DISCUSSION

S. dysenteriae type 1 is a serious bacterial pathogen responsible for bloody diarrheal cases which occur in third world and receives special attention in developing countries due to its role in causing epidemic dysentery outbreaks (WHO, 2005a). The situation is becoming grimmer because of lack of vaccine against *S. dysenteriae* and emergence of drug resistance to the clinical antibiotics used for the treatment of shigellosis (WHO, 2005b). *S. dysenteriae* type 1 has been categorized as bio-terror agent in category B by centre for disease and prevention (CDC). Approximately, 10-100 cells of *S. dysenteriae* can lead to infection in humans, hence, low inoculum size place it in high risk infectious organism (Sur *et al*., 2004). Main line of concern lies in paediatric mortality and morbidity due to their under developed gut associated immunity (Hoque *et al.*, 1994; Foye *et al*., 2012).

In this era of modern world, increased incidences of gastrointestinal infections, resultant of disturbed microflora and acute dependency on antibiotics, has led to the implementation of alternative methods to control infections. World health organization has strongly recommended the exploitation of prophylactic as well as therapeutic potential of probiotic strains (Bengmark *et al*., 1998; WHO, 2002). Lactic acid bacteria being part of normal intestinal microflora of humans, particularly lactobacilli, are increasingly employed as a potential probiotics (Mitsuoka, 1992; Walter *et al*., 2000; Denli *et al*., 2003). Various *Lactobacillus* strains such as *L. acidophilus*, *L. rhamnosus*, *L. casei* and *L. fermentum* have been reported in literature possessing anti-*Shigella* activity (Moorthy *et al*., 2007; Nawaz *et al*., 2011). However, there are limited numbers of reports on the use of probiotics for the control of *S. dysenteriae*.

In present study, lactobacilli were isolated from healthy breast fed infants' stool. It is well documented, that promising probiotic microorganisms can be isolated from infants stool as they possess nascent flora composed of lactobacilli and bifidobacteria least affected by intake of drugs and antibiotics (Patel *et al*., 2004; Borchers *et al*., 2009).

One hundred lactobacilli isolates were obtained from 50 stool samples based on morphological, biochemical and PCR based identification. All the 100 lactobacilli isolates were vigorously screened for probiotic attributes such as tolerance to lysozyme mediated proteolysis in mouth, harsh acidic conditions prevailing in stomach, tolerance against bile salts secretion and ability to adhere and colonize GIT and possessing antimicrobial activity. It has been well documented that enteric lactobacilli isolates possess inherent ability to tolerate

harsh environment existing in human GIT and are more likely to adhere and colonize intestinal tract (Gilliland, 1979; Charteris *et al*., 1998; Patel *et al*., 2004).

Lysozyme is an important component of human saliva and possesses antibacterial property. Lysozyme levels in saliva and stomach varies from 10-100ug/ml, therefore ability of microorganisms to survive at these levels of lysozyme, act as one of the criteria for selecting a probiotic strain to be administered orally (Puniya *et al*., 2012). Out of 100 lactobacilli isolates, 45 isolates isolated from human infants' stool samples showed complete tolerance to lysozyme which is in accordance with findings of Kozakova *et al*. (2005), Mandal (2006) and Puniya *et al*. (2012).

Probiotic microorganisms exhibit their probiotic action in intestine, and to effectively render its action they should arrive in adequate number and in viable form (Mainville *et al*., 2005). Deprivation in probiotic count and viability occurs as it passes GIT starting from mouth to intestine, but most of losses occur on exposure to acidic environment persisting in the stomach and bile salts secreted in the duodenum and small intestine (Mainville *et al*., 2005).

Parietal cells of stomach produce acid that makes stomach highly acidic (pH 1.5-3) that creates inhospitable environment for microorganisms, severely limiting their numbers that reach to the intestine (Ashraf *et al*., 2009). In our study, most of the lactobacilli isolates were killed at low pH (2.0) conditions, while 10 isolates namely RT1-34, RT4-54, RT5-2, RT9-10, RT16-2, RT21-4, RT21-24, RT26-6, RT27-17 and RT48-15, showed remarkable tolerance to harsh acidic environment with high percentage survivability (≥90%). Our results are in accordance with the reports published by Dhewa *et al*. (2010), Mourad and Nour-Eddine (2006) showing enhanced potential of *Lactobacillus* strains to survive well at low pH.

