

Bacterial taxonomic representation of Hypothyroid Breast Milk and Infant Faecal samples with OTU Heat Maps and Rare Faction Curves

D. Esther Lebonah¹, J. PramodaKumari^{2*}

¹Department of Biotechnology, Sri Venkateswara University, Tirupati-517502, A. P, India

²Department of Microbiology, Sri Venkateswara University, Tirupati-517502, A. P, India

*Corresponding Author: pramodakumarij@gmail.com

Available online at: www.isroset.org

Received: 20/Oct/2020, Accepted: 21/Nov/2020, Online: 31/Dec/2020

Abstract- The alterations of gut bacterial communities provoke disease conditions in humans. Gut bacterial dysbiosis is one of the causes of hypothyroidism. Maternal gut bacteria migrate towards the mammary glands via an endogenous cellular route and later inhabit the gastrointestinal tract of the breast-fed neonate through the entero-mammary pathway. So, it is necessary to expose the abundance and richness of bacteria inhabiting both in breast milk and infant's gut. The study showed that the high number of OTUs (Operational Taxonomic Units) related to the many classes of bacteria and rarefaction curves related to some fraction of unidentified species diversity in all the samples.

Keywords: Hypothyroidism, breast milk, infant faeces, bacterial communities, OTUs, Rarefaction curves.

I. INTRODUCTION

Some bacteria guard humans against foreign organisms by presenting physical barriers like intestinal mucosa and sustaining the immune system with their properties called competitive exclusion and building of antimicrobial substances [1, 2]. Colonization of commensal bacteria in the gut commences as soon as the birth of a baby and progressively builds up a highly diverse ecosystem all over the growth [3]. They show a beneficial association [4]. Gut bacteria play a vital role in protein and lipid homeostasis and also in the synthesis of required nutrients and vitamins [5] through the production of short-chain fatty acids (SCFAs) and finally furnish energy to the human intestinal epithelium [6].

The heat map is a creative put on view that concurrently discloses row and column hierarchical cluster structure in a data matrix. It consists of a rectangular tiling with each tile shaded on a colour scale to signify the value of the corresponding element of the data matrix. The rows (columns) of the tiling are ordered such that similar rows (columns) are near each other. On the vertical and horizontal margins of the tiling, there are hierarchical cluster trees. This cluster heat map is a synthesis of several divergent graphic displays developed by statisticians over more than a century [7]. As well as, rarefaction is a method that spots on lopsided sample size, which is regularly a significant issue in comparisons of assorted variety. The rarefaction curve is the normal number of higher scientific classifications, for example, families or genera correspond to in an arbitrary assortment of lower taxonomic units like genus and species. The position of these curves is explored by locating the most ideal uniform upper and lower limits for a fixed number of units and

groups. The situation of rarefaction curves between these cut off points gives a characteristic proportion of evenness of diversity [8]. This technique was first recommended by Sanders [9]. Both these OTU heat maps and rarefaction curves help bacterial taxonomic representation from phylum to species. The abundance of the bacteria by OTU heat maps and the computation of species richness by rarefaction curves explored. This is useful for the exploration of diversity and identity of bacterial communities inhabiting inside the human body. Therefore, the present study focussed on the OTU heat maps and rarefaction curves to explore the bacterial diversity of breast milk and breast-fed infant faeces.

II. RELATED WORK

Gut bacteria have an impact on human health. Dominant bacteria in the gut are Bacteroidetes and the Firmicutes. 90% of known bacteria were found and the remaining 10% is still needed to be classified [10]. As well as dysbiosis causes ill-health like inflammatory bowel disease, obesity, cancer, thyroid problems and autism [11]. Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Bacteroidetes and Fusobacteria were the major phyla found in the gut [12].

Healthy thyroid conditions are the reflection of gut flora. Bacterial gut composition minimizes the possibility of contracting thyroid nodules and cancer. As already known the relationship between gut bacteria and thyroid function, there were some studies in humans that had attempted to manipulate the gut bacteria. The probiotic treatment improved thyroid health by increasing blood plasma thyroid hormones. This was achieved by supplying broiler chickens with lactic acid bacteria [13]. Likewise,

Supplementation of *Lactobacillus reuteri* improved thyroid health in humans [14]. In order to find the bacterial connection between women with hypothyroidism and their breast-fed infants, the present study was accomplished.

