Chapter 5: Discussion
5. Discussion

Increased incidence of lifestyle diseases, increased life expectancy and inadequate nutrition due to current lifestyle, nutraceuticals gaining importance and emerged as a part of daily diet all over the world. The Nutraceuticals are segmented into dietary supplements and functional food and beverages. Probiotic fortified food is a type of functional food which is currently 4 billion dollor in 2018 and expected to reach 18 billion dollor in 2025 as per the joint report by ASSOCHAM-MRSS, New Delhi, 2018. Although globally nutraceutical market is full of different products in the form of food supplements, dietary supplements, probiotic fortified foods and non-food supplements attracting consumers and helping the health improvements; there is demand of more product. As gut microbiota significantly affected by the age, food habits, geographic locations and genetic makeup of individual. Different human gut microbiome study of individual from different geographical locations shows difference in microbiome composition. One of the study shown the difference in microbiota composition of Indian population and western population (Bhute et al., 2016) and contribute separately in health and diseases. Based on this scientific approach although presence of different nutraceuticals product, market (producer and consumer) demands for indigenous, population specific probiotic fortified functional food targeting Indian population. Attention need to be paid for development of natural non-dairy probiotic product targeting the vegetarian, lactose intolerance person and person on cholesterol restricted diet (Martins et al., 2013).

The human gastrointestinal tract is a dynamic ecosystem inhabited by different microbial species which remain in a complex equilibrium. Gut microbiota has significant impact on human health influencing human physiology and metabolism to a greater extent (Shreiner et al., 2015). Various clinical conditions such as diarrhoeal diseases, inflammatory bowel syndrome, food allergies etc. are associated with imbalances of the gut microbial community (dysbiosis). In humans, LAB are key players in maintaining stability and diversity of the gut microbiome (Servin, 2004). LAB have been explored for their probiotic potential and health benefits due to their therapeutic activity against pathogens (Dicks & Botes, 2010).

The conventional sources used for isolation of LAB are milk and milk products, human faeces, food products, fermented drinks etc. Increasingly, probiotics from alternative sources particularly other than dairy products are being selected for use in lactose intolerant people. Many of the widely studied potential probiotic strains are of human or
animal intestinal origin whereas the probiotic properties of bacteria from non-intestinal and non-dairy fermented foods are less explored (Sornplang & Piyadeatsoontorn, 2016). In the present study, LAB were isolated from fresh vegetable sources such as cauliflower, gherkins, cluster beans, fenugreek, cow pea, bitter gourd, french beans, tomato, ridged gourd, cucumber and bottle gourd. They were screened for basic probiotic properties and also for additional bioactive properties including antioxidant, antimicrobial activity, anti-inflammatory activity and short-chain fatty acid synthesis ability for potential application of these bacteria in functional foods.

The present study reports one of the less exploited sources for isolation of LAB-fresh vegetables. The plant phyllosphere is densely colonized by a complex and highly diverse microbial population exhibiting pronounced differences with respect to difference in plant species. One of the key influencing factors determining the type and density of microbes colonizing the plant phyllosphere is phytochemical concentration and composition (Ruppel et al., 2008; Barnard et al., 2013). Diverse species of LAB such as Enterococcus munditii, Lactobacillus plantarum, Pediococcus pentosaceus, Lactococcus lactis and Streptococcus sp. populated the plants but Lactobacillus plantarum stably dominated both epiphyte and endophyte throughout all the phonological stages (Lamont et al., 2017; Pontonio et al., 2018) In agreement with this in the present study Lactobacillus plantarum was the predominant species isolated from fresh vegetables. Fresh vegetables harbour LAB and the predominant genera in the identification were found as Lactobacillus, Enterococcus and Weissella. LAB colonization appeared to preferably occur on tomato, cauliflower, fenugreek, and gherkins. Interestingly, all these vegetables are rich source of dietary fibres and antioxidants (Meghwal et al., 2012; Erba et al., 2013; Ahmed et al., 2013). Plant probiotics are the plant associated bacteria which benefits the plants; these microorganisms can emerge from surrounding environmental ecosystems and it’s survival is regulated by the plant (Berlec 2018) The nutritional contents, plants secondary metabolites and phyllosphere provide favourable conditions for the survival and growth of LAB on plants (James et al., 2003). The present study highlight that the fresh vegetables harbour the LAB predominantly Lactobacillus sp.