Normally, 0.3% of bile salts are present in duodenum and small intestine, but once food is ingested and during first few hours of digestion concentration of bile salts rises to a level between 1.5-2.0% in human intestine (Gotcheva *et al*., 2002). Surprisingly all the human lactobacilli isolates were completely tolerant to bile (1.5% oxgall). This could be attributed to the presence of bile salts hydrolase activity, through which hydrolysation of conjugated bile salts takes place (Du Toit *et al*., 1998). Though the mechanism of bile salt resistance by our isolates was not investigated, but this property has been associated with the presence of bile salts hydrolase activity in lactobacilli (Dhewa *et al*., 2010). Bile salt hydrolase activity has been mostly found in lactobacilli isolated from stool or intestine of animals and humans (Tanaka *et al*., 1999). This suggests that, all the lactobacilli isolates presented in this study being of human origin possess inherent ability to resist toxic effects of bile.

Bacterial adhesion to intestinal mucosa is a key process responsible for bacterial survival and colonization in the GIT (Jankowska *et al*., 2008). In present study, cell surface hydrophobicity in range from 8 to 50% was observed. These differences could be credited to the variation in expression levels of cell surface protein amongst these isolates (Ramiah *et al*., 2007; Kaushik *et al*., 2009). Eleven isolates namely RT4-54, RT5-2, RT6-10, RT9-6, RT9-10, RT12-26, RT16-2, RT20-2, RT25-6, RT26-6 and RT27-17 showed remarkable hydrophobicity (≥40%), amongst all other lactobacilli isolates indicating their capability to adhere to intestinal epithelial cells as stated by Rosenberg *et al*. (1980), higher the hydrophobicity more is the ability to adhere intestinal epithelial cells.

Based on probiotic attributes, six potential probiotic lactobacilli were screened in present study. These lactobacilli isolates RT4-54, RT5-2, RT9-10, RT16-2, RT26-6 and RT27-17 were identified as *L. paraplantarum* (LP1), *L. pentosus* (LP2), *L. pentosus* (LP3), *L. pentosus* (LP4), *L. plantarum* (LP5), *L. rhamnosus* (LP6). *L. rhamnosus, L. plantarum* and *L. pentosus* holds special attention as being promising probiotic candidate exhibiting number of metabolic functions that are beneficiary for human health (Alander *et al*., 1999; Klingberg and Budde, 2006; Puniya *et al*., 2012).

In current study, Caco-2 cell monolayer, which is human colon adenocarcinoma epithelial cell line was used to analyse bacterial adhesion to epithelial cells. Caco-2 cells differentiate as normal small intestine epithelial cells and express characteristics of mature enterocytes having apical hydrolases with brush border microvilli (Fogh *et al*., 1977; Greene and Klaenhammer, 1994). All the six lactobacilli isolates exhibited worthy adherence (16-25%; adhesion score: 450-1550) on Caco-2 cells. Our findings are in agreement with others who also showed high adherence capability of *L. pentosus* and *L. plantarum* strain to intestinal cells (Tulumoglu *et al*., 2005; Kaushik *et al*., 2009; Duary *et al*., 2011). Highest adherence to Caco-2 cells was exhibited by LP6 followed by LP5, LP4, LP2, LP3 and LP1.

Potential probiotic strain must possess anti-microbial activity against pathogens causing enteric infections in humans (Suskovic *et al*., 2010). It is well documented that *L. rhamnosus* and *L. plantarum* possess anti-microbial activity against *Bacillus cereus, Clostridium sporogenes, K. pneumoniae, S. typhimurium, S. sonnei* and *S. aureus* but very few reports are published on anti-microbial potential of *L. pentosus* (Isolauri *et al*., 2004; Baccigalupi *et al*., 2005; Suskovic *et al*., 2010). In current study, out of six probiotic lactobacilli isolates, LP4, LP1 and LP6 possessed broad spectrum *in vitro* anti-microbial activity against commonly found clinical pathogens and were successfully able to co-aggregate these pathogens. There

are numerous reports on anti-microbial activity of lactobacilli against enteropathogens that supports our findings (Tambekar and Bhutada, 2010; Ren *et al*., 2013). It has been well documented that ability of probiotic strains to coaggregate pathogens helps in preventing their colonization in gut (Vlkova *et al*., 2008; Zhang *et al*., 2011). Our results are in accordance with Ren *et al*. (2013) and Xu *et al*. (2009) who also demonstrated coaggregation of various pathogenic bacteria by lactobacilli speces.

Competitive riddance of exogenous pathogen takes place when indigenous microorganisms and exogenous pathogenic microorganisms compete for space to adhere to colonic mucosa and nutrients availability (Ohashi and Ushida, 2009). Probiotic microorganisms physically block pathogen colonization due to nonspecific steric hindrances of receptors that play role in identifying pathogenic bacteria (Bernet *et al*., 1994; Chichlowaski *et al*., 2007). In current study, all the six lactobacilli isolates successfully competed with pathogenic bacteria (*A. baumanii, E. faecalis, E. coli, K. pneumoniae, P. vulgaris, S. typhimurium, S. flexneri, S. aureus* and *S. maltophilia*) and resulted in significant ($p<0.05$) decrease in percentage adhesion of pathogens on Caco-2 cells. Maximum riddance of pathogens was exhibited by LP6 followed by LP4, LP1, LP5, LP3 and LP2. Our observations are in agreement with Fooks and Gibson (2002) who reported *Lactobacillus* strains mediated inhibition of intestinal adhesion of various pathogens. Similarly, detachment of enteropathogen such as *Salmonella*, *Shigella* and *Clostridium* has been well documented in literature due to competing probiotic strains (Isolauri *et al*., 2004; Baccigalupi *et al*., 2005).

