CHAPTER – V
**SUMMARY**

*E. coli* Nissle 1917 played a major role in probiotic field after *Lactobacillus*. *E. coli* Nissle1917 is one of the oldest, most well-characterised probiotic agents and has shown promising results in treatment of various intestinal diseases. *E. coli* can survive extreme acid stress conditions. Proteomic studies of gut microflora explain the molecular mechanisms, expression patterns of proteins and enzymes in response to dietary components and therapy provide a rationale for the development of new active ingredients.

The study showed that the application of proteomic tools provided an overview of the proteins present in *E. coli* Nissle under *Cocos nucifera* sap and wine stress conditions. It confirmed that proteins are involved in various biological functions under stress and defense reactions. The aim of the study is to understand the structure prediction and functions of the differentially expressed stress proteins of *Escherichia coli* under *Cocos nucifera* sap and wine stress.

Probiotic *E. coli* especially *E. coli* Nissle 1917 might be involved in the induction of protective antimicrobial agents, which involved in the maintenance of indigenous microflora of intestine. Hence in the present study *E. coli*Nissle 1917 was collected from culture collection center Germany, maintained on Nutrient broth 50 μl of overnight cultures were transferred into 50 ml broth and grown until OD reached 0.5 (McFarland standard). Cells were adjusted to equal OD = 0.05 in nutrient broth supplemented with 100 mg/L ampicillin. Test samples of cocoti sap and cocoti wine were collected from rural area of Tirupati and it was filtered with vacuum pump filtration by using 0.02μ Nitrocellulose membrane filters, then analysed the physico-chemical characters of the sample. Various concentrations of cocoti sap and wine were added into the broth, then allowed for incubation at room temperature for 5 hours. Measured the growth curves of experimental organism for every half-an-hour by using spectrophotometer (Techcomp) at 620nm.

Alcohol is a frequently abused drug and an important cause of morbidity and mortality. In addition to its effects on the tubular gastrointestinal tract, alcohol affects the physiologic motor action of the esophagus, stomach and intestine. In low concentrations, it has a direct stimulating effect on gastric acid secretion, although contrary to conventional
wisdom, it is not considered to have a pathogenetic influence on the development of peptic disease. Regardless of the type and dosage of beverages such as alcohol facilitates the development of gastroesophageal refluxes disease. In the present investigation, the minimum inhibitory concentration (MIC) of cocoti sap and wine was determined by Macro-dilution method. Growth was monitored using spectrophotometer in every half an hour at 670nm. Which concentration shows approximately 50% growth inhibition was selected as stress concentration for the subsequent proteomic analysis.

The role of the probiotic proteins may involve in the colonization of gastrointestinal tract, adhesion to host tissues, or immunomodulation of the host immune system. The identification of probiotic proteins predicted to be involved in such interactions can pave the way towards well targeted studies of the protein-mediated contacts between bacteria and the host, with the goal to enhance the understanding of the mode of action of probiotic bacteria. Proteins were extracted from bacterial cells by the method of Trizol protein extraction method, centrifugation and sonication techniques were used for cell lysis. Quantify the protein sample by Bicinchoninic acid (BCA) method. 2-D gel electrophoresis was carried out to study differentially expressed proteins under cocoti sap and cocoti wine stress conditions. pH 4 to 7 (Bio-Rad)range IPG strips were used for Isoelectric focusing. 500μg of rehydration buffer and 2% Ampholytes were mixed with the purified protein sample. Then 320μl of sample were loaded in strips and allowed for rehydration. After rehydration, we carried out 6-stepped focusing which was as follows: phase 1, linear gradient up to 250 Volts in 15 minutes; phase 2, linear gradient up to 500 Volts in 30 minutes; phase 3, rapid gradient up to 1000 Volts in 1 hour; phase 4, linear gradient up to 5000 Volts in 3 hours; phase 5, linear gradient up to 10,000 Volts in 3 hours; and phase 6, linear gradient up to 10,000 Volts in 12 hours. Working temperature was set to 20ºC in IEF. After Isoelectric focusing, proteins were separated in the second dimension by using 12% SDS-PAGE gel and 80V was maintained through while running the second dimension. After second dimension, gels were stained with coomassie blue stain. We noticed over all 800 proteins in our gel, 370 spots were visualized clearly. Gels were digitized using gel scanner (Typhon Variable Mode imager), and allowed for gel analysis by using Image master 2-D platinum 6.0 software. It quantify the protein spots and showed the variation between control and treated gel samples, the spot size indicates, up and down-regulation of the
protein. The expressed protein spots were separated by using spot cutter and these spots can be analysed by MS- for protein identification. The protein spots were dehydrated with acetonitrile and dried at 40°C, the samples were digested with proteolytic enzyme (trypsin) cleaved protein sample mixed with matrix (3,5 dimethyl-4- hydroxyl cinnamic acid) compound, prepared a sample slide contains digested protein sample and matrix compound air dried for ten minutes.

Peptide detectability is defined as the probability that a peptide is identified in an MS/MS experiment and has been useful in providing solutions to protein inference and label-free quantification. Mass spectrometry was performed and the spectra measured for unknown peptides were compared against the mass peaks derived from calibration of internal standards. Spectra were collected over the mass range of 800-3500Da. Generated spectra values were converted into Mascot Generic Format (MGF), MGF is a simple human readable format for MS/MS data. MASCOT search engine was used for spot identification by querying for fixed modifications of protein by Carbamidomethyl of cysteine and oxidation of Methionine, database NCBInr, taxonomy *E.coli*, and enzyme trypsin were selected for reference, peptide tolerance, peptide charge, mass tolerance were changed based on protein. Number of missed cleavage sites was allowed up to one. Searching for high percentage sequence coverage, number of related sequences to find a related protein.

Bioinformatics is a rapidly developing interdisciplinary science, new and improved versions of the software and data banks are released very frequently. Mostly used methods in proteomic field such as sequence analysis and comparative proteomics may provide valuable aid and provide insights to different areas of research. Mainly used software tools are MASCOT, SWISS-MODEL, FUGUE, PYROL2, PDB Viewer, Rampage validation tool. Of these bioinformatics tools SWISS-MODEL is one of the best tools for alignment of template and target proteins. Swiss Model (http://swissmodel.expasy.org/) alignment mode were selected for template alignment, 3-D model was generated by using phyre-2 software (http://www.sbg.bio.ic.ac.uk/) used to obtained Protein Data Bank(PDB) file, RasMol software (http://rasmol.org/) were used to analyse the 3-D structure of protein sample. The final structure was analyzed by Ramachandran’s plot drawn by using Rampage Ramachandran’ssserver (http://mordred.bioc.cam.ac.uk/). Further analysis of pdb structure by
using pdb validation tool, Z-score and error value of structure were analysed by QMEAN server (http://swissmodel.expasy.org), finally the protein physico-chemical characters, like molecular weight, molecular formula, half-life of the protein, extinction coefficient, grand average of hydropathicity (GRAVY), total number of atoms and instability index were determined by using ProtParam server.