One of the primary selection criteria for new probiotic functionality is its ability to survive and colonize in the stressful gut environment with tolerance to acidic pH, bile toxicity and pancreatic enzymes. LAB isolates in this study had probiotic potential better than the reference strain Lactobacillus plantarum MCC2156. The isolates predominantly belonging to the genera Lactobacillus and Enterococcus were more tolerant and one isolate
Weissella sp. ID10V had also exhibited significant tolerance to gastric conditions; reflecting their suitability as a probiotic culture. These results are in accordance with the studies revealing the ability of the Lactobacillus strains isolated from dairy products to be viable when exposed to low pH (Maragkoudakis et al., 2006). One study reports the probiotic potential of Lactobacilli isolated from fermented olives (Argyri et al., 2013). In our study, Lactobacillus plantarum AG40V exhibited significant viability when exposed to low pH and bile toxicity, whereas other research suggests about strong bile tolerance but considerably lower viability at acidic pH of Lactobacillus plantarum isolated from spontaneous fermented wheat bran (Manini et al., 2016). The common resistance mechanisms in Lactobacillus species, to withstand the effect of bile acids on cell physiology, are found to be active bile efflux system, bile salt hydrolysis, changes in central metabolic pathways and changes in the design/composition of cell membrane and cell wall (Ruiz-May and Rose, 2013). Our results are in agreement with the study where majority of the Lactobacillus isolates obtained from traditional Greek dairy products and meat products were found to be highly resistant to bile salts even after 4 hours of exposure (Pavli et al., 2016). This shows that isolates from unconventional sources particularly non-intestinal isolates are equally potent in their probiotic properties as compared to the isolates of intestinal origin. Besides these basic probiotic abilities, isolates were also assessed for bioactive properties such as antioxidant activity and antagonism against human and plant pathogenic bacteria (antimicrobial activity). They were also tested for genotoxicity, susceptibility to antibiotics, milk curdling and food preservation ability.

Lipid oxidation reduces nutritional value and causes development of rancidity in lipid-rich foods. The antioxidant ability of cell free supernatant of Lactobacilli was determined for their possible applications in preservation of foods rich in fatty acids. It is noteworthy that antioxidant activity of LAB isolated from fenugreek was higher than LAB isolated from other vegetables used in the study. This can be attributed to their origin, where fenugreek is a rich source of dietary fibres and antioxidants. Fenugreek leaves contains different phytochemicals; saponins, alkaloids, flavonoids which helps to enhance it’s antioxidant capacity (Meghwal and Goswami 2012; Bano et al., 2016; Venkata et al., 2017). Ultraviolet-radiation has been reported to cause lipid peroxidation. In natural conditions due to presence of UV radiation in the sunlight, high oxidative stress might be generated as a result of lipid peroxidation and this may be the reason why LAB isolated from fenugreek might have evolved with maximum antioxidant potential in response to this increased oxidative stress. One study reports about high antioxidant ability of culture
supernatant and intracellular extract of probiotic strain *Enterococcus durans* LAB18s (Pieniz *et al.*, 2014). Our study had shown similar results with respect to antioxidant properties of *Enterococcus* isolates.

