CHAPTER-V

Discussion
5. DISCUSSION:

5.1. pH Sensitive Growth of *Lactobacilli*:

The gastric environment is very harsh on microbes for the very high acidic environment it harbours. As a result any probiotic microbe which has to show its effects must pass this testing environment and reach downstream having a comparatively favourable condition for survival and growth. *Lactobacillus* species have been considered intrinsically resistant to acid (Tannock, 2004) which makes them a potential probiotic candidate.

The results in this study have shown growth at comparatively low pH values indicative of a probiotic character. The seven strains that showed growth at low pH have all shown a tendency of increased sensitivity with decreasing pH values which has been a normal characteristic data in many studies (Jin *et al.*, 1998; Ronka *et al.*, 2003). But the initial phase of growth in all strains have been quite good which may be due to the acid tolerance of *Lactobacilli* being attributed to the presence of a constant gradient between extracellular and cytoplasmic pH.

The growth of the strains have been restricted with increasing time while the sensitivity to very low pH can be seen with the growth going drastically towards zero within two to three hours of the time passage. It has been observed in various research findings that when the internal pH reaches a threshold value, cellular functions are inhibited and the cells die. One of the internal mechanisms may be the F₀F₁-ATPase which is a known mechanism that gram-positive organisms use for protection against acidic conditions (Charteris *et al.*, 1998). The decreasing growth
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pattern as seen in the present findings with respect to time and pH might have been due to decreased power to resist by the inherent mechanisms of the cells with the passing of time. This may have been due to the homeostatic mechanisms being overburdened and efflux of the hydronium ions being reduced leading to decreased intracellular pH. This, thus, leads to the death of the cells and ultimately reducing the colony counts at increased intervals of time particularly at low pH values.

Two of the strains have shown considerable growth under very low pH conditions. These again reach the death point after 5 or 7 hours at pH between 2 to 3. But their growth, albeit being very low CFU counts, show their potential to pass the testing environment of the stomach. Again, this characteristic growth can be attributed to the quality of the specific strain to acclimatise and thrive in the acidic microenvironment of the fermented food product of their origin — *L. plantarum* from fermented bamboo shoot and the *L. fermentum* from the indigenously made curd in the bamboo stem covered with banana leaf.

The reduction in GAPDH activity in a specific *Lactobacillus* strain caused survival to decrease by $8.30 \log_{10} \text{CFU ml}^{-1}$ in the presence of glucose. The data indicate that glucose provides ATP to F0F1-ATPase via glycolysis, enabling proton exclusion and thereby enhancing survival during gastric transit. So, in the case of this particular study these strains must have derived specific carbohydrates from their rich environment and utilised their inherent mechanisms in tandem with nutrients available to survive and thrive. During the growth in low pH condition in the MRS
broth, the exploitation of rich media within the acidified MRS medium may provide protection to bacteria by providing energy and metabolic precursors (Corcoran et al., 2005). These findings of Corcoran may be attributed to the results of the present study wherein the support to grow at low pH may have been provided by the rich growth medium MRS.

Thus, these two abovementioned strains in particular show good potential as probiotics in terms of growth in the stressed acidic condition. Moreover they are present in huge quantities in the food. When ingested their very high population and protection and shielding by the food materials enables them to pass the testing environment of the gastrointestinal tract and exert their beneficial effects by colonising the congenial environs downstream.

5.2. Growth in Bile of *Lactobacilli*:

Bile plays an important role in the physiology of intestinal bacteria, thus conditioning their functionality. This is particularly important for probiotic bacteria, since their beneficial effects must be generated in the presence of this biological fluid. In fact, it is known that the activities of intestinal *Lactobacilli* are deeply influenced by the presence of bile salts, and even some of them, such as cholesterol assimilation, have been directly correlated with bile salt metabolism in these bacteria. Activation of molecular machinery to counteract oxidative and acid stresses are common responses to bile stress, as well as utilization of bile efflux systems and bile modification through bile salt hydrolases (Lorena et al., 2013). The ability of the *Lactobacilli strains* in the present study to thrive at higher concentrations of bile can thus be
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contributed to such mechanisms of survival. These strains have also shown growth at low pH which might also have been of additive value to their survival as shown in the above research findings.