III. METHODOLOGY

4.1 Collection of Samples

Healthy and hypothyroid breast milk samples were collected in sterile containers by manual expression according to the instructions of Bhatt *et al.*, and Collado *et al.*, [15, 16]. Fresh faeces samples were collected from the breastfed infants of the considered mothers with care and followed the collecting protocol [17]. All the samples were collected at Government Maternity Hospital, Tirupati, India.

4.2 Bacterial DNA extraction and Further Analysis on Illumina Platform

Xcelgen Blood gDNA Kit was used to extract bacterial DNA from the breast milk and Stool DNA Isolation Kit was used to extract bacterial DNA from stool samples by Stool. The Nextera XT Index Kit (Illuminacatalog # FC-131-1001) was used for the preparation of amplicon libraries in accordance with the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). Based on V3-V4 hyper-variable region, primers were designed for the amplification of 16S rDNA gene of bacteria [18]. The 16S rDNA gene was sequenced to examine the diversity of bacteria. Quantitative Insights Into Microbial Ecology (QIIME), a bioinformatics tool was used to generate data on illumina platform. Based on the 16S rDNA sequence similarity, Operational Taxonomic Units (OTUs) were picked. A representative OTU was picked and assigned using reference databases. OTU Abundance Heatmaps and Rarefaction curves were prepared by QIIME Analysis. Through the Virtual Box, the OTU heatmaps were generated to display OTU counts per sample which represents high and low OTU counts [19].

IV. RESULTS AND DISCUSSION

Heatmap images were generated to visualize the OTU table at the class level. In the heatmap given below, each row shows an OTU, and each column corresponds to a sample. The more intense colour at the subsequent position in the heatmap corresponded to the higher the relative abundance of an OTU in a sample. By default, OTUs (rows) were clustered by UPGMA hierarchical clustering, and sample (columns) was presented in the sort in which they appear in the OTU table. Purple contributes to the high number of OTUs while red contributes to the low number of OTUs.

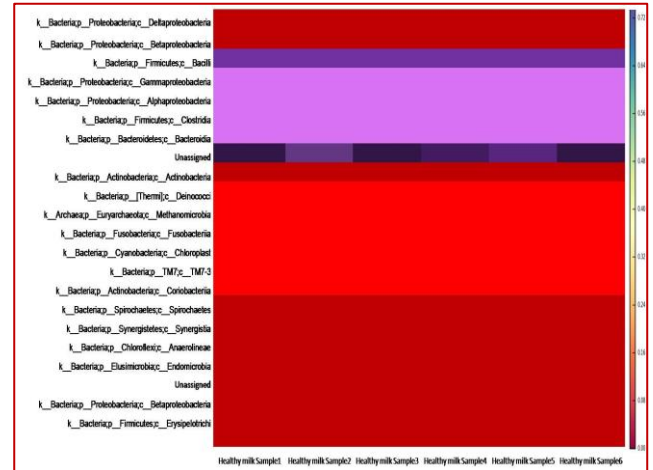


Fig 1: An OTU table heat map showing taxonomy assignment for each OTUs for Healthy Breast Milk Samples.

The OTU heatmap presents OTU counts per sample, where the counts are coloured on the basis of the contribution of each OTU to the total OTU count present in the sample. Based on the taxonomy assignment, the table was filtered the OTU table by number (500) of counts per OTU. Here, the OTUs for Deltaproteobacteria and Betaproteobacteria were high in number in comparison to Gammaproteobacteria, Alphaproteobacteria, Clostridia, Bacteroidia and unassigned classes. The number of OTUs for the remaining classes represented in the image was in between high and low.

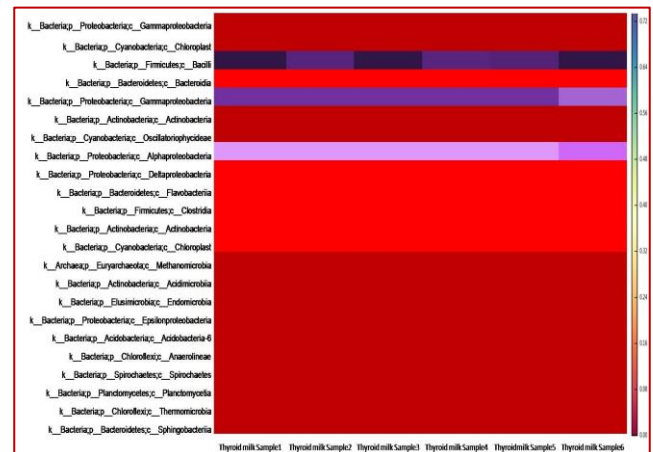


Fig 2: An OTU table heat map showing taxonomy assignment for each OTUs for Thyroid Breast Milk Samples.