LAB synthesize different metabolites such as organic acids, hydrogen peroxide, diacetyl, ethanol, acetaldehyde, bacteriocins etc. (Šušković *et al.*, 2010). LAB having potent antimicrobial activity can be an effective alternative for antibiotics and can be useful in urogenital and gastrointestinal infection therapies (Sanz *et al.*, 2007; Mokoena *et al.*, 2017). Probiotics also serve to enhance the intestinal barrier function through competitive exclusion and antagonism against pathogenic microorganisms (Bron *et al.*, 2017). The antimicrobial activity of LAB isolates was tested against representative human pathogens such as *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Citrobacter freundii* and *Enterobacter cloacae*. Antimicrobial activity of *Enterococcus* sp. ID11V and ID19V was found to be higher amongst all *Enterococcus* isolates, whereas *Lactobacillus* sp. J129V, J23V and ID12V were found to be inhibitory when tested against the above mentioned representative human pathogens. *Lactobacillus* sp. J129V had shown maximum inhibitory activity against *Klebsiella pneumoniae*- one of the important nosocomial pathogen showing multi-drug resistant phenotype. Cell free supernatant of lactobacilli isolated from curd and human milk samples did not exhibit inhibitory activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Klebsiella pneumoniae* (Sharma *et al.*, 2017); whereas in our study, most of the LAB isolates were found inhibitory against all the tested clinically important human pathogens.

In addition, antagonism was also tested against representative plant pathogens like *Ralstonia solanacearum*, *Erwinia carotovora*, *Xanthomonas campestris* and *Pseudomonas aeruginosa*. These pathogens are responsible for crop damage ultimately resulting in severe economic loss. There is need of control on chemical pesticides in agricultural sectors. The abuse of chemical pesticides creates negative effects on health and environment (Nicolopoulou-stamati, 2016). It was observed that LAB isolated from fenugreek and tomato were superior in their antimicrobial potential. This study highlights their potential application as bio-control agents against phytopathogens. Three strains selected for functional screening based on their bioactive potential, they were *Lactobacillus plantarum* AG40V, *Enterococcus sp.* ID11V and *Enterococcus sp.* ID19V.

These three isolates were selected for screening of short chain fatty acids (SCFA) synthesis to find probable health applications. SCFA plays vital role in physiological functioning of colonic function, reduction in inflammation, offer protection against colon
carcinogenesis (Gim et al., 2012). Both butyric acid and propionic acid activates the apoptosis in colon cancer cells, helps in mineral absorption, mineral uptake resulting in recovery from diarrhoea (Topping et al., 2003), propionic acid may inhibit the synthesis of cholesterol in liver (Zhao et al., 2006). In the present study the SCFA synthesis ability of the probiotic bacterium was tested in vitro in MRS growth medium with the glucose as a carbon source. The chromatographic separation indicates the secretion of SCFA propionic acid and acetic acid from all three selected probiotic bacterium but the butyric acid synthesis was minimum. The synthesis of the SCFA is dependent on the source of carbohydrates. The previous reports observed no SCFA synthesis in presence of glucose but 10 fold higher production in presence of pectin (Nazzaro et al., 2012). The synthesis of SCFA is also strain specific; Pessione (2015) observed difference with 14 different Lactobacillus plantarum strains in production of butyric acid and propionic acid (Pessione et al., 2015). Many previous reports suggests the highest variability in synthesis of butyric acid and propionic acid (Mcfarlane et al., 2003; Mills et al., 2000). The secretion of SCFA is also dependent on the type of diet and the metabolism pattern followed by the bacterium such as heterofermenters are less SCFA producer whereas the facultative homofermenters produce higher amount of SCFA (London et al., 1990; Axelsson et al., 1998 and Pessione et al., 2015). Our strains showed the production of SCFA in the growth medium. After the study of metabolites secreted the strains further studied for any adverse genotoxic effect.

