and kept at 4 °C until analysis. Collected raw meat and finished meat products were subjected for bacterial population analysis by serial dilution method. Furthermore, these isolates were analyzed for biochemical and molecular characterization.
Isolation of Bacteria from Raw meat and Finished Meat Products

Raw mutton samples such as Mutton, Sheep Liver, Minced Mutton, Sheep Lungs, Sheep Head, Sheep Intestine & Sheep Feet and finished products viz Mutton Samosa, Mutton Cubes, Mutton Kofta, Mutton Burger, Mutton Sheek, Mutton dried kabab & Mutton briyani were collected from four different markets (Russel Market, Tannery Market, K.R Market and Johnson Market) in Bangalore, using sterile plastic bag and transported to laboratory in aseptic conditions.

Serial dilution

The microbial populations in the above collected samples were quantitatively enumerated by standard serial dilution method. Five test tubes with 10 ml sterile distilled water and 4 other with 9ml sterile distilled water each, were taken.

1 gm of each collected samples (Raw meat) was taken and homogenized with 10ml of sterile distilled water. 1 gm of each collected samples (Finished product) was taken and homogenized with 10ml of sterile distilled water.

Serial dilutions was carried out by transferring 1ml of the $10^{-1}$ dilution to the $2^{nd}$ tube which contains 9ml of sterile water to get $10^{-2}$ dilution. Dilutions were made from $10^{-1}$ to $10^{-5}$.These dilutions were used for the isolation of microorganisms.
Spread plate method

Using the spread plate method, a small volume of bacterial suspension was distributed evenly over the surface of an agar plate. 200ml of nutrient agar was prepared in a conical flask and autoclaved. After sterilization, the media was poured into the five sterile petriplates and was allowed to solidify. After solidification, 0.1 ml of each dilution (10^{-1} to 10^{-5}) of all samples was taken and spread on nutrient agar media using a sterile L-rod by standard spread plate technique. Inoculated plates were incubated at 37°C for 24-48 hours.

Cultural characteristics of the microorganism

1) Colony Characteristics

Determination of the morphology of a single colony growing on the surface of a plate culture is considered as an important tool in the description and identification of microorganisms.

Morphological characteristics such as form, elevation, margin, pigment production, opacity, text and the colony size were observed for the individual colony.

2) Identification by Gram’s staining

The Gram staining was used as the initial characterization and classification of bacteria. It was used as a key procedure in the identification of bacteria based on staining characteristics, morphology
and arrangement using a light microscope. All the isolated individual colonies were selected and Gram’s staining was performed.

1. A loop of the colonies were transferred with one drop of water and spread by circular motion of sterile inoculating loop to make uniform bacterial smear, air dried and then gently heat fixed with indirect flame.

2. The slides were put on staining tray and the smear was flooded with the primary stain ‘crystal violet dye’ for 1 minute, rinsed with direct stream of tap water and dried.

3. Then the mordant grams iodine was added to the smear and kept for 1 minute, and washed gently with indirect tap water.

4. The slides were decolorized with 95% ethanol for 30 seconds and blot dried.

5. Slides were then counter stained with safranin dye for 2 minutes and air dried.

6. The slides were examined under 40X light microscope.

**Pure culture and maintenance**

The isolated culture was maintained by pure culture methods following the procedure outlined here under

1) Streak plate method

2) Nutrient slant preparation
1) **Streak plate method**

100ml of nutrient agar was prepared in a conical flask and autoclaved. The media was then poured into the sterile petriplates and allowed to solidify. After solidification, a loop full of culture was taken and streaked onto the media for the isolation of individual pure single colonies and incubated at 37°C for 24 hr in an inverted position.

2) **Nutrient slant preparation**

50ml Nutrient agar was prepared and taken into five clean test tubes and autoclaved. The tubes were then kept in a slanting position and allowed to solidify. A loop full of individual isolated pure colonies was taken and subcultured on the slant. This was incubated at 37°C for 24hr and observed for the growth. Well grown culture was stored at 4°C for further studies.