Microorganism to be inculcated in food products for human use should not possess antibiotic resistance pattern in order to qualify the safety parameters (Mourad and Nour-Eddine, 2006). Transmission of antibiotic resistance genes to unrelated and potential pathogenic bacteria should be minimized (Mourad and Nour-Eddine, 2006). Lactobacilli selected in present study, showed sensitive profile against commonly used antibiotics (amikacin, amoxicillin-clavulanic acid, cefoperazone, ciprofloxacin, cotrimoxazole, gentamicin and nalidixic acid). Similar results were obtained for *L. plantarum* isolated from olives by Mourad and Nour-Eddine (2006). In current study, only one of the isolate LP3 was found to be resistant to gentamicin which is in agreement with Tulumoglu *et al*. (2013) who documented resistance of lactobacilli against gentamicin. Hence, all the six lactobacilli isolates being sensitive to most of the clinically used antibiotics, qualify one of the main safety criteria to be used as

probiotic strain. All the six lactobacilli isolates were completely biocompatible to each other, hence can be successfully used together in cocktail form for future studies.

Based on functional attributes, out of six probiotic lactobacilli namely LP1, LP2, LP3, LP4, LP5 and LP6, three probiotic lactobacilli namely LP1, LP4 and LP6 showed superior probiotic functional characteristics along with broad spectrum antimicrobial activity were considered for further studies.

Shigella, being facultative intracellular pathogen, has prominent specificity for primate hosts mainly humans (Schroeder and Hilbi, 2008). Pathogenesis of *Shigella* is mediated by its ability to adhere intestinal epithelial cells, followed by invasion of colonic epithelium where multiplication of *Shigella* takes place and reaching macrophages present in lamina propria (Schroeder and Hilbi, 2008). Macrophages acts as a defensive structure against *Shigella* outspread, but *Shigella* being one of the intracellular pathogens, has developed means to escape macrophage arbitrated defence such as phagocytic killing (Zychlinsky *et al*., 1996). *Shigella* survivability in macrophages is attributed to triggering of macrophage apoptosis and evading phagocytosis mechanism (Zychlinsky *et al*., 1992). Macrophage death leads to initiation of inflammatory reaction in response to liberation of pro-inflammatory cytokines such as TNF-α and IL-8. This further results in acute intestinal inflammatory response that contributes to colonic epithelial destruction leading to dysentery (Moorthy *et al*., 2010; Fiorentino *et al*., 2014). During infective state, oxidative stress leads to production of ROS that targets DNA, RNA, lipids and proteins (Beckman *et al*., 1990).

It has been well reported that viable and cell free extracts of *Lactobacillus* speces increase phagocytic killing of pathogens in macrophages (Perdigon *et al*., 1986; Valdez *et al*., 2001; Rishi *et al*., 2009). But very little studies have been carried out to observe the effect of lactobacilli on *S. dysenteriae* type1 infected macrophages. Based on this, in the present study various treatment groups consisting of lactobacilli cell suspension and LCFS of individual *Lactobacillus* (T1-T3), cocktail of two lactobacilli (T4-T6) and cocktail of all the three lactobacilli (T7) were designed to evaluate most effective treatment for treating *Shigella* infected macrophages. Amongst all the treatment groups, probiotic cocktail of all the lactobacilli was the most proficient in significantly increasing intracellular killing of *S. dysenteriae* by peritoneal macrophages. This might have resulted from synergistic effect of probiotic cocktail on augmentation of phagocytic killing of pathogen by macrophage.

Generation of ROS results in peroxidation of lipids in cell membrane (Chanana *et al*., 2006). High levels of MDA were observed in macrophages infected with *S. dysenteriae,* which clearly indicated cellular damage of macrophages. Subsequently, when probiotic treatment was provided to infected macrophages, a decrease in levels of MDA was observed. Out of three lactobacilli used for treating infected macrophages, LP6 was most effective. Although the three lactobacilli reduced levels of LPO, the probiotic cocktail group (T7) was most effective due to synergistic effect of all the three probiotic lactobacilli. In support of our findings, Rishi *et al*. (2009) have reported lactobacilli mediated reduction in lipid peroxidation in *Salmonella* infected rat peritoneal macrophages.