In vitro genotoxicity testing of probiotic strains targeted for health application is important both for safety assessment as well as for evaluating their potentiality to confer protection against oxidatively induced DNA damage. Several infections and disease conditions are either aggravated or are a result of DNA damage due to reactive oxygen species (Nawani et al., 2010). In the present study the Lactobacillus sp. isolated from three different sources (Lactobacillus sp. Ha1-HF from human faeces, Lactobacillus sp. Ba1-FB from fermented food and Lactobacillus plantarum AG40V from vegetable source) were tested for genotoxicity effect on peripheral blood mononuclear cells (PBMCs). The protective effects of LAB cell lysate on cell damage were assessed by alkaline comet assay (Singh et al., 1988). The extent of DNA damage is measured by the percent DNA in the comet tail, a higher percentage indicates greater DNA damage. In safety assessment studies, it was evident that none of the Lactobacilli induced DNA damage in PBMCs. Their values of percent DNA in tail did not differ significantly from that of untreated PBMCs, validating the safety of these Lactobacilli. Overall, the percent DNA in tail did not exceed 15% for PBMCs treated with the Lactobacilli strains chosen for safety assessment. The
ability of LAB cell lysates to confer protective effects on PBMCs from oxidative DNA damage was also determined. The protection offered by Lactobacillus sp. Ha1-HF was highest followed by Lactobacillus sp. Ba1-FB and Lactobacillus plantarum AG40V. Lactobacillus sp. Ha1-HF was a potential strain as it could minimize the DNA damage to value significantly different than other Lactobacilli and offered two-fold protection than that given by quercetin. Lactobacillus sp. Ha1-HF displayed better lipid peroxidation inhibition ability than Lactobacillus sp. Ba1-FB and Lactobacillus plantarum AG40V, which suggests that Lactobacillus sp. Ha1-HF may be having additional antioxidant abilities. This can be attributed to their origin where they may be exposed to a higher cellular oxidative stress as compared to other two sources of isolation. Lactobacillus sp. Ba1-FB and Lactobacillus plantarum AG40V offered protection from DNA damage at par than that given by quercetin that has been reported to inhibit hydrogen peroxide induced DNA damage (Min & Ebeler, 2009). The protective effects of Lactobacillus acidophilus isolated from Korean-fermented vegetable kimchi; against H₂O₂ induced genotoxicity on human derived cell line HT-29 were reported in previous study (Chang et al., 2010). Such probiotic bacteria may be of particular relevance to pharmaceutical industry. As the strains found synthesizing various active metabolites and found non toxic and exhibiting protective effect on oxidative damage are desirable for the use of these strains in health applications. Before the in vitro health application of the strains in probiotic formulation the strains were tested on intestinal cancerous cell line Caco-2. In this study the probiotic bacterial metabolites were tested for gene modulatory effect having health benefits.

In order to study gene modulatory effect and it’s therapeutic application of these three strains Lactobacillus plantarum AG40V, Enterococcus sp. ID11V and Enterococcus sp. ID19V were exposed to intestinal cancerous cell line Caco-2. In-vitro Caco-2 culturing and exposure to conditioned medium of probiotic cell mimicking the intestinal inflammatory condition of cancerous patients. Gene modulation pattern due to metabolite was studied with key functioning in inflammation and apoptosis. Caco-2 cell line exposed to conditioned medium of probiotic Lactobacillus plantarum AG40V, Enterococcus sp. ID11V and Enterococcus sp. resulted in secretion of different chemokine and cytokine expressing immune modulatory function. Along with inflammation few genes targeted NF-κB activation mediator of apoptosis process. This induction of apoptosis previously reported by Ruemmele et al. (2003) due to secretion of butyrate, tyramine (Ai et al., 2016), stachyose (Huang et al., 2014) in Caco-2 cells by Lactobacillus and Enterococcus in line to this as we observed significant increase in genes in NF-κB mediated, FAS receptor
ligand activated, BCL2 mediated apoptosis in all three cultures. Significant upregulation of FAS receptor in Enterococcus sp. ID11V metabolite found to be important in activation of apoptosis in Caco-2 cells. As intestinal cancerous cells are observed with apoptosis indicates promising application of these strains in colorectal cancer treatment. Along with apoptosis many other genes such as GBP-1, TBX21, LMD1, TGFB etc found to be involved in different types of inflammation and in case of IBD. Downregulation of these genes found important in secretion of anti-inflammatory metabolites by these three strains. Different cytokine and chemokines secreted in Caco-2 in presence of Lactobacillus and Enterococcus is in line to the observation by Jiang et al. (2012) with Lactobacillus acidophilus. Another set of gene found to be expressed in intestinal integrity function. Bron et al., (2016) found that probiotics modulates human diseases by impating intestinal barrier function in line to this exposure of the metabolites found differential expression in CLDN12 and PCDH1 which are involved in calcium dependent cell-cell adhesion indicating metabolites roll in intestinal adhesion. These positive gene modulatory effect of selected probiotic bacterial metabolite was further confirmed by animal studies. According to FSSAI and WHO any strain before dosing to the animals need to be tested for pattern of antibiotic susceptibility.