Some studies have shown the acquisition of resistance to be associated to metabolic shifts which may be the reason wherein the strains got static and then acclimatised to slowly shift to other metabolic pathways which provide them with more ATP production and hence energy to survive and push out the bile molecules diffusing into the cell (Sánchez et al., 2007b). Since bile acts as a detergent solubilizing the lipid membrane, the number of strains in the present study showing growth at higher bile concentrations decreased drastically. The inherent machinery of these strains could not contain the increasing pressure of bile and thus were injured beyond repair. Those that survived must have adjusted accordingly by shifting to other metabolic pathways as described in the abovementioned research findings and were able to survive. Some strains had shown growth in a very slow manner which can be associated with time lag in the acclimatization to the stress.

Other studies have shown that bile adaptation resulted in higher glucose consumption and lactic acid formation (Burns et al., 2010). Since MRS contains glucose as one of its constituents, it must have been utilised as an anchoring material in metabolic shifts to gain energy and hence adapt to the new and increasing pressures. Metabolic shifts have been shown to aid in evading the pressure of increasing bile acids of the upper region of the gastrointestinal tract (Ruas-Madiedo et al., 2005). This may be of great use in vivo as a selective advantage within the distal colon where glucose is not
available and the maltose present can be preferred over glucose. Thus the
*Lactobacillus* strains in the present study should have a better survival rate if these
findings are considered which make them of a greater probiotic value.

Across the spectrum of available data there has been more of discordance than the
similarities (Begley *et al.*, 2005). The lack of accordance may be due to the
differences in experimental strategy adopted, the type of bile and the way it was
administered as well as the time of exposure to the stress.

Several candidate signalling molecules from various experiments have now been
identified in vitro wherein bile has been implicated as a potential signaling molecule
that would indicate to a bacterium that it had entered the small intestine (Kristi *et al*.,
2008). Such a signal could serve to stimulate the organism to adapt its physiology to
optimize growth and survival in the GI tract. The strains in this study have shown
growth at higher bile concentrations which may help them to survive much better to
exert their probiotic character by using such signals to produce metabolic shifts
under stress in the gastrointestinal tract.

Whatever may be the reason of the survival of these strains of *Lactobacilli* under
present study, it is obvious that those coming from food products having higher salt
concentrations as well as from a low pH environment are able to cope better with the
stress of increasing bile concentration. This may be due to the acclimatization in the
niches of stress in their local environment which helped them adapt show resistance
when exposed to the purified bile acid (Ox bile). Thus it can be said that such better
adapted strains are potential probiotic microbes which when given the canopy of the food material to survive and thrive in the gastrointestinal tract do express their probiotic characters in vivo, a reason that the fermented food being used across the globe.

5.3 Growth of *Lactobacilli* under antibiotics stress:

Antimicrobial agents or antibiotics are of enormous value for combating infectious diseases, but their efficacy has been made vulnerable by development of microbial resistance. Presently, there is concern over the possible spread of resistance determinants (from the food chain) to antimicrobials. Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes that could be transmitted to pathogens, either in the food matrix or, more importantly, in the gastrointestinal tract. The production of fermented dairy products from raw milk in antibiotic challenged environments may select antibiotic-resistant LAB harbouring transmissible resistance genes. In fact, horizontal gene transfer is essential for bacteria to survive and adapt to new environments. Strains intended for the use in food systems as starters or probiotics should therefore be carefully examined for antimicrobial susceptibility, especially those isolated during the so called “post-antibiotic era” (Teuber *et al.* 1999) (Perreten *et al.* 1997) (Kurland *et al.* 2003).

Potentially transferable genes conferring resistance to one or more of these antibiotics have been characterized in several LAB species. Antimicrobial resistances alone cannot be considered virulence factors, but they can complicate the treatment of opportunistic infections. Probiotics are always used in huge numbers per dose of...
consumption and hence the presence of effective gene transfer mechanisms in members of this genus can thus be very troublesome (Ana et al., 2005).

The main aims of knowing the behavior of exogenously applied *Lactobacillus* under the effect of antimicrobial substances are to have an approach of the response of *Lactobacilli* being administered to patients subjected to some kind of antibiotic therapy and to consider the concomitant use of *Lactobacilli* and an antibiotic to restore the disrupted ecological environment (Virginia et al., 2006).

Antimicrobial resistance of candidate probiotic *Lactobacilli* was found to be not associated with extra chromosomal elements, as plasmids were not found in the strains, by applying the technique of Maniatis et al. This observation would indicate a low probability of antibiotic resistance transmission to pathogenic microorganisms.