**Biochemical characteristics**

On the basis of *Bergey’s Manual of Determinative Bacteriology* different media were used for the biochemical characterization of the isolated and selected bacteria for their identification (Robert et al. 1957).
Starch hydrolysis

\[ \text{Starch} \xrightarrow{\text{Amylase}} \text{Maltose} \xrightarrow{\text{Maltase}} \text{glucose} \]
**Procedure**
Sterile starch agar plates were prepared.
A single line of streak of the organisms was made across the centre of the starch agar plate.
The plates were incubated at 37°C for 24 hours.
After incubation the plate was flooded with gram’s iodine solution.
Hydrolysis was indicated by clear zone formation around the microbial growth and unchanged starch gives as brown color.

**Voges-Proskauer (VP) Test**

\[ O_2 \]

[Glucose → Acetoin → Diacetyl → Red Dye]

KOH  \[ α\text{-naphthol} \]

**Procedure**
Isolated bacteria was inoculated into MR/VP broth and incubated for 48 hours.
0.6 ml of alpha-naphthol was added to the test broth and shaken.
0.2 ml of 40% KOH was added to the broth, shaken and allowed to stand for 15 minutes.
Appearance of red color was taken as a positive test.
The negative tests were kept for one hour for confirmation (since maximum color development occurs within one hour after addition of reagents).

**Citrate Utilization Test**

\[
\text{citrate} \rightarrow \text{citrate permease} \rightarrow \text{citrate inside cell} \\
\text{citric acid} \rightarrow \text{citrase} \rightarrow \text{oxaloacetic acid} \\
\text{CH}_3\text{COOH} + \text{CHOOH} \rightarrow \text{acetic acid} + \text{formic acid} \\
\text{alkaline pH} \rightarrow \text{pyruvic acid} \\
\text{CO}_2 + \text{2Na}^+ + \text{H}_2\text{O} \rightarrow \text{Na}_2\text{CO}_3 \text{ (Alkaline pH)}
\]

**Alkaline pH**

- Bromothymol Blue \rightarrow Green to Prussian Blue
Procedure

Bacterial colonies were inoculated into slope of Simmon’s citrate agar slant.

They were incubated at 37°C for 72-96 hours.

The change in the colour of the medium from green to blue indicated the positive test for citrate utilization.

Indole production test

\[
\text{H}_2\text{O} + \text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{Pyruvate} + \text{Ammonium}
\]

Tryptophan undergoes deamination and hydrolysis by bacteria that show tryptophanase activity.

Tryptophan + water = indole + pyruvic acid + ammonia

The prime requirement for this test was the presence of tryptophan in the medium. The presence of indole with the microbe grown in the medium demonstrates the capacity to degrade tryptophan. Production of indole depends upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye rosindole.
**Procedure**

Tryptone broth was prepared in test tube and autoclaved.
Sterilized tryptone broth was inoculated with a small amount of the pure culture and incubated at 35°C for 24-48 hours.
To test for indole production, 5 drop of Kovacs reagent was added directly to the tube.
A positive indole test was indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent.

**Methyl red test**

![Chemical equation and diagram]

**Procedure**

20ml of MR-VP broth was prepared in test tubes and autoclaved at 121°C for 15 minutes.
Sterilized MR-VP broth was inoculated with a loop full of the pure culture and incubated at 37°C for 48 hrs.
After incubation, 3-5 drops of Methyl Red was added to each broth including the control. After the addition of methyl red indicator the tubes were observed for colour change which turns red from yellow at pH <4.0. Red color indicated the mixed acid fermentation.