Lactobacillus plantarum AG40V, Enterococcus sp. ID11V and Enterococcus sp. ID19V were further selected for preparation of formulations and hence were tested for their antibiotic susceptibility pattern. As per the FSSAI and WHO guidelines, the probiotic bacterium used in the formulation should be sensitive to different antibiotics; as genes conferring antibiotic resistance should not be transferred to the host consuming probiotic formulation; considering global concern for the emergence and dissemination of antibiotic resistance. According to EFSA, drug resistance mediated by intrinsic mechanisms and chromosomally acquired gene mutations exhibits low risk of clinical ramifications and such probiotic strains should be regarded as safe for human consumption, whereas acquired resistance determinants on mobile genetic elements represents a serious safety issue for public health (Gueimonde et al., 2013). In the previous study, five strains of LAB were found to be resistant to gentamycin. Most of the tested strains showed lower resistance to chloramphenicol. The reference strain Lactobacillus casei Shirota was resistant for chloramphenicol (Pavli et al., 2016). In our study, Lactobacillus plantarum AG40V exhibited resistance to gentamicin whereas Enterococcus sp. were found sensitive to gentamicin. All the tested strains were sensitive to chloramphenicol. Conclusively, Lactobacillus plantarum AG40V was found sensitive to β-lactam group of antibiotics.
whereas *Enterococcus* sp. ID11V and *Enterococcus* sp. ID19V exhibited sensitivity towards \( \beta \)-lactam and aminoglycoside antibiotics; indicating their safety for probiotic formulation preparation.

After successful *in vitro* testing of vegetable isolates for probiotic and bioactive properties the potent strains representing different genus; *Lactobacillus plantarum* AG40V, *Enterococcus* sp. ID11V and *Enterococcus* sp. ID19V were selected for probiotic formulation preparation. As a probiotic formulations are suitable method for delivery of probiotic bacterium (Govender *et al*., 2014) this method was chosen for dosing of animals. The majority of probiotic bacteria do not have the capacity to survive under the adverse conditions such as the strong acidity and the presence of bile salts in the gastrointestinal tract (GI). In order to overcome this drawback, different probiotic formulations were paprepared and tested for their survival rate in GI tract.

In India as per Food Safety Standards Act of India (FSSAI, 2006) probiotics are concerned as a special third category ‘Food for Special Dietary uses /Functional Foods /Nutraceuticals/Health supplements’. The present study targets the preparation of stable probiotic formulation with probiotic bacterium consortium and testing the effectiveness in suitable animal model.

After *in vitro* safety assessment the potential strains were selected for probiotic formulation preparation. In present study selected plant probiotic potential was compared with the isolates obtained from other sources such as fermented food and human sources on suitable animal model. The selected plant probiotics for probiotic formulation preparation were *Lactobacillus plantarum* AG40V, *Enterococcus* sp. ID11V and *Enterococcus* sp. ID19V. Although *Lactobacillus* sp. enjoying GRAS status, *Enterococcus* sp. is emerging as probiotic bacterium but not yet recognized under GRAS status by FSSAI. The present study is evident for the use of *Enterococcus* sp. in probiotic formulation preparation and positive health benefits on animals. *Lactobacillus plantarum* AG40V found best player from all other *Lactobacillus* strains so selected and was common in all four probiotic formulations. *Enterococcus* sp. ID11V and ID 19V was the best candidates fulfilling all basic criteria and proved safe *in vitro* was selected from vegetable source for preparation of probiotic formulations. To compare the plant probiotic potential with other sources two strains *Lactobacillus* sp. HO17HF (Human source) and *Lactobacillus* sp. SK2FB was chosen in formulation preparation. FSSAI approved prebiotic ingredients were used in probiotic formulations. After preparation stability study of probiotic formulation is extremely necessary for consistent efficacy of the product; all
probiotic formulations from the present study was maintained successfully at 4°C for 60 days and all found stable.

