6.1 SUMMARY

Human microbiome consists of numerous host adaptive commensal bacteria that adopt various adaptation and evolution trends in order to adapt to different niches of the human body. In order to achieve so, they learn to evade host defense mechanisms, attain symbiosis with human body and adapt to diverse physiological environmental requirements of different body sites. In this study, three of the adaptation mechanisms, adaptation to humans as hosts, adaptation to niche as well as adaptation of commensal bacteria to pathogenic lifestyle was studied to undermine the different aspects of evolution. For this purpose, probiotic species, *Lactobacillus reuteri,* a model organism for studying host adaptation, *Lactobacillus* species, predominant in most of the niches of human body like oral cavity and opportunistic pathogen and skin commensal, *Staphylococcus epidermidis* were used.

6.1.1 Tracing evolutionary adaptation trends in *Lactobacillus reuteri,* by a taxonomic outlier

Host adaptation of *L. reuteri* was done by its comparative analysis with a putative new species *Lactobacillus* sp. M31, LM31, which is a closest known species and *L. vaginalis,* the other closely related species. LM31 was received as the ancestral species of LM31. Genome based taxonomy revealed it to be a putative new species and high genetic makeup among LM31 and *L. reuteri* DSM20016 (T). Our analysis revealed that LM31 is an important outlier species for comparative studies unlike other closely related bacterial species. Conserved proteins tree of *L. reuteri* strains with LM31 isolates explains ancestry of the species, marking rodent III lineage as the ancestral lineage, followed by porcine V lineage and human II lineage, while, porcine IV lineage, rodent I lineage and poultry/human VI lineage are contemporary and probably allochthonous lineages. This evolution pattern is in accordance of the evolution time line of their hosts. The tree also manifests that there is a recent host jump of human lineage strain to poultry giving rise to poultry/human lineage.

Most the genes shared by *L. reuteri* from its ancestral species and genes gained by it after diversification were mobile genetic elements, hinting their role in diversification of *L. reuteri.* Presence of even higher number of mobilomes and subsequent absence of a CRISPR array in human lineage strains also indicates role of mobilomes in adaptation of human lineage. Presence of even higher number of ISs in human strains as well as

subsequent absence of a CRISPR array indicates role of mobilomes in adaptation of human lineage of *L. reuteri*.

This study also revealed role of some important candidate gene clusters in host adaptation of *L. reuteri*. *pdu-cbi-cob-hem* cluster, known to produce reuterin and vitamin B12, is present in *L. reuteri*. Due to this feature, the species was named 'reuteri', since, the cluster was considered unique to this species and has been previously reported to be conserved in human lineages. However, this study busted the uniqueness of *L. reuteri* for having 'reuterin *(pdu-cbi-cob-hem)*' cluster by showing that the cluster is ancestral to the lineages, as it is also present in LM31 in complete synteny. Other well characterized gene clusters, urease and secA-secY2, which are indirectly involved in biofilm formation and were observed to be absent from human lineage and conserved in rodent lineage was present in LM31 genome, indicating their lost in human II lineage, but ancestral to *L. reuteri*. In addition, other biofilm forming clusters, SPS1 and SPS2 present in rodent lineage with their homologs in other lineages except human lineage, were absent in LM31, indicating their horizontal gene transfer to the strains. Another cluster, Nitrate reductase-molybdenum cofactors-Iron transport cluster, which is not studied till now in *L. reuteri* was introduced in this study. The cluster harbors levansucrase gene, known for biofilm formation, was seen present in all lineages of *L. reuteri* and LM31, but was absent in human lineage. This study shows absence of most of the biofilm forming genes in human lineage and hence, hints other process for colonization of the lineage. Interestingly, candidate gene clusters are inherited by lineages, irrespective of their evolutionary time-line and is strictly according to their hosts. In addition, my study also rejects *L. reuteri* human lineage adaptation as an outcome of reductive evolution. As no drastic change in genome sizes of *L. reuteri* strains of different lineages was observed.