This trend is quite good in terms of probiotics as being sensitive to antibiotics prevents them from being used as vectors of antibiotic resistance for pathogens since probiotics are always given at a higher dose and there is always the possibility of gene transfer to pathogens. This is also quiet good for the use of probiotics as adjuvants for drug or vaccine release or delivery without producing any adverse effect on the health of the individual.

### 5.4 Antipathogenic activity of the *Lactobacilli*:

Beneficial urogenital normal flora playing an important role in inhibition of uropathogens and preventing urinary tract infection has now been known for almost
a century (Newman D, 1915). This concept of beneficial normal flora is nothing but probiotics.

The present study involves bacterial strains from a variety of fermented food products and they show promising result in terms of antipathogenic activity against the clinically pathogenic isolates of *E.coli* and *Salmonella*. Various methods have also been adopted to screen out the most effective isolates. A few strains have shown a really promising effect.

The antipathogenic effect is the reason that the fermented food product is popular among the general population. This can ameliorate a lot general as well as certain chronic problems. These microbes can be of great use in decreasing the antibiotic use and prevent the generation of superbacteria which are multi-resistant and cause intractable infections that are difficult to treat. The use of drugs to control infection particularly in children can lead to liver or renal problems or can even leave a debilitating effect in the long run. Hence children are now being generally treated with probiotics, particularly for cases like diarrhoea. Early weaning of mother’s milk also leaves them from development of a normal flora wherein the population of microbes preventing the growth of infectious and pathogenic ones are reduced. As a result the children keep falling prey to opportunistic infections. Such situations can be dealt with by giving regular doses of such pure probiotic antipathogenic strains which shall thrive in the gastrointestinal tract of the children and exert their antipathogenic effect against the pathogens.
Among the adults bacterial vaginosis (BV) is an increasing problem among women and has been associated with the decrease in the *Lactobacilli* count in the vagina (Nikhil *et al.*, 2011). The increasing number of cases of antibiotic-resistant pathogenic microorganisms has led to the extensive study of the use of probiotics for the treatment of BV. The use of such antipathogenic effect producing probiotics for the treatment of BV has provided a ray of hope of natural and nontoxic treatment modality (Dover *et al.*, 2008). The antipathogenic probiotics may offer cost-effective treatment of BV.

The samples of pathogens in the present study were virulent clinical isolates of *E.coli* and *Salmonella spp.* which have been inhibited by the various *Lactobacillus* strains isolated from different fermented food products. The development of inhibition zones shows the effect of the strains which may be due to the production of various kinds of bactericidal compounds such as lactic acid, H$_2$O$_2$ or any antimicrobial produced by the bacteria like the S-layer proteins from kefir *Lactobacilli* have been shown to antagonize biological effects of bacterial toxins (Carasi *et al.*, 2012). The antimicrobial metabolites produced by the probiotics have shown great potential not only to restrict the growth of anti-microbial resistant strains but also kill the same. Such antipathogenic character makes them viable as potential probiotics for use not only in human but can be used to a great effect among animals.

Despite numerous studies which demonstrate antipathogenic properties of probiotics, great variability exists in their reported effectiveness in reducing intestinal infection
(Eutamene et al., 2007; Patterson and Burkholder, 2003) and this variability may depend on which probiotic organism is used, as well as health status of the host.

5.5 Molecular identification of the Lactobacilli:

The carbohydrate fermentation pattern has been a favourable mode of species level identification in connection with Lactobacilli in the older studies. But it involves a lot of time and money and is quite cumbersome in terms of procedural ease. The new version of molecular identification has almost replaced the older processes and involves the 16S rRNA sequence as well as collaborating it with the sequences of specific regions of certain housekeeping genes. We have compared our sequence based result obtained via BLAST search from the sequence database on the NCBI website and found it in complete consensus with the RFLP pattern generated from a different set of PCR as was published by Vethachai Plengvidhya et al. (Vethachai Plengvidhya et al., 2007).

The values in the tree show the different isolates to be clustered in different groups and with different strengths of bonding in terms of sequence homogeneity. G1 has shown great variation in sequence with respect to sequences of other strains and hence appears highly divergent and showing least bonding with the other Lactobacilli strains. Though the tip of this strain on the tree is not much distant in terms of evolutionary time of the horizontal line from the basic root of the phylogenetic tree, yet the branching at the nodes largely differentiates it from the other clusters of the Lactobacilli strain clusters. G6 and G7 form a closer cluster showing greater sequence homogeneity. G4 shows more closeness to this cluster and
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all these three strains show greater homogeneity to strain G5 as compared to the cluster of strains G2 and G3 in terms sequence homogeneity as visible by the branching at nodes and the bootstrap values generated.