These four probiotic formulations were tested for acute, sub-acute and efficacy on suitable animal model. Inline to many studies proved health applicability of probiotic formulation like one which was prepared by Cha et al. (2014) with Lactobacillus casei, L. plantarum, L. rhamnosus and Bifidobacterium lactis successfully suppressing the chemically induced inflammation in mice. Despite Lactobacillus carries GRAS status and Enterococcus found safe (Mansour et al., 2014) and used in different commercially used probiotic formulations. Lactobacillus isolates L. coryniformis CECT5711 and L. gasseri CECT5711 found safe on BALB/c mice (Villoslada et al., 2007). Another study with L. rhamnosus HN001 and L. acidophilus strains found safe on BALB/c mice (Zhou et al., 2000) Even the Enterococcus sp. is normal resident flora of human gut; safety of the genus is controversial and strain specific safety testing is more essential. Enterococcus faecium 2C found safe in study done by Khalkhali & Mojgani, (2018) Inline to this probiotic Formulation 1 Lactobacillus plantarum AG40 and Enterococcus sp ID11V and Formulation 2 Lactobacillus plantarum AG40 and Enterococcus sp IDI9V. Contrast results were obtained in study done by Uzoke and group on different strains of Enterococcus sp., they found toxic effect Enterococcus ratti, suspicious results with Enterococcus avium, Enterococcus gallinarum, Enterococcus faecium and Enterococcus faecalis while Enterococcus porcinus, Enterococcus dispar, Enterococcus munditi, Enterococcus hirae and Enterococcus cecorum as safe tested on Albino rats (Uzokwe et al., 2014). In our study all four probiotic formulations does not caused any mortality, no weight loss and no abnormalities in gross necropsy were found in tested on Swiss albino mice in animal studies.

According to OECD guidelines subacute oral toxicity was recommended for 28-days to study the effect of repeted dosing of probiotic formulation on host. To monitor effect of repeated dose oral toxicity exposure of probiotic bacterium for 28 days on Sprague-Dawley rat was carried out with four probiotic formulations individually. None of the orally dosed formulation caused any sign of toxicity at a dose of 1x10⁹ CFU/kg BW/day. No toxicological significant difference, no changes in feed consumption and general and behavioral conditions were observed on Sprague-Dawley animals dosed with probiotic formulation and control not dosed with probiotic formulation. In present study average weekly feed consumption was similar for male and female animals in all study groups. Formulation 1 Lactobacillus plantarum AG40V + Enterococcus sp. ID11V and
Formulation 2 *Lactobacillus plantarum* AG40V + *Enterococcus* sp. ID19V was found to be more safe and no adverse effects was observed in male and females rats.

Previous study done by the Starke et al. (2015) showed *in vitro* effects of *Enterococcus faecium* NCIMB 10415 co-culture with *Lactobacillus* spp. indicating enhancement of growth of probiotic *Lactobacillus* spp. supports the present study. Different studies by Botes et al. (2008), Guo et al., 2016 and Khalkhali & Mojgani, 2018 indicated *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423, *Enterococcus faecium* 2C safe for human and animals. Probiotic formulation 3 was combination of *Lactobacillus plantarum* AG40V + *Lactobacillus* sp.HO17HF and formulation 4 *Lactobacillus plantarum* AG40V + *Lactobacillus* sp.SK2FB passed the acute and subacute oral toxicity. The strains used in formulation 3 *Lactobacillus* sp.HO17HF (human faeces) and *Lactobacillus* sp.SK2FB (fermented food) in formulation 4 were from different sources. Plant probiotic strains were used in Formulation 1 and 2 such as *Lactobacillus plantarum* AG40V, *Enterococcus* sp. ID11V and *Enterococcus* sp. ID19V found at par in their safety and potential with isolates from human and fermented food source.