Nitrite is estimated as an indirect measure of NO due to its short half-life of merely few seconds. Nitric oxide is a significant signalling entity that regulates various physiological processes (Rishi *et al*., 2011). Increased levels of NO_2 were observed in macrophages infected with *S. dysenteriae*. Subsequently, reduction in level of NO_2 was achieved on treatment with probiotic lactobacilli cell suspension and their cell free extract. Maximum reduction was incurred in cocktail group (T7) of all the three lactobacilli followed by treatment groups (T4-T6). It has been suggested that the reduction in the levels of NO_2 could be due to reduced expression of TNF-α and IL-8 in presence of probiotics, in return reducing expression of nitric oxide synthase which is responsible for production of NO (Rishi *et al*., 2011).

Probiotics are reported to demonstrate anti-oxidative ability by enhancing enzyme SOD and non-enzymatic substance GSH (Haest *et al*., 1997; Xing *et al*., 2006) in the host cells. SOD protects cell from toxicity caused by free radicals by catalytically cleaning superoxide radicals (Xing *et al*., 2006) and GSH is crucial cellular antioxidant that protects cell from lipid peroxidation and oxidation of sulfhydryl groups present in proteins (Haest *et al*., 1997). In the present study, increased levels of SOD and GSH were achieved in infected macrophages upon treatment with lactobacilli cell suspensions and LCFS. This corroborates the earlier findings of Rishi *et al*. (2009) that documented lactobacilli mediated increase in anti-oxidant activity in host cell. In the present study, highest level of SOD prevailed in probiotic cocktail treatment group (T7) followed by treatment group composed of two lactobacilli (T6). Amongst individual treatment groups (T1-T3), LP6 (T3) was the most efficient in enhancing SOD activity compared to non-significant increase in levels of SOD in treatment groups comprising LP4 (T1) and LP1 (T2). Similarly, levels of GSH were also

greatly increased in macrophages infected with *S. dysenteriae* on treating them with probiotic cell suspension and its cell free extracts. Out of all treatment groups, probiotic cocktail T7 group led to the maximum increase in level of GSH in infected macrophages followed by T6. Probiotic cocktail (T7) consisting of all the three lactobacilli was found to be the best treatment as it led to the significant alteration in macrophage functions in comparison to the treatment groups containing cocktail of two lactobacilli (T4-T6) or individual treatments (T1-T3). The enhanced effect observed using cocktail of three isolates could be attributed to the synergistic effects of anti-*Shigella* factors present when all the three lactobacilli were taken together.

In order to combat *Shigella* mediated intestinal infection, *Shigella* adherence and internalization by intestinal epithelial cells should be targeted (Moorthy *et al*., 2010; Fiorentino *et al*., 2014). In the present study, probiotic cocktail of all the three lactobacilli (T7) maximally reduced *S. dysenteriae* adherence and internalization by 61.2% and 74.6% to intestinal epithelial Caco-2 cells. Synergistic effect of all the three probiotic lactobacilli led to the highest reduction in *S. dysenteriae* adherence and internalization to intestinal epithelial Caco-2 cells in comparison to individual lactobacilli treatment (T1-T3). Our results are in accordance with Moorthy *et al*. (2010) who observed lactobacilli (*L. acidophilus* and *L. rhamnosus*) mediated reduction in *S. dysenteriae* adherence and internalization. In addition to it, there are several other reports documenting ability of probiotic lactobacilli to inhibit pathogenic adherence and invasion of colonic epithelium (Bernet *et al*., 1994; Hudault *et al*., 1997; Gopal *et al*., 2001; Lee and Puong, 2002; Lee *et al*., 2003). There are numerous reports on potential of *L. rhmanosus* to inhibit adhesion of pathogens to intestinal surfaces (Gopal *et al*., 2001; Lee *et al*., 2003) but ability of *L. pentosus* and *L. paraplantarum* is reported for the very first time, hence needs to be explored further. Moreover, it has been reported that indigenous strains of lactobacilli are more effective as they possess strong adhesion ability for bacterial cell surface as compared to other dairy strains commercially available in market (Chauviere *et al*., 1992; Sarem *et al*., 1996; Wang *et al*., 2008; Duary *et al*., 2011).

The mechanism behind inhibition of adherence and internalization of *S. dysenteriae* on Caco-2 cells has been attributed to competition for space and nutrient uptake on intestinal lining in presence of probiotic lactobacilli (Moorthy *et al*., 2010). There are numerous reports signifying the ability of probiotics to inhibit adherence and internalization of entropathogens on intestinal epithelial cells (Gorbach *et al*., 1987; Coconnier *et al*., 1993; Alander *et al*.,

1999; Collado *et al*., 2005; Gueimonde *et al*., 2005; Mappley *et al*., 2011). Additionally, probiotic lactobacilli are reported to stimulate mucus production by goblet cells and strengthens intestinal barrier that plays critical role in controlling EPEC/EHEC like pathogenic infections (Caballero-Franco *et al*., 2007; Bergstrom *et al*., 2010).