The OTU heatmap presents OTU counts per sample, where the counts were coloured based on the contribution of each OTU to the total OTU count present in the sample. Based on the taxonomy assignment, the table was filtered the OTU table by number (500) of counts per OTU. Here, the OTUs for Deltaproteobacteria and Chloroplast were high in number in comparison to Alphaproteobacteria, Gammaproteobacteria and Bacilli. The number of OTUs for the remaining classes represented in the image was in between high and low.

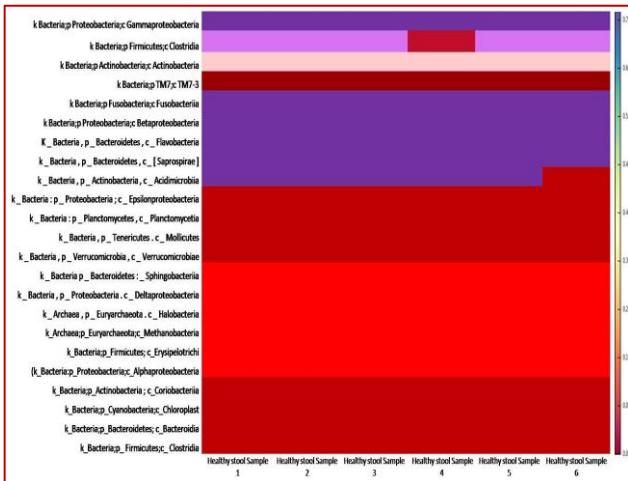


Fig 3: An OTU table heat map showing taxonomy assignment for each OTUs for Faeces Samples of infants who breastfed Healthy Mother's Milk (indicated in the image as Healthy Stool Samples).

The OTU heatmap exhibits OTU counts per sample, where the counts were coloured on the basis of the contribution of each OTU to the total OTU count present in the sample. Based on the taxonomy assignment, the table was filtered the OTU table by number (500) of counts per OTU. Here, the OTUs for TM7 were high in number in comparison to Clostridia. The number of OTUs for the remaining classes represented in the image was in between high and low.

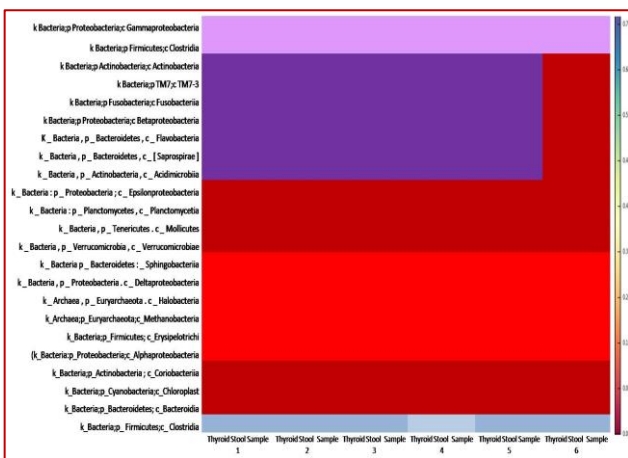
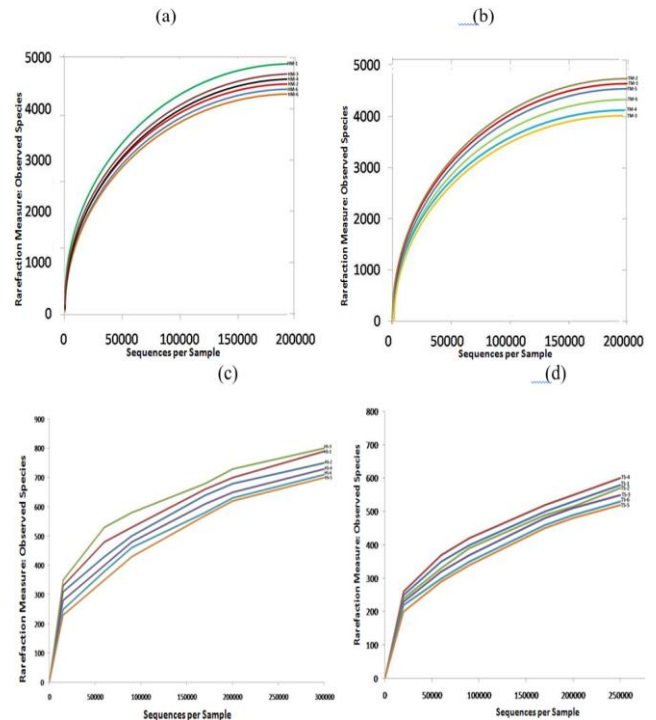