6.1.2 Understanding role of IS*200* in host adaptation of *Lactobacillus reuteri*

Since, my previous study (Section 6.1.1), suggested role of insertion elements in host adaptation of *L. reuteri,* an initiative was taken to design a study to narrow down the effect. For this purpose, IS*200,* which was present in all strains of *L. reuteri,* was considered. Preliminary studies suggest expansion in human adapted strains of *L. reuteri*. Hence, is important to carryout systematic and large-scale studies on this IS element.

IS*200* is a small (700 bp) insertion sequence. with restricted transposase activity but is widespread in the members of eubacteria, hence, is considered ancestral in the population. This study utilizes many methods to find out the IS*200* copy number in diverse lineages of *L. reuteri* and also standardized the library preparation protocol for high read lengths for low GC species. IS*200* was found to be absent in ancestral strain, LM31 and *L. vaginalis* hinting their horizontal gene transfer to *L. reuteri.* Various methods to find out IS*200* copy number in draft genomes revealed comparable results and revealed expansion of IS*200* in human lineage strains, which were further confirmed by complete genomes available for the strains. However, conservation of the locations of the IS*200* copies in synteny, along with their high ANI (revealing their clonal nature) and same SNPs in IS*200* copies, hints that this expansion is happened recently in all strains, and might have been before emergence of different clones (now strains) of human lineage of *L. reuteri.* This conclusion can also be drawn for porcine V lineage (ATCC53608, I5007 and ZLR003), where strains have high ANI, indicating clonal nature. In this case, ZLR003 strain has four copies, where two of its copies shares similar location as that of other strains. Rodent I lineage proved to be a prodigy in this scenario, as it contains some strains having high ANI and sharing same location with each other, indicating presence of these copies as ancestral. However, at the same time, it also carries some strains having random locations indicating their independent gain by horizontal gene transfer.

Expansion in porcine lineage IV was also observed by draft genomes analysis and RT-PCR. Present study provided the first complete genome sequence of porcine IV lineage strain, further confirming the copies and locations of IS*200*. However, unlike human strains, porcine IV lineage strains shows random locations of IS*200,* hinting independent gain of the insertion element. Interestingly, none of the IS*200* copy was found on any plasmid.

One locus of IS*200* was found conserved in human, porcine IV and porcine V lineage, which was inserted in NiMoI cluster carrying the levansucrase gene (section 6.1.1), hinting ancestral origin of this insertion element in the strains. High number of mobilomes (insertion elements and phages) in LR20.2 genome as well as at the random locations of IS*200,* speculate invasion of IS*200* in porcine/human lineage, where there is an accidental host jump of some porcine strain to humans, trying to adapt there by gaining mobilomes, forming porcine IV lineage.

6.1.3 Comparative phylogenomic insights into adaptation strategies of skin commensal, *Staphylococcus epidermidis*

Ecological features and environmental factors greatly shapes the selection pressure among bacterial isolates and thereby, microbial diversity. Understanding pathogenicity of *S. epidermidis* by incorporating pathogenic strains has been done in past. However, this is the first systematic study emphasizing on the emergence of pathogenic strains from the already existing commensal strains. In addition, this is the first attempts to assess the problem from phylogenomic aspect.

In this study, twenty-eight *S. epidermidis* isolates of Indian origin (SEI) were analysed with another twenty-one isolates of individuals of western origin (SEA). Phylogenetic analysis depicts simultaneous diversification of the two lineages (PG1 and PG2), independent of individual, site and geography of the isolate. SEI isolates of PG1 lineage in a single group depicting their clonal nature, inspite of the fact that they were isolated from different individuals. MLST analysis depicted it as a new ST, ST 691, unique to Indian population.

In silico search of putative commensal markers (*fdh, arsD)* and pathogenic genes (IS*256*, *icaA* and *mecA*) in all 49 isolates, revealed presence of *fdh* gene in all isolates of PG1 lineage and their complete absence from PG2 lineage. No pathogenic marker was present in PG1 lineage, while, PG2 consists of pathogenic RP62A strain. Only *fdh* gene was the differential marker between both lineages.