**Catalase test**

\[2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2\]
Procedure
The sterile clean slides were made grease free using alcohol. A loop of the colonies from each culture were transferred on to the slide and spread by circular motion of sterile inoculating loop to make uniform bacterial smear. The bacterial smear was allowed to air dry and then gently heat fixed with indirect flame. Using a Pasteur pipette, 1-2 drop of 3% $\text{H}_2\text{O}_2$ was added onto the organism and bubble formation was observed as the positive result.

Oxidase test

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase activity. In the presence of an organism with cytochrome oxidase activity, the reduced colorless reagent becomes an oxidized dark blue colored product.
Procedure

Filter Paper Spot Method
A loop full fresh 18 to 24 hour culture of isolated individual colonies were picked and spread onto a small piece of filter paper.
Colonies in the filter paper was covered with 1 or 2 drops of 1% Gordon and McLeod oxidase reagent.
Oxidase positive microorganisms changed the color to dark purple within 60 to 90 seconds.

Urease test

Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{NH}_2 \\
\text{C} & \quad \rightarrow \quad \text{urease} \\
\text{H}_2\text{O} & \quad \rightarrow \quad 2\text{NH}_3 + \text{CO}_2
\end{align*}
\]

\[(\text{NH}_2)_2\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3\]

Urease test media contained 2% urea and phenol red indicator.

Procedure

Christensen's Urea Agar was prepared and autoclaved at 121°C for 15 minutes.
A full loop of fresh 18- to 24-hour inoculum from pure culture was streaked on to the entire slant of christensen's urea agar with a color control.
Inoculated tubes were incubated at 37°C for 2-6 days. Positive results were with appearance of bright pink colour at 6 hr, 24 hr everyday was observed upto 6 days.

**Nitrate reduction test**

\[
2e^- + 2H^+ + NO_3^- \rightarrow NO_2^- + H_2O
\]
Nitrate reduced to nitrite

Procedure

Nitrate reduction medium was prepared in test tubes having Durham tubes and autoclaved at 121°C for 15 minutes. Sterilized medium was inoculated with the heavy loop of the inoculum from well-isolated colonies of the test organism along with the control and incubated at 37°C for 24 to 48 hr. The culture was tested for reduction of the substrate observed for gas production in the Durham tube. 2ml of each reagent A and B were mixed in a test tube with broth culture and mixed well. If the test organism has reduced the NO$_3^-$ to NO$_2^-$, a red color appears within 2 minutes, indicating the presence of NO$_2^-$ in the tube.

Caesin Hydrolysis test

Proteins as macromolecules, cannot be directly taken up by the microorganisms into their cells. Thus the bacterium produces extracellular caesinase to cleave the peptide linkage of protein releasing the amino acid residues. The formation of zone on utilizing casein indicates the proteolysis process.
Procedure

The identified individual bacterial isolates were screened for caseinase production by spot inoculating onto the skim milk agar plates. Incubation was carried out at 37°C for 48 hours. Proteolytic activities of the isolated organisms were detected by clear zone of hydrolysis.

A clear zone of casein hydrolysis gave an indication of an extracellular secretion of caseinase by the organisms.

Motility test

Motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms. Motility in bacteria can be provided by a variety of mechanisms, but the most common involve flagella. The presence of flagella occurs primarily in bacilli but there are a few flagellated cocci, thus motility is a very important means of identification in the family Enterobacteriaceae. Motility test medium with triphenyltetrazolium chloride provides an easy method for determining
motility. TTC in its oxidized form is colorless. As bacteria grow in the presence of TTC, the dye is absorbed into the bacterial cells where it is reduced to the insoluble red-colored pigment formazan. Growth is indicated by the presence of the red color, and as motility occurs, small to very large regions of color can be observed around the area of inoculation.

**Procedure**

The medium for motility test was prepared in test tubes and autoclaved at 121°C for 15 minutes.

To test for motility, using a sterile needle a well-isolated colony was picked up and stabbed into the medium till 1 cm from the bottom of the tube.