Fig 4: An OTU table heat map showing taxonomy assignment for each OTUs for Faeces Samples of infants who breastfed Thyroid Mother's Milk (indicated in the image as Thyroid Stool Samples).

The OTU heatmap shows OTU counts per sample, where the counts were coloured based on the contribution of each OTU to the total OTU count present in the sample. Based on the taxonomy assignment, the table was filtered the OTU table by number (500) of counts per OTU. Here, the OTUs for Bacteroidia, Chloroplast, Coriobacteria, were high in number in comparison to Clostridia. In addition to that, high number of OTUs found for Actinobacteria, TM7-3, Fusobacteria, Betaproteobacteria in thyroid stool sample-6. The number of OTUs for the remaining classes represented in the image was in between high and low.

The computation of species richness for samples based on the construction was provided by rarefaction curves. This curve represents a plot of the number of species as a function of the number of samples. On the left, the steep slope specifies that a large fraction of the species diversity yet to be discovered. The vertical axis shows the diversity of the community, although the horizontal axis shows the number of sequences regarded to the diversity calculation.



Graph 1: The rarefaction curves showing the species richness and species diversity of (a) Healthy Breast Milk Samples (HS-1,2,3,4,5,6), (b) Thyroid Breast Milk samples (TM-1,2,3,4,5,6), (c) Faeces Samples of infants who breastfed Healthy Mother's Milk (HS-1,2,3,4,5,6), (d) Faeces Samples of infants who breastfed Thyroid Mother's Milk (TS-1,2,3,4,5,6).

From the graph, it can be inferred that (a) the slight steep slope indicates that some fraction of the species diversity remains to be discovered in Healthy Breast Milk Samples, (b) the steep slope signifies that a large fraction of the species diversity yet to be discovered in Thyroid Breast Milk Samples, (c) and (d) very less steep slope found and it indicates the maximum discovered bacterial species diversity and less undiscovered species diversity (generally at genus and species level) in both groups of faeces samples. But the species richness and species diversity were slightly varied from sample to sample in all milk and faeces samples. The presence of unclassified bacteria was supported by Li [20] in milk samples and by Liu [21] in faeces samples.

Our results were supported by Martín *et al.*, [22] and Oddi *et al.*, [23]. Cataloguing the bacterial taxa found in healthy and diseased conditions by Illumina sequencing will allow further closed-ended molecular studies for targeting both cultivable and as yet uncultivated ones, revealing numerous new disease-causing pathogens involved as with perilous effects of the types of diseases. Nowadays, the

human breast is also prone to some types of cancers [24], therefore it is necessary to find out the diversity of bacterial communities in addition to the cytotoxicity testing.

V. CONCLUSION AND FUTURE SCOPE

The study concluded that the high number of OTUs was related to the classes of Deltaproteobacteria and Betaproteobacteria in healthy breast milk samples, Chloroplast in thyroid milk samples, TM7 in healthy infant samples and Bacteroidia, Chloroplast in thyroid infant faeces. Rarefaction curves showed some fraction of the species diversity remains to be discovered in all the samples. Species richness and species diversity were somewhat wide-ranging from sample to sample despite milk and faeces taken. This variation was found between healthy and thyroid samples. Nevertheless, bacterial diversity in healthy and diseased conditions will play prominent role in the future treatment of chronic diseases. Even though there were many studies on bacterial pathogens till now endows with a depiction of how these populations of bacteria involved in the health condition of human beings, but with an insufficient resolution to see how exactly they act upon was not yet distinguished. So, the exploration of the bacterial diversity of healthy and diseased people will give a broad spectrum to observe the relationship between human beings and microbes. The present study may pave the way for future studies regarding the connection between human health and bacteria.