The seven *Lactobacilli* strains under study shows a segregated group and very distant from *Acinetobacter bouvettii* (HQ413146.1) and shows a very different bootstrap value at the branching point from the rest of the reference strain sequences of a variety of bacteria including those of the *Salmonella sp.*, *Bifidobacterium sp.*, *Pseudomonas sp.*, *Bacillus sp.* and *Enterococcus sp.*

The *Lactobacilli* show a group arrangement with other *Lactobacilli* data incorporated from external database from internet. The *L. plantarums* show slight heterogeneity in terms of grouping together and show a little proximity with the *L. casei*. However they have not deviated to the extremes of distance from the main cohort of the *plantarum* group. The *L. fermentum* strains under study have shown great proximity with their group and have been together and showing homogeneity with the data imported from internet for the tree formation. These results thus are in concurrence with available database on the internet showing the validity of our strains under study.

5.6 mRNA expression profile of immune-modulation:

It is well established fact that interferon (IFN)-γ is a pleiotropic cytokine capable of potent immunomodulatory effects on a variety of immune cells both in vitro and in vivo. There have been several studies carried out in mice lacking the expression of IFN-γ or its receptor, which has pointed out that IFN-γ is one of the most significant
factors in the host defence against infectious agents. In particular, IFN-γ exerts important activities on monocytes/macrophages and lymphocytes, which generally result in macrophage activation and T cell differentiation toward a T helper cell type 1 (Th1) of immune response (Patrizia et al., 2005).

In the present study the Lactobacillus strains have not been able to induce extensively the expression of IFN-γ at the transcriptional level. But at the same time there is consistent increase and many folds the control in the initial days particularly in the fifth and 10\textsuperscript{th} day. Though the amount of increment and expression of IFN-γ as whole is not very high as that of IL-2, yet, the expression of this pluripotent inflammatory cytokine is noteworthy in terms of E.coli as it is a clinically pathogenic isolate showing the determination of the immune system to throw it out of the system and ease out the infection. But the expressions of the same in terms of both the strains of Lactobacilli administration have not been enough of an inflammatory type. This is quite favourable in terms of probiotic potential as they need not elicit a response from the immune system of the host that kills them before expressing their beneficial effects.

Interleukin-2 (IL-2), a cytokine mainly produced by activated T lymphocytes, plays a fundamental role in sustaining the growth of T, B, and NK cells. During the last phases of antigen-specific T cell response, this cytokine contributes to the maintenance of T cell homeostasis by promoting activation induced cell death of effector T lymphocytes. In keeping with the pivotal regulatory role exerted by IL-2 in the immune system, IL-2 deficient mice show a generalised immune system deregulation (Narumi et al., 1990).
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Interleukin 2 (IL-2) has shown a huge increase in the early days as compared to the control and has maintained above normal level in due course of time as compared to control in E. coli administered animals. This effect is also positive in terms of the cleansing the system of the pathogenic microbes. This cytokine is known to help in proliferation of cellular immunity of T, B and NK cells which is helpful in taking up the pathogenic antigens and producing a cascade for the clearance of infection. But when the expression is observed in the Lactobacilli administered animals, it shows an initial increase but tapers down gradually. This is good again for probiotics as they need to survive within the animal to show their beneficial effects.

It has been reported that interleukin 2 (IL-2) and interferon gamma (IFN-gamma) production by CD4+ T cells and IFN-gamma production by CD8+ T cells from naive mice in response to soluble anti-CD3 and antigen-presenting cells (APCs) were strikingly inhibited by culture in the presence of IL-4. It has also been shown that IL-2 induces IFN-γ mRNA expression and protein secretion in peritoneal macrophages (PM) from different mouse strains. In one study it was demonstrated that IFN-γ and IL-2 synergize for the production of IL-1β in human monocytes and TNF-beta in peripheral blood mononuclear cells. Furthermore, IFN-γ and TNF-alfa may synergize with respect to activating host defence functions in human monocytes and anti-tumor activity in murine macrophages (Narumi et al., 1990).

Thus, it's quite clear that IL-2 not only induces the expression of IFN-γ but they also work synergistically to induce other pro-inflammatory cytokines as well as organizes the host defence against any pathogenic effect. It was observed from the present study that there was an increase of both the cytokines in pathogenic E. coli
administered animals. But there has been only an initial increase followed by a reduction in the expression of both in the *Lactobacilli* administered animals.