It was incubated at 37°C for 18 hours or until growth was evident

A positive motility test was indicated by a red turbid area extending away from the line of inoculation.

**Gelatin hydrolysis test**

Gelatin is a protein derived from the connective tissues of vertebrates, that is, collagen. It is produced when collagen is boiled in water. Gelatin hydrolysis detects the presence of gelatinases. Gelatinases are proteases secreted extracellularly by some bacteria which hydrolyze or digest gelatin. This process takes place in two sequential reactions
Gelatin: \(-\text{Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-}\)

Extracellular fluid \(\rightarrow\) gelatinase: breaks peptide bonds between amino acids.

Amino Acids: Ala Gly Pro Arg Gly Glu 4Hyp Gly Pro

Bacterial Cell

\begin{align*}
\text{Gly} & \quad \text{Pro} \\
\text{Respiration or fermentation} & \quad \text{Citric Acid cycle}
\end{align*}

In the first reaction, gelatinases degrade gelatin to polypeptides (6).

\[
\text{H}_2\text{O} \quad \text{ASM MicrobeLibrary} \odot \text{ dela Cruz and Torres}
\]

\[
\text{Gelatin} \quad \text{Gelatinase} \quad \text{Polypeptides}
\]

Then, the polypeptides are further converted into amino acids (6).

\[
\text{H}_2\text{O} \quad \text{ASM MicrobeLibrary} \odot \text{ dela Cruz and Torres}
\]

\[
\text{Polypeptides} \quad \text{Gelatinase} \quad \text{Amino Acids}
\]
**Procedure**

A heavy inoculum of 18 to 24 hour-old test bacteria was stab-inoculated into tubes containing sterile nutrient gelatin. The inoculated tubes and an uninoculated control tube were incubated at 25°C for up to 1 week and checked every day for gelatin liquefaction. The tubes were immersed in an ice bath for 15 to 30 min. Afterwards, the tubes were tilted in an angle to observe if gelatin was being hydrolyzed. The positive result indicated liquification of the medium after exposure to cold temperature (ice bath), while the uninoculated control medium remained in solid form.

**Triple sugar iron (TSI) test**

Triple sugar iron (TSI) agar is a tubed differential medium used in determining carbohydrate fermentation and H2S production. TSI differentiates bacteria based on their fermentation of lactose, glucose, sucrose and on the production of hydrogen sulfide. TSI is most frequently used in the identification of the Enterobacteriaceae, although it is useful for other gram-negative bacteria.

![Chemical Reaction](image)
**Procedure:**

TSI agar test medium was prepared in test tubes and autoclaved at 121°C for 15 minutes.
To test for sugar fermentation, using a sterile needle a well-isolated colony was picked up and stabbed into the medium till the bottom of the tube and streaked till the top of the tube.
It was incubated at 37°C for 48 hours or until growth is evident
Positive result was observed with a change in red to yellow.

**Mannitol salt agar**

Mannitol salt agar (MSA) is both a selective and differential medium used in the isolation of staphylococci. It contains 7.5% sodium chloride and thus selects for those bacteria which can tolerate high salt concentrations. MSA also distinguishes bacteria based on the ability to ferment the sugar mannitol, the only carbohydrate in the medium.

**Procedure**

Mannitol salt agar was prepared and autoclaved at 121°C for 15 minutes.
Sterile autoclaved media was poured into the plates and allowed to solidify.
A plate of mannitol salt agar was streaked with appropriate culture using the quadrant streak plate method to obtain isolated colonies. Well-isolated colonies provided the best results in the biochemical differentiation in bacteria using MSA.
Positive results were observed with the change in colour from pink to yellow.
**Bile Esculin test**

Esculin is a glycoside composed of glucose and esculetin. Many bacteria can enzymatically hydrolyze esculin, but a few can do so in the presence of bile. Organisms that hydrolyze esculin molecules and use the liberated glucose as the energy source, release esculetin into the medium. The free esculetin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar slants from dark brown to black.