In the current study, the impact of probiotic lactobacilli addition in comparison to *S. dysenteriae* addition to Caco-2 cells was also evaluated. Out of three treatment sets, limitative set was most successful in inhibiting/reducing adherence and internalization of *S. dysenteriae* to Caco-2 cells followed by competitive set and displacement set. Our results are in line with Moorthy *et al*. (2010) who also observed that probiotic lactobacilli addition one hour prior to *S. dysenteriae* seeding of Caco-2 cells is most effective in reducing adherence and internalization of *Shigella* to Caco-2 cells.

Moreover, cell free supernatant of each lactobacilli isolates was also analysed for its potential to reduce adherence and internalization of *Shigella* on Caco-2 cells. Reduction in both adherence and internalization of *Shigella* on Caco-2 cells was observed but less in comparison to viable probiotic lactobacilli treatment. Our results were found to be contradictory to the findings of Moorthy *et al*. (2010) who documented, that only viable lactobacilli cells can lead to suppression in *Shigella* adherence and internalization in Caco-2 cells. On the other hand, our results are in accordance with Ingrassia *et al*. (2005) and Abedi *et al*. (2013) who also have documented, the ability of LCFS containing certain biomolecules to reduce pathogenic adherence on Caco-2 cells. The present observation can be justified as probiotic lactic acid bacteria secrete certain metabolites such as bacteriocins, fatty acids, butyrate, organic acids and hydrogen peroxide that inhibit or kill bacterial pathogens rendering them to attach to epithelial cells (Rooj *et al*., 2010).

Caco-2 cells upon *S. dysenteriae* infection, exhibited only 30% of cells' survivability. On the other hand, when Caco-2 cells were co-cultured with *S. dysenteriae* and probiotic lactobacilli, enhanced survivability of Caco-2 cells was observed. Probiotic cocktail of all the three lactobacilli led to maximum survivability of Caco-2 cells infected with *S. dysenteriae.* Our results are in accordance with Moorthy *et al*. (2010) who also documented that lactobacilli strains led to decrease in Caco-2 cell cytotoxicity caused by *S. dysenteriae*. Similarly, there are reports on the potential of lactobacilli to reduce pathogens mediated epithelial cell's cytotoxicity (Maudsdotter *et al*., 2011).

Probiotic cocktail of all the three probiotic lactobacilli exhibited most significant and promising effects in reducing *S. dysenteriae* mediated cytotoxicity and invasion of intestinal epithelium. Therefore, probiotic cocktail was used for further studies to evaluate immunomodulatory effect on *S. dysenteriae* infected Caco-2 cells.

Host immune response generated against *Shigella* invasion of colonic epithelium contributes to over induced inflammatory response imperative for disease development (Fiorentino *et al*., 2014). In present study, keeping in mind quest for understanding host microbe interaction on mucosal immunity, expression levels of various cytokines and TLRs was evaluated. Expression levels of TLRs (*TLR2, TLR4* and *TLR9*), transcriptional factor (*NF-κβ*), cytokines (*1L-1β, IL-8, TNF-α, IFN-γ, IL-10, TGF-β, IL-12, IL-18, IL-23*) genes was evaluated by performing real time PCR (qPCR) in *S. dysenteriae* infected Caco-2 cells in absence and presence of novel probiotic cocktail.

The intestinal epithelial cells possess TLRs that recognize particular microorganisms (Ozinsky *et al*., 2000; Galdiero *et al*., 2002). Toll like receptors play pivotal role in inducing intestinal inflammation (Villena *et al*., 2014). In current study, expression levels of *TLR2* recognizing Gram positive bacteria (Kelly and Conway, 2005), *TLR4* recognizing lipopolysaccharides (LPS) of Gram negative bacteria (Kelly and Conway, 2005) and *TLR9* recognizing CpG islands in bacteria (Rachmilewitz *et al*., 2004) were evaluated. In the present study, mRNA expression levels of *TLR2, TLR4* and *TLR9* in intestinal epithelial Caco-2 cells upon *S. dysenteriae* infection was significantly (infection group; $p<0.05$) increased in comparison to uninfected Caco-2 cells. Our results are in accordance with Massari *et al*. (2006) and Raja *et al*. (2011) who documented that TLR2 is associated with recognizing shiga toxin produced by *S. dysenteriae* and TLR4 is associated with recognizing LPS of *S. dysenteriae* contributing to the initiation of infection. Increased expression of *TLR9* in intestinal epithelial Caco-2 cells upon *S. dysenteriae* infection is not reported till date, hence needs to be explored further. On the other hand, when Caco-2 cells were co-seeded with *S. dysenteriae* and probiotic cocktail, significant (treatment group; $p<0.05$) decrease in expression of *TLR4* by 83.6% and significant ($p<0.05$) increase in expression of *TLR2* by 99.1% and *TLR9* by 95.1% was observed. Our findings are in line with Rizzo *et al*. (2013) and Villena *et al*. (2014) who reported use of lactobacilli for combating intestinal infection by down-regulating *TLR4* expression. Whereas increased expression of *TLR2* and *TLR9* in intestinal epithelial cell upon incubation with lactobacilli has also been reported by Kitazawa