SEI isolates belonging to ST 691 of PG1 lineage encodes for a novel lantibiotic and a sactipeptide, further suggesting commensal status of these isolates and highlighting diversity between SEA and SEI isolates of same phylogenetic lineage. Presence of these antimicrobial peptides in SEI isolates hints their role in ecological fitness of the isolates and their adaptation to environmental stresses evident in Indian conditions.

Considering all these factors, my study defines the two lineages as 'true commensals' for PG1 lineage isolates and 'potential pathogenic status' for PG2 lineage, which can be identified by '*fdh*' marker.

6.1.4 Niche adaptation strategies of *Lactobacillus* sp. isolated from a subgingival plaque in oral cavity

Numerous *Lactobacillus* species are present throughout niches of human body. This study evaluates differences in adaptation strategies of *Lactobacillus* species present in oral cavity as compared to the same species present in other niches of the human body. In addition, few oral *Lactobacillus* are reported until now and there are limited understanding of adaptation strategies in oral cavity.

Four *Lactobacillus* isolates were isolated from subgingival plaque of a healthy individual, from which DISK7 came out to be a new species, closely related to *L. gasseri*. Five other strains of *L. gasseri* have high ANI and dDDH with DISK7 as compared to type strain of *L. gasseri*, indicating misclassification of the strains. Comparison of DISK7 to *L. gasseri* isolates form gut, vaginal and breast isolates revealed many signatures for oral niches. DISK7 shares highest number of genes with vaginal isolate, JVV03. This may be because of similar physiological environment of the two niches. Similar physiological environment is marked by: four PTS systems, glycosyltransferases, *gtfA*, *gtfB* genes, required for biofilm formation, *sugE* gene, which is responsible for conferring resistance to cetylpyridinium, cetyldimethylethyl, ammonium and cetrimide cations present in antiplaque or antiseptic agents and potABCD operon (which is used in uptake of polyamines) which is helpful in growth, cell wall formation and acid resistance. Unique genes of DISK7 signifies the genes required for oral cavity adaptation. These includes, more PTS systems for various sugars, since, oral cavities loaded with many sugars, capsular polysaccharide biosynthesis proteins (EPS formation), fluoride resistance gene, biofilm formation cluster, nicotinamide metabolism (nicotinamide is a dietary supplement used in oral health medicines) and ECF transporter for transport of sugars. To combat biological stress and inhibition of pathogenic strains in oral cavity, DISK7 contains a bacteriocin biosynthetic cluster that yielded an active bacteriocin inhibiting the growth of various bacteria and yeast. This study manifests the adaptation mechanisms of *Lactobacillus* species in oral cavity and highlights the role of the adaptation features to the ecology.

6.2 FUTURE PRESPECTIVES

My work has shown insights into diverse adaptation mechanisms of commensal host adaptive bacteria of human microbiome. This study proves role of ecological features, environmental factors and selection pressure in adaptation of bacteria to a host, niche and lifestyle.

1. This work had suggested presence of alternative mechanisms for colonization in human gut by human lineage strains, attempts would be made to find alternative colonization mechanisms for human colonization.

2. Role of genes and clusters defining lineages from different hosts was considered in this study. However, genomic differences between two different lineages of same hosts, i.e. porcine IV and porcine V; rodent I and rodent III, as well as other genomic differences in lineages by comparative analysis can be studied in order to have in-detail perspective on adaptation of the lineages of *L. reuteri*.