![Chemical Reaction Diagram](image)

**Procedure**

Bile Esculin test medium was prepared in test tubes and autoclaved at 121°C for 15 minutes.

Using a sterile inoculation loop, a well-isolated colony was picked up and inoculated into the agar slants.

It was incubated for 18-24 hours at 37°C in an aerobic atmosphere.

Positive results were observed for the change in colour from dark brown to black.
cAMP test

**Procedure**

5% sheep blood agar was prepared and autoclaved at 121°C for 15 minutes. Sterilized blood agar was poured into the petriplates and allowed to solidify. Using sterile inoculation loop, a good loop of well grown culture was streaked onto the plates. The positive control organism was streaked parallel to and approximately 1 in from the unknown organism. It was incubated for overnight at 35°C.
Coagulase test

The tube coagulase test is performed by mixing bacterial cells into a larger volume of plasma in a small test tube. As the bacteria multiply in the plasma, they secrete staphylocoagulase. Staphylocoagulase initiates blood coagulation by activating prothrombin, Staphylocoagulase adheres to fibrinogen, forming a complex that cleaves fibrinogen into fibrin, bypassing the blood clotting cascade and directly causing a clot of fibrin. Formation of a clot will be noted within 24 hours for a positive response.

Procedure

Using the pipette, aseptically transferred 0.5 ml of the reconstituted plasma into the sterile test tube.
Selected colonies of the bacteria were collected using the sterile loop.
Emulsification of the bacteria in the 0.5 ml of plasma was carried out and placed in the incubator at 37°C.
It was observed at intervals over the next 4 hours for evidence of the clot.
Clot formation was the positive result.

DNA Isolation

For isolation of genomic DNA, by SDS the cell membrane was disrupted in order to release the DNA in the extraction buffer. DNA thus is protected from endogenous nucleases by chelating Mg\(^{2+}\) ions using EDTA. Phenol and chloroform were used to denature and separate proteins from DNA. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold ethanol or isopropanol.
**Procedure**

1. Isolated and identified bacterial cultures were inoculated overnight in Nutrient rich broth.
2. 2 ml of the well grown culture was transferred to a centrifuge tube and centrifuged at 6000rpm for 2 min.
3. The supernatant was decanted.
4. The pellet was collected and resuspend in 467 μl TE buffer.
5. 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K was mixed and incubated for 1 hr at 37 °C.
6. Equal volume of phenol-chloroform mixture was added and mixed gently to avoid shearing the DNA by inverting the tube until the phases are completely mixed at 10000rpm for 10 min. at 4°C.
7. Transferred the upper aqueous layer to a fresh tube and again an equal volume of phenol-chloroform mixture was added.
8. Transferred the upper aqueous phase to a fresh tube and added 1/10 volume of sodium acetate.
9. The mixture was centrifuged at 10000rpm for 10 min. at 4°C.
10. To the supernatant 0.6 ml of isopropanol was added and mixed gently until the DNA precipitated.
11. Pooled DNA was washed gently in 1 ml of 70% ethanol for 30 sec followed by centrifugation at 10000rpm for 10 min. at 4°C.
12. The pellet was resuspended with 200 μl TE buffer and stored at 4°C.
DNA Quantification by Nanodrop

Nucleic acids absorb light at a wavelength of 260 nm. If a 260nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For double-stranded DNA, an Optical Density (OD) of 1 at 260 nm correlates to a DNA concentration of 5ng/μl, so DNA concentration can be easily calculated from OD measurements. 2 μl of extracted genomics DNA was taken on to the plates of nano drop and buffer was taken as control. Then the OD was measured at 260 nm.

PCR amplification of 16S rRNA gene

16S rRNA gene was amplified by using following primers; 27F AGAGTTTGATCMTGGCTCAG, 1492RTACG GYTACCTTGTTACGACTT, 518F CCAGCAGCCGCGGTAATACG, 800R TACGGGTATCCT AATCC and the amplified 1.5 kbp gene fragment DNA was purified by running on the agarose gel.