et al. (2008), Alvarez *et al*. (2009b) and Salva *et al*. (2010), suggesting role of TLR2 and TLR9 in recognizing Gram positive lactobacilli by intestinal epithelial cells that competes with pathogen. Therefore, increased expression of *TLR2* and *TLR9* and decreased expression of *TLR4* in probiotic treated Caco-2 cells in comparison to *S. dysenteriae* infected Caco-2 cells plays crucial role in delimiting invasion of *S. dysenteriae* and promoting probiotic lactobacilli colonization on intestinal epithelium.

TLR4 mediated activation of nuclear transcriptional factor NF-κβ further signals secretion of pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α leading to chronic inflammation in host cells (Jobin and Sartor, 2000; Haller *et al*., 2004; Villena *et al*., 2014a). In present study, expression levels of *NF-κβ, IL-1β, IL-8* and *TNF-α* in *S. dysenteriae* infected intestinal epithelial cells was significantly (infection group; $p<0.01$) enhanced in comparison to uninfected Caco-2 cells. Our results are in line with Sansonetti *et al*. (1999), Pedron *et al*. (2003), Kohler *et al*. (2002), Roselli *et al*. (2006), Hormannsperger and Haller (2010) and Moorthy *et al*. (2010) who documented increased expression of *NF-κβ* that further up-regulates expression of pro-inflammatory cytokines such as *IL-1β, IL-8* and *TNF-α* in epithelial cells, leading to intestinal lining dysfunction and tissue damage. Interestingly, it was noted that Caco-2 cells when co-seeded with *Shigella* and probiotic cocktail (treatment group) showed significant decrease in gene expression of *NF-κβ, IL-1β, IL-8* and *TNF-α* genes in comparison to Caco-2 cells seeded with only *S. dysenteriae*. There are several reports, suggesting potential of lactobacilli to down-regulate gene expression of *NF-κβ* and its associated pro-inflammatory cytokines that controls mucosal inflammation induced by enteropathogen (Villena *et al*., 2005; Roselli *et al*., 2006; Salva *et al*., 2010; Shimazu *et al*., 2012; Arora *et al*., 2014; Kitazawa *et al*., 2014). Moreover, Moorthy *et al*. (2010) documented lactobacilli mediated down-regulation of *IL-8* and *TNF-α* in *S. dysenteriae* infected Caco-2 cells.

Another pro-inflammatory cytokine *IFN-γ* gene expression was also studied. It was observed that levels of *IFN-γ* were highly elevated in Caco-2 cells which were co-seeded with *S. dysenteriae* and probiotic cocktail (treatment group) followed by expression levels of *IFN-γ* in Caco-2 cells infected with *S. dysenteriae* (infection group). This high increase in *IFN-γ* expression level in Caco-2 cells in treatment group might play crucial role in protecting intestinal epithelial cells from acute inflammatory damages. This could be attributed to the IFN-γ mediated activation of natural killer cells and T lymphocytes that directly targets clearance of *S. dysenteriae* from site of infection (Raqib *et al*., 1997). Higher levels *IFN-γ*

were observed in initial phase of *S dysenteriae* infection but as the infection becomes more acute depletion in *IFN-γ* in epithelial cells was observed by Raqib *et al.* (1997) that supports our observation. This suggests role of IFN-γ in protecting epithelial cells from intestinal infections. But till date, there is no report on potential of *Lactobacillus* strains to elevate expression levels of *IFN-γ* during infection, hence changes in expression levels of *IFN-γ* upon probiotic lactobacilli treatment needs to be explored further. However, there are certain reports that suggest role of *Lactobacillus* in stimulating IFN-C and IL-12p70 which are potent inducer of IFN-γ, activating host cellular immunity leading to the pathogen clearance (Arokiyaraj *et al*., 2014).