ACKNOWLEDGEMENTS

We are grateful to the staff members of Government Maternity Hospital for helping us to find specific people by providing their records all over the Tirupati and its rural areas. We are very much happy by seeing rural people who were voluntarily given their breast milk samples.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this original research paper.

REFERENCES

- [1]. LV Hooper, T.S. Stappenbeck, C.V Hong, J.I. Gordon, "Angiogenins: a new class of microbicidal proteins involved in innate immunity". *Nature Immunology*, Vol. 4, Issue., 3, pp. 269-273, 2003.
- [2]. D. Bouskra, C. Brézillon, M. Bérard, C. Werts, R. Varona, I.G. Boneca, G. Eberl, "Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis." *Nature*, Vol. 456, Issue. 7221, pp. 507-510, 2008.
- [3]. E.W. Rogier, A.L Frantz, M.E. Bruno, L. Wedlund, D.A. Cohen, A.J. Stromberg, C.S. Kaetzel, "Lessons from mother: long-term impact of antibodies in breast milk on the gut microbiota and intestinal immune system of breastfed offspring." *Gut Microbes*, Vol. 5, Issue. 5, pp. 663-668, 2014.
- [4]. J.L Round, S.K Mazmanian. "The gut microbiota shapes intestinal immune responses during health and disease." *Nature Reviews of Immunology*, Vol. 9, Issue. 5, pp. 313-323, 2009.
- [5]. M.J. Morowitz, E.M. Carlisle, J.C. Alverdy, "Contributions of intestinal bacteria to nutrition and metabolism in the critically ill." *Surgical Clinics*, Vol. 91, Issue. 4, pp. 771-785, 2011.
- [6]. S.H. Duncan, P. Louis, J.M. Thomson, H.J. Flint, "The role of pH in determining the species composition of the human colonic microbiota." *Environmental Microbiology*, Vol. 11, Issue. 8, pp. 2112-2122, 2009.
- [7]. W. De Pauw, G. Ammons. U.S. Patent No. 8,954,859. Washington, DC: U.S. Patent and Trademark Office, 2015.
- [8]. J.J Gart, A. F. Siegel, R.Z. German, "Rarefaction and taxonomic diversity." *Biometrics*, pp. 235-241, 1982.
- [9]. H.L Sanders. "Marine benthic diversity: a comparative study." *American Naturalist*, Vol. 102, Issue. 925, pp. 243-282, 1968.
- [10]. P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, D.A. Relman, "Diversity of the human intestinal microbial flora". *Science*, Vol. 308, Issue. 5728, pp. 1635-1638, 2005.
- [11]. C. Zhang, A. Yin, H. Li, R. Wang, G. Wu, J. Shen, M. Zhang, L. Wang, Y. Hou, H. Ouyang, Y. Zhang, "Dietary modulation of gut microbiota contributes to alleviation of both genetic and simple obesity in children." *EBioMedicine*, Vol. 2, Issue. 8, pp. 968-984. 2015.
- [12]. M. Groussin, F. Mazel, E.J. Alm, "Co-evolution and co-speciation of host-gut bacteria systems" *Cell Host & Microbe*, Vol. 28, Issue. 1, pp. 12-22, 2020.
- [13]. G. Riezzo, G. Chimienti, A. Orlando, B. D'Attoma, C. Clemente, F. Russo, "Effects of long-term administration of *Lactobacillus reuteri* DSM-17938 on circulating levels of 5-HT and BDNF in adults with functional constipation." *Beneficial microbes*, Vol. 10, Issue. 2, pp. 137-147, 2019.
- [14]. M.U Sohail, M.E. Hume, J.A. Byrd, D.J. Nisbet, A. Ijaz, A. Sohail, M.Z. Shabbir, H. Rehman, "Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress." *Poultry science*, Vol. 91, Issue. 9, pp. 2235-2240, 2012.
- [15]. V.D. Bhatt, Y.H. Vaidya, P.D Kunjadia, R. Patel, "Isolation and characterization of probiotic bacteria from human milk." *International Journal Pharmacy and Science and Health*, Vol. 3, pp. 62-70, 2012.
- [16]. M.C. Collado, S. Delgado, A. Maldonado, J.M. Rodríguez, "Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR." *Letters in Applied Microbiology*, Vol. 48, Issue. 5, pp. 523-528, 2009.
- [17]. R. Balamurugan, B.S Ramakrishna, "Estimation of faecal carriage of *Clostridium difficile* in patients with ulcerative colitis using real time polymerase chain reaction." *Indian Journal of Medical Research*, Vol. 127, Issue. 5, pp. 472-477, 2008.
- [18]. A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F. Glöckner, "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies." *Nucleic acid Research*, Vol. 41, Issue. 1, pp. 1-11, 2013.
- [19]. J. Kuczynski, J. Stombaugh, W.A. Walters, A. González, J.G. Caporaso, R. Knight, "Using QIIME to analyze 16S rRNA gene sequences from microbial communities." *Current Protocols in Microbiology*, Vol. 27, Issue. 1, pp. 7-10, 2012.
- [20]. S.W. Li, Watanabe, K., Hsu, C. C., Chao, S. H., Yang, Z. H., Lin, Y. J., ... & Tsai, Y. C. "Bacterial composition and diversity in breast milk samples from mothers living in Taiwan and mainland China." *Frontiers in Microbiology*, Vol. 8, pp. 965-980, 2017.
- [21]. J.H. Liu, M.L. Zhang, R.Y. Zhang, W.Y. Zhu, S.Y. Mao, "Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows." *Micro Bio*, Vol. 9, Issue., 2, pp. 257-268, 2016.
- [22]. R. Martín, S. Langa, C. Reviriego, E. Jiménez, M.L. Marín, J. Xaus, L. Fernández, J.M. Rodríguez, "Human milk is a source of lactic acid bacteria for the infant gut." *The Journal of pediatrics*, Vol. 143, Issue. 6, pp. 754-758, 2003.