Gel electrophoresis

Agarose gel electrophoresis is a procedure used to separated DNA fragments based on their molecular weight.
The technique consisted of 3 basic steps:
1. Preparation of agarose gel
2. Electrophoresis of DNA fragments
3. Visualization of DNA fragments.
Preparation of Agarose Gel

Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution is then poured into a mould and allowed to solidify. As it cools, agarose undergoes polymerization i.e. sugar polymers cross-link with each other and cause the solution to gel, the density or pore size of which is determined by concentration of agarose.

Electrophoresis of DNA fragments

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates towards the anode. Migration of DNA through the gel is dependent upon

- Molecular size of DNA
- Agarose concentration
- Conformation of DNA
- Applied current

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps the movements of larger DNA fragments as the spaces between the cross-linked molecules is more. The progress of gel electrophoresis is
monitored by observing the migration of a visible dye (tracking dye) through the gel. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.

**Visualization of DNA fragments**

Since DNA is not naturally colored, it will not be visible on the gel. Hence the gel, is stained with a dye ethidium bromide to the DNA. An intercalating dye like ethidium bromide is added to agarose gel and location of bands determined by examining the gel under UV trans illuminator.

**Procedure**

1. Prepared 1X TAE by diluting appropriate amount of 50X TAE buffer.
2. 0.4g of agarose was taken and to that was added 50ml of 1X TAE.
3. The solution was kept on a boiling water bath to obtain a clear solution.
4. The combs of electrophoresis unit was set such that it is approximately 2 cm away from the cathode.
5. Added 10μl ethidium bromide per 100ml of agarose gel, once the temperature of the agarose solution reached 60°C.
6. Poured the agarose gel in the central part of the tank gently to avoid fermentation of air bubble.
7. The gel was kept at room temperature and allowed to solidify.
8. Poured 1X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
9. Gently lifted the combs, ensuring that wells remain intact.
10. The power supply to the electrophoretic unit was connected as and set to 60V.

**DNA sequencing**

The 16S rRNA sequences of the isolated DNA was carried out at “Applied Biosystems, Bangalore”.

**Safety measures**

**Cold Storage**

100g of raw meat samples (Raw Mutton, Sheep liver, Minced Mutton, Sheep Lungs, Sheep Head, Sheep Intestine, Sheep Feet) were taken and kept at different temperatures (-5°C, -10°C, -15°C, -20°C) for cold storage preservation. 10g of each raw meat samples in triplicates were taken for the bacterial contamination through serial dilution method. Samples were collected at different intervals such as 0 day, 5 day, 10 day, 15 day.

**Pickling**

100g of each raw meat samples (Raw Mutton, Sheep liver, Minced Mutton, Sheep Lungs, Sheep Head, Sheep Intestine, Sheep Feet) were taken for pickling using 2%, 4%, 6%, 8% salt. 10g of each raw meat samples were taken for bacterial contamination using serial dilution method. Samples were collected at different intervals Viz: 6hrs, 12hrs,
18hrs, and 24hrs. Subsequently, the isolated bacterial colonies were subjected to 16S rRNA gene amplification and sequencing. Identification of bacteria was done by 16S rRNA gene sequence through BLAST analysis (Azhar & Sarangi, 2013).

**Combinations of cold storage and pickling**

In order to investigate the combination effect of pickling and cold temperature on the meat preservation, raw meat samples were taken and kept under following combinations; 4 °C + 2% salt; 4 °C + 4% salt; 4 °C + 6% salt and 4 °C + 8% salt in triplicates. After 35 hr of incubation, meat samples were taken for analysis of bacterial contamination.

**Statistical Analysis**

Microsoft word was used to draw the tables. Microsoft Excel was used to plot the graph with error Bars.