Inflammatory damage of intestinal lining can be further restricted by the action of anti-inflammatory cytokines such as IL-10 and TGF-β that specifically helps in limiting inflammatory reactions in host cell and maintaining gut homeostasis (Letterio and Roberts, 1998; Sanchez-Munoz *et al*., 2008). In the present study, significant increase in expression of anti-inflammatory genes in Caco-2 cells infected with *S. dysenteriae* was observed. This increase in anti-inflammatory genes during infection can be attributed to play role in preventing complete intestinal damage so that bacteria can survive and multiply in intestinal epithelium in order to establish infection (Chen *et al.*, 2005; Kim *et al*., 2005; Ingersoll and Zychlinsky, 2006). However, the mRNA levels of *IL-10* and *TGF-β* gene were highly enhanced in Caco-2 cells co-seeded with *S. dysenteriae* and probiotic cocktail of lactobacilli (treatment group) in comparison to mRNA levels in *S. dysenteriae* infected Caco-2 cells (infection group). Our findings are in accordance with Dieleman *et al*. (2003), Otte *et al*. (2004), Corr *et al*. (2007) and McClemens *et al*. (2013) who reported that increased expression of anti-inflammatory genes in host epithelial cells upon interaction with probiotic lactobacilli helps in combating number of enteric infections. Moreover, it has been widely reported that IL-10 and TGF-β specifically down-regulates expression of *NF-κβ* and pro-inflammatory cytokines in intestinal epithelial cells, therefore preventing excessive mucosal inflammation during diseased condition (Roselli *et al*., 2007; Letterio, 2005; Foye *et al*., 2012; McClemens *et al*. 2013).

In addition to above mentioned cytokines, expression levels of *IL-12, IL-6, IL-18* and *IL-23* were even evaluated. IL-6 has been generally categorized as pro-inflammatory cytokine and contributes to the tissue injury and augments local inflammatory response (Raqib *et al*., 1995). But there are certain reports in literature that states IL-6 as multifunctional cytokine having both pro-inflammatory and anti-inflammatory properties and plays role in generating

host response against enteropathogen, terminal differentiation of immune cells and hematopoeisis (Weinstein *et al*., 1997; Goodrich and McGee., 1998; Ng *et al*., 2003). In present study, expression levels of *IL-6* were found to be increased significantly in Caco-2 cells co-seeded with *S. dysenteriae* and probiotic cocktail (treatment group) in comparison to Caco-2 cells infected with *S. dysenteriae* (infection group). Our findings were in contrary to those who reported that lactobacilli strains generally down regulate IL-6 production in intestinal epithelial cells which were co-challenged with LPS (Shimazu *et al*., 2012; Takanashi *et al*., 2013). However, Vinderola *et al*. (2005) and Duersteler *et al.* (2010) showed increase in expression of *IL-6* in intestinal epithelial cells upon stimulation with *Lactobacillus* speces, that supports our findings. It has been reported that IL-6 up-regulation further leads to increase in IgA production in intestinal lamina propria that play crucial role in exclusion of enteropathogens (Perdigon *et al*., 1991; Matar *et al*., 2001). Further, observations made by Miller and McGee (2003) on anti-inflammatory effects of IL-6 signalling down-regulation of LPS induced *TNF-α* and *IL-1* gene expression supports our findings on IL-6 mediated protection in probiotic cocktail treated Caco-2 cells in presence of *S. dysenteriae*.

On the other hand, high expression level of *IL-12* was observed in *S. dysenteriae* infected Caco-2 cells (infection group) in comparison to un-infected Caco-2 cells. Interleukin-12 being one of the potent cytokine that signals for production of pro-inflammatory cytokines such as TNF-α might play crucial role in *S. dysenteriae* mediate mucosal inflammation (Sansonetti *et al.,* 1996). Our results are in line with Sansonetti *et al*. (1996) who also observed production of IL-12 in piglets infected with *Shigella.* However, when Caco-2 cells were co-seeded with *S. dysenteriae* and probiotic cocktail (treatment group) significant decrease in *IL-12* gene expression was noticed. Our results are in accordance with Pena *et al.* (2005) who reported that combination of different *Lactobacillus* strains can significantly reduce *IL-12* gene expression which in return regulates mucosal inflammation in mouse colitis infection model.

For the very first time in the present study, role of IL-18 and IL-23 in *S. dysenteriae* mediated intestinal epithelial infection is assessed. Expression levels of both *IL-18* and *IL-21* were significantly enhanced in Caco-2 cells infected with *S. dysenteriae* (infection group) in comparison to un-infected Caco-2 cells. However, Caco-cells co-cultured with *S. dysenteriae* and probiotic cocktail (treatment group) indicated increased expression of *IL-18* and *IL-21*, compared to *S. dysenteriae* infected Caco-2 cells. This increased expression of *IL-18* and *IL-21* might play role in regulating mucosal inflammation. Our findings are backed upon by

