Chapter: 7

Summary and Conclusion
SUMMARY AND CONCLUSION

The notable findings obtained in this investigation are summarized as follows

- Based on selective screening employing 4 μg/ml of erythromycin, the ER\textsuperscript{r} microflora from diverse food samples ranged from 5.6-50%. From a total of 171 representative food samples, 102 LAB cultures were isolated.

- Among the isolated cultures, 60 LAB isolates were further selected with their ability to grow in presence of 8 and 16 μg/ml of erythromycin.

- The isolates were tentatively identified using biochemical and physiological tests and differentiated based on the finger printing using molecular methods such as random amplified polymorphic DNA. This method was useful in rapid grouping of similar cultures.

- The confirmation of the representative ER\textsuperscript{r} LAB was performed through 16S rRNA gene sequencing. The identified cultures encompassed the species of \textit{Lactobacillus}, \textit{Leuconostoc}, \textit{Enterococcus} and \textit{Pediococcus} suggesting ER\textsuperscript{r} is a wide spread mechanism among LAB.

- The phenotypic erythromycin resistance was initially detected using the macrolide, lincosamide and streptogramin B double disc diffusion test “D zone test” and the isolates were categorized as constitutive (cML\textsubscript{S}B), inducible (iML\textsubscript{S}B), keyhole (KH), lincosamide (L), intermediate (I), and synergistic intermediate (SI) phenotypes. This was followed by minimum inhibitory concentration (MIC) values for erythromycin.
Determining cross resistance among different MLS antibiotics through multiple disc diffusion method, pristinamycin was found to induce clindamycin resistance in *E. faecium* IB6-2B culture. This observed phenotype was similar to that of iMLS$_B$ phenotype generally detected in bacteria with erythromycin induced clindamycin resistance.

The isolates displayed a minimum inhibitory concentration (MIC) ranging from 8 to 256 $\mu$g/ml of erythromycin. For most of the isolates obtained from traditional fermented foods, the MIC values for erythromycin were lower (8-32 $\mu$g/ml) compared to that of the isolates from fermented dry sausages. This high level resistance was due to the constitutively expressed ER$^I$ genes as detected through cMLS$_B$ phenotype.

Positive PCR amplifications were obtained for methylase encoding gene *erm*(B) in 88% of the total LAB isolates indicating the wide spread occurrence of the gene. Besides *erm*(B), the macrolide efflux gene *msr*(C) was found in *E. durans*, *P. pentosaceus*, *E. lactis* and *Lb. fermentum* species. None of the isolates were positive for *erm*(A), *erm*(C) or *mef*(A).

The evaluation of all the ER$^I$ isolates for tetracycline resistance (TC$^r$) displayed MIC values ranging from 8- 256 $\mu$g/ml. TC$^r$ determinants [*tet*(M), *tet*(W), *tet*(S) *tet*(O), *tet*(K) and *tet*(L)] demonstrated their presence in *Lactobacillus*, *Pediococcus* and *Enterococcus* species with simultaneous occurrence of genes with same or different mechanisms.
Resistance to clindamycin was observed among isolates using double disc diffusion test. Two isolates with cMLS\textsubscript{B} phenotypes such as \textit{En. faecium} strain (IB6-2B) and \textit{Lb. salivarius} CHS-2E were found positive with typical clover leaf like inhibition zone. This phenotypic observation was also substantiated with overlay assay, where the clindamycin sensitive \textit{M. luteus} ATCC 9341 culture was found to grow around the test culture \textit{En. faecium} IB6-2B.

The genetic basis of the clindamycin inactivation phenotype in \textit{En. faecium} IB6-2B and \textit{Lb. salivarius} CHS-2E was also corroborated by PCR analysis, wherein a positive amplification for \textit{lnu}(B) gene was obtained. None of the other isolates were found positive either with phenotypic or genotypic analysis. This assay was found to be simple and efficient to detect clindamycin resistance or enzymatic inactivation mechanism among LAB.

Plasmid profiling of LAB isolates displayed plasmids of size ranging from 1.5 to >21 kb suggesting diverse nature of plasmids in ER\textsuperscript{r} LAB. PCR was carried out to identify the mobile genetic elements of Tn916/Tn1545 and Tn917 family of transposons and only \textit{xis} gene of Tn916 transposon was detected indicating their possible role in spreading of ER\textsuperscript{r}.

Long PCR technique was employed to study the linkage between the genes encoding resistance to erythromycin and tetracycline resistance. It was found that these genes coexisted in 3 \textit{Lactobacillus} and 1 enterococcal strain. This linkage was further confirmed by Southern hybridization,
where *erm*(B) and *tet* genes (*tet*(M), *tet*(W) and *tet*(L)) and *lnu*(B) genes were detected on single high molecular weight plasmid indicating its R-plasmid nature.

- Southern hybridization with all *En. durans* strains harbored the efflux pump encoding gene *msr*(C) on chromosome, while it was detected both on chromosome and plasmids in *En. faecium* and *P. pentosaceus* isolates.

- The erythromycin methylase encoding gene *erm*(B) was detected in chromosome and on either single or multiple plasmids of enterococci, as well as *Lactobacillus* and *Pediococcus* isolates.

- The phenotypic induction studies performed with macrolides displayed cross resistance or synergistic effect among macrolide lincosamide and streptogramin B antibiotics suggesting the presence of unidentified ER' and/or mutations in the identified *erm*(B) gene.

- Expression studies carried out using RT-PCR analysis showed that *tet*(W) was inducible even at concentration of 64 μg/ml indicating its possible role in deciphering high level resistance to tetracycline.

- *In vitro* conjugation experiments confirmed that three *Lactobacillus* strains could transfer either only erythromycin or both erythromycin resistance (ER') and TC' to recipient strain *Enterococcus faecalis* JH2-2 with an average frequencies of $1 \times 10^{-4}$, $3.8 \times 10^{-3}$ and $2 \times 10^{-3}$ per donor cell, respectively.
The filter mating experiments carried out with three *E. durans* strains IB9-4, IB9-1 and IB9-2 were successful where they could transfer the erythromycin resistance gene *erm*(B) with an average transfer frequencies of $1.40 \times 10^{-7}$, $1.77 \times 10^{-4}$ and $1.00 \times 10^{-6}$, respectively. This indicates the conjugative nature of the ER$^\text{r}$ phenotype.

ER$^\text{r}$ obtained from different food products of Indian origin indicated the prevalence of antibiotic resistant bacteria in the food chain though it is not exposed to antibiotics. Furthermore, it indicates that food matrices such as fermented foods provide a suitable environment to support the spread of antibiotic resistance.

The detection of ER$^\text{r}$ and TC$^\text{r}$ genes in LAB isolates underline the prevalence of acquired antibiotic resistance in LAB of food origin. Hence, a selection criteria need to be followed before considering such environmentally isolated LAB as starter cultures or probiotics.

The results of the present study indicated that LAB from fermented foods harbored acquired ER$^\text{r}$ and TC$^\text{r}$ coexisting on plasmids as well as chromosome associated with mobile genetic elements. These results suggested the possibility of AR gene transfer among bacteria, which is an undesirable trait for those candidates indented for use as probiotic or starter cultures.