- [23]. S. Oddi, A. Binetti, P. Burns, A. Cuatrin, J. Reinheimer, S. Salminen, G. Vinderola, "Occurrence of bacteria with technological and probiotic potential in Argentinian human breast-milk." *Beneficial Microbes*, Vol. 11, Issue. 7, pp. 685-702, 2020.
- [24]. S. Satish, G. Gore, A. Ganpule, S. Srivastava, M. Athavale, "Comparative Cytotoxicity of Standard of Care Treatment Drugs on Human Breast, Prostate, Colon, Cervical and Hepatic Cancer cell lines" *International Journal of Scientific Research in Biological Sciences*. Vol.7, Issue.5, pp.70-81, 2020.

AUTHOR'S PROFILE

Ms. D. Esher Lebonah submitted Ph.D thesis in Biotechnology in SV University, Tirupati. Her area of research is NGS technology. She qualified many National Exams. At present, she's focussing on Food technology.



She has a total of 11 international research papers and some of them are from Elsevier, Springer journals with Pubmed citation. She has 7 years of research experience and 5 years of post graduate teaching experience. She guided 10 postgraduate projects. Currently, she's working for DST-SERB project related chitosanase production. She is good at molecular biology techniques, fermentation kinetics and techniques, biochemistry & biophysical and biochemical techniques, plant cell cultures and bioprocess engineering and technology.

Dr .J.Pramoda Kumari is working as Assistant Professor, Dept. Microbiology, S V University. She did M.Sc, M.Phil, Ph.D, Dip. in Yoga. She received awards from ICMR,SRF(1986-89); Young Scientist by DST (1989-91); RA by CSIR (1991-96). Her area of research is Molecular Microbiology, Proteomics of Probiotics, Phytoremediation and NGS Technology. She has 26 years research and teaching experience. She published research papers both in National and International journals and 4 international books. She presented many papers in India and one paper in International Symposium organised by PSRC centre at Singapore in the year-2012. She guided 6 Ph.Ds and 1 M.Phil and many M.Sc projects. She organized one National Conference. She is the reviewer of Journal of Bioinformation, UK, Journal of Microbiology, Biotechnology and Food Sciences, Germany, BANWA-Research Journal, Philadelphia, USA and Current Trends in Biotechnology, India. She acted as Chair person of 4 National Symposiums. She's the active member of many Professional bodies and every year she conducts campus interviews for post graduate students.