Chen *et al*. (2005) and Brisbin *et al*. (2010) who also reported that lactobacilli strains tend to induce secretion of IL-18 and IL-21 to control intestinal infections. Increased expression of *IL-18* gene can be attributed to its role in activation of NK cells to remove bacterial pathogen and stimulation of IFN-γ (Way *et al*., 1998; Le-Barillec *et al*., 2005) that can further play role ion protection of intestinal cells from *Shigella* infection. On the other side, increased expression of *IL-21* might have contributed to the maintenance of intestinal cell proliferation during infection to maintain epithelial barrier integrity (Parrish-Novak *et al*., 2000, 2002). Main aim of present study was to analyse effect of probiotic cocktail of *L. pentosus*, *L. paraplantarum* and *L. rhamnosus* on intestinal epithelial inflammatory reaction induced by *S. dysenteriae*. Results of present study, demonstrated that probiotic cocktail potently down regulated pro-inflammatory pathway by targeting NF-κβ pathway induced by *S. dysenteriae* and generation of anti-inflammatory cytokines that play role in shut down of pro-inflammatory action. Together this study provides better understanding of how probiotic lactobacilli being part of commensal microflora can contribute to host intestinal homeostasis.

Viable lactobacilli cell suspension and LCFS of all the three probiotic lactobacilli successfully inhibited growth of *S. dysenteriae*. Lactobacilli cell free supernatant of all the three probiotic lactobacilli was characterized for evaluating a) minimum inhibitory concentration and b) time dependent killing of *S. dysenteriae*. Highest MIC of 16 against *Shigella* was observed with LP6 followed by MIC 8 with LP4 and LP1. Synergistic effect of LCFS of all the three probiotic lactobacilli was exhibited in terms of MIC 64 against *S. dysenteriae*. Similarly, LCFS cocktail of all the three probiotic lactobacilli impeded *S. dysenteriae* growth maximally followed by LCFS of *L. rhamnosus, L. pentosus* and *L. paraplantarum*. It was further observed that LCFS inhibited *S. dysenteriae* during its initial growth phase suggesting that LCFS targets growing cells of *Shigella* and obstructs their cell division as suggested by Rishi *et al*. (2011).

All the three probiotic LCFS retained anti-*Shigella* activity on exposure to high temperature (45-110 ˚C), treatment with detergent (SDS, tritonX100, tween 20 and tween 80) and various proteolytic enzymes, respectively. On the other hand, anti-*Shigella* activity was lost when pH of the LCFS was maintained above pH 6. Therefore, from above observation it is clearly understood that LCFS of LP1, LP4 and LP6 exhibited its anti-*Shigella* activity predominantly due to acidic component and certainly not by bacteriocins which is contrary to reports that reported bacteriocins to play major role in anti-microbial activity (Tolinacki *et al*., 2010; Mohankumar *et al*., 2011). In literature, the anti-microbial activity of lactobacilli has

attributed to the presence of bacteriocin, lactic acid and H_2O_2 in the culture filtrates (Suskovic *et al*., 2010). The organisms isolated in this study produced lactic acid and H_2O_2, but not bacteriocin. Our findings are in agreement with others who also indicated the prominent role of on anti-microbial effect of lactic acid and H_2O_2 on bacterial pathogens like *E. coli* and *S. enterica* (Tomas *et al*., 2003; Pridmore *et al*., 2008). Presence of other molecules like aldehydes and smaller peptides may also be responsible for anti-microbial activity. In the current study, primarily lactic acid (120-180mg/ml) and H_2O_2 (70-90µg/ml) produced by *Lactobacilli* were responsible for killing *S. dysenteriae in vitro*. Lactic acid and hydrogen peroxide production was maximally achieved during 36-48 hours of *Lactobacilli* growth. Out of the three *Lactobacilli* used in present study, LP6 produced highest amount of lactic acid and H_2O_2 followed by LP4 and LP1.

Our findings on anti-microbial effect of lactic acid and H_2O_2 produced by *Lactobacilli* are in accordance with previous reports (Pridmore *et al*., 2008; Maudsdotter *et al*., 2011). Bacteriocin activity was not found in above mentioned *Lactobacilli* against *S. dysenterie,* suggesting that anti-microbial proteins produced by these *Lactobacilli* directly don't kill *S. dysenteriae* but might play role in blocking attachment and internalization of *S. dysenteriae* on intestinal cells. Moreover, Singh *et al*. (2013) reported that bacteriocin such as nisin produced by LAB are capable of killing Gram negative microorganisms only in presence of EDTA or some other agents like essential oils, citrate or antibiotics.

Probiotic cocktail of *L. pentosus* (KJ802481), *L. paraplantarum* (KJ802482) and *L. rhamnosus* (KJ802483) has been for the first time reported to be effective against *S. dysenteriae.* This cocktail can be further used as a natural alternative for treating *S. dysenetriae* infection especially in infants for whom stringent antibiotic treatment is not recommended. Along with protection against *S. dysenteriae*, Probiotic cocktail can also develop overall health of infants and enhance their immune system to combat other infections.