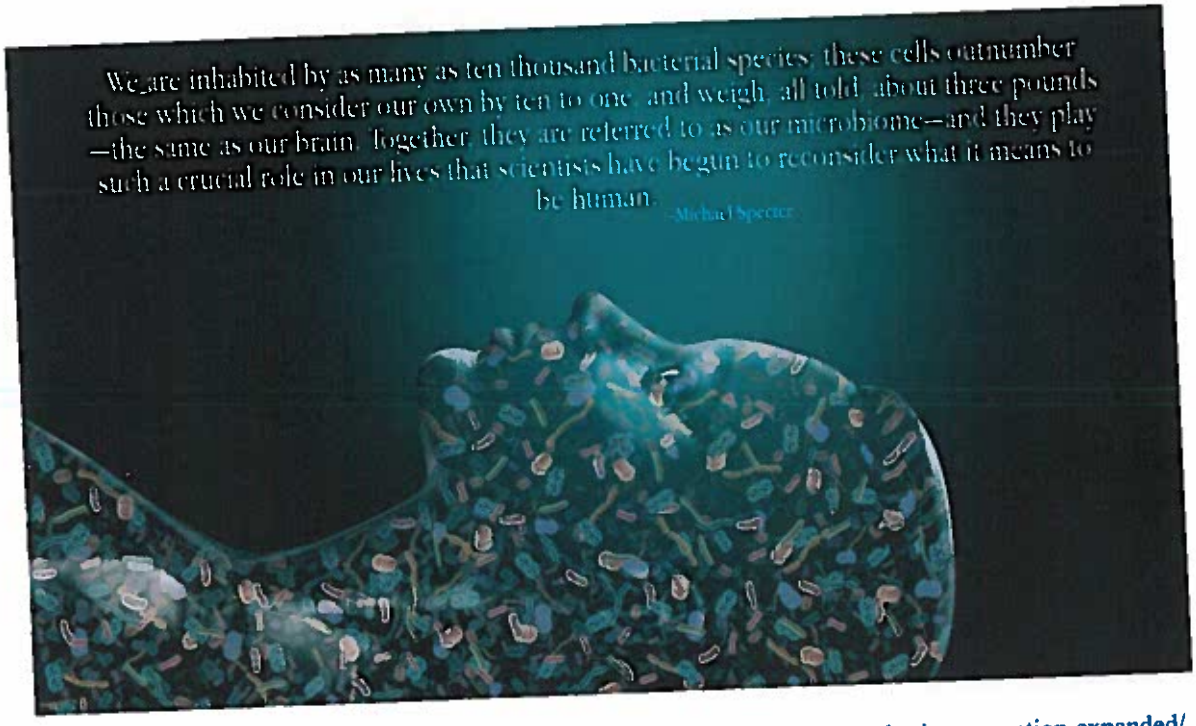
3. Host adaptation is well examined in *L. reuteri*. However, such adaptation mechanisms are also possible in other *Lactobacillus* species like, *L. salivarius* and *L. ruminis*. Attempts would be made to find out the parallels in adaptation mechanisms of these species and signatures required human gut adaptation will be drawn out.

4. Gut colonization by *L. reuteri* human lineage strains has not been successful in the past studies. This may be due competitive inhibition of the incorporated strains by already adapted and colonized strains in gut and hence, attempts will be made to evaluate colonization of human lineage *L. reuteri* strains in neonatal gut, devoid of pre- colonized gut bacteria.

5. Attempts will be made to generate complete genomes of more strains of rodent III and porcine IV lineage to have in-depth view of IS*200* copy number, location and orientation, which will further explains the role of IS*200* in host adaptation of the lineages. In addition, the exact mechanism underpinning the expansion of IS*200* in porcine IV and human II lineage is still unknown and hence, efforts are required to understand these mechanisms by tracing the flow of IS*200* to *L. reuteri*.

6. Synthesis of bacteriocin produced by novel ST691 of *S. epidermidis* and its experimental validation on various pathogenic strains is required.

7. My work shows presence of potential pathogenic *Staphylococcus epidermidis* strains among commensal isolates and distribution of markers defining their pathogenicity. Further efforts are required to find out the phylogenomic status of *S. epidermidis* lineages (PG1 and PG2) in pathogenic isolates as well by comparative analysis of *S. epidermidis* isolates from patients.



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