In the *E. coli* administered animals also, the reduction in expression of both IL-4 and IL-10 have been quite low. This can be due to the skewing down of their effect by the combined effect of the Th1 pro-inflammatory cytokines IL-2 and IFN-γ. But the expressions of IL-4 and IL-10 have been quite high in the potential probiotic *Lactobacilli* strains. This shows that indeed there has been a scuttling effect on Th2 cytokines by the Th1 cytokines in the pathogenic condition. But there has been an opposite reaction in the *Lactobacilli* administered animals where the roles both in terms of expression and dominance has been reversed as well the effect being much stronger. This simply supports and strengthens the positive effects of potentially probiotic microbes.

The cell wall structures of pathogenic Gram-negative bacteria as well as of non-pathogenic Gram-positive microorganisms act as excellent inducers of inflammatory cytokines (Kitazawa *et al.*, 1992; Heumann *et al.*, 1994; Breuninger *et al.*, 1994; Mancuso *et al.*, 1994). According to these previous studies, our results may suggest that the surface properties of LAB are important in determining an immune response and that the different immune responses observed among the LAB assayed may be due to differences in their cell wall structures which not only the lipids but all kinds proteins embedded within the cell wall. Since we have used the cell lysate the cytoplasmic proteins shall account as well. The most remarkable effect was observed for the production of anti-inflammatory cytokines such as IL-4 or IL-10.
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In vitro experiments have shown the production of TGF-β beta by the cultured human intestinal cells in response to stimulation with microbial antigens (Yoshioka et al., 2001), and that some probiotic species stimulate production of IL-10 in cord blood (Young et al., 2004).

This again supports our assumption for the diversity and level of expression of the Th1 and Th2 cytokines in our study.

Interleukin-10 (IL-10) is a pluripotent cytokine and the most significant anti-inflammatory cytokine found within the human immune response (Alejandra et al., 2011; Asadullah et al., 2003). It inhibits cytokine synthesis in Th1 cells, and received as such the designation of cytokine synthesis inhibition factor (CSIF) (Lalani et al., 1997; Howard et al., 1992; Opal et al., 1998). IL-10 is generally considered an immunosuppressive molecule with its main biological function being the limiting and termination of inflammatory responses. It inhibits the production of pro-inflammatory mediators while enhancing the production of anti-inflammatory mediators (Asadullah et al., 2003). IL-10 has an effect on survival, proliferation, and differentiation of human B cells as well as inducing IgA and IgG production by B cells. Regarding the effects on T cells, IL-10 inhibits the production of IL-2 and IFN-γ by Th1 cells (Del Prete et al., 1993; de Waal et al., 1991).

Thus the many fold increase in the mRNA expression of IL-10 is a sign of non-pathogenic character and inclined towards creation of a non-inflammatory environment in the Lactobacilli administered animals. The reduction in level of Th1
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inflammatory cytokines in these animals shows their inhibition and skewing effect of the IL-10 on them.

One of the studies found a remarkable effect for the production of anti-inflammatory cytokines such as IL-4 or IL-10. The high levels detected for IL-4 and the knowledge that Th1 cells stimulate IgG2a antibodies production, whereas IgG1 antibodies are induced under control of Th2 (cytokine IL4), led the researchers to analyse the antibody isotype against ovoalbumin induced after oral administration of LAB. It was then shown that L. casei, L. delbrueckii ssp. bulgaricus and L. acidophilus affect the systemic humoral immune response. This finding was then proposed to be considered in the selection of Lactobacilli strains as vectors which would alleviate the negative aspects of gut inflammation through their incorporation in some oral vaccines (Perdigón et al., 2002).

Interleukin 4 has also been shown to suppress interleukin 2 and interferon-γ production by naive T cells stimulated by accessory cell-dependent receptor engagement (Toshio et al., 1993).

Hence we understand that the probiotic characters of the Lactobacilli strains are concomitant with the continuous increase of IL-4 in the Lactobacilli administered animals as well the increase in IL-10. Both these anti-inflammatory cytokines have skewed down the effect of the inflammatory Th1 cytokines thereby generating an environment where the probiotic bacteria can make the environment friendly for its survival so that it can survive, propagate and colonise as commensals by competing
out the pathogens to exert its beneficial effects as a GRAS microbe helping its consumer positively.