Antibiotic sensitivity study is important to check the probiotic toxicity on host as horizontal gene transfer is another major cause of pathogenesis. Although very few, some cases are reported before of bacteraemia associated with probiotic therapy (Cannon et al., 2005; Land et al., 2005). Study on safety assessment of *Lactobacillus* probiotic strains done by Lara-Villoslada and group (2007) observed few bacterial cells from liver and spleen, but the bacterial incidence was from probiotic treated mice and control mice both indicating the translocation not associated with probiotic treatment. In the present study the blood and tissue extracts of organs were screened for presence of bacterial translocation as indicator of pathogenicity. In present study the oral dosing of the probiotic formulation does not indicated any bacterial translocation in the blood and different organs and absence of histologically detected pathologies with all four probiotic formulation. Similar observations, absence of bacterial translocation, in a study done by Songisepp and group (2012) while studying safety of probiotic cheese containing *L.plantarum*.

Faecal sample analysis of probiotic formulation fed on SD rats for cultivable bacteria was done on MRS agar to study the probiotic bacterial survival pattern. Formulation 1 displayed the survival of *Lactobacillus* and *Enterococcus* in GI tract and successive increase in count of LABs probiotic formulation feeding progresses. Indicating *Lactobacillus plantarum* AG40V and *Enterococcus* sp. ID 11V and ID 19V isolated from vegetable surface successfully shown the probiotic effect in Sprague-Dawley
rats. Similar results were obtained from the isolates selected from different sources; human source (Lactobacillus sp. HO17HF) and fermented food (Lactobacillus sp. SK2FB). Apart from successful passage through GI tract of Sprague-Dawley rats all probiotic formulations exhibited the inhibition toward two common pathogens E.coli and Klebsiella sp.

Efficacy of all probiotic formulations was studied in chemically (TNBS) induced animals. Ethanol along with TNBS was used for induction of inflammation in colon, to study the anti-inflammatory effect of the probiotic formulation on animal model. Efficacy of probiotic formulation was compared with standard drug (Prednisolon). There was no statistically significant difference (p<0.05) among the groups for food intake. Animals from these groups exhibited weight loss and/or altered stool consistency (watery diarrhea) and/or blood in stool after a day of TNBS induction, indicating inflammation in intestine. Control group received neither TNBS induction nor the drug treatment no sign and symptoms of disease such as weight loss and changes in stool consistency etc. during the study period. Observations are consistent with other authors who used chemical (TNBS) method for disease induction (Morampudi et al., 2014; Szalai et al., 2014; Antoniou et al., 2016). After successful disease induction the animals from group (standard and all four test formulations treated group) exhibited recovery after feeding of standard drug and probiotic formulations, respectively for the period of 21 days. Disease induction and recovery due the respective drug treatment was analysed using different markers such as nitric oxide level, catalase, LPO, MPO, fibrinogen (Bossuyt, 2006). GI tract is a key source of reactive oxygen species production; in IBD the intestinal epithelial cells, neutrophils and macrophages produces inflammatory cytokines producing oxidative stress. In IBD the inflammatory mediators are released due to destruction of tight junction and in increased permeability gastrointestinal epithelial cells (Moura et al., 2015).

In Inflammatory bowel disease the nitric oxide produced by macrophages is important parameter in the pathophysiology of tissue injury, inflammation and tissue damage. Serum nitrite concentration is measured as biomarker indicating active phase of both ulcerative colitis (UC) and Crohn’s disease (CD) (Keshavarzian, 2003; Avdagic et al., 2013). In present study nitric oxide level was monitored; to evaluate successful induction of IBD in SD rats and to study the effect of probiotic formulations. Significant increase in DC male and female rats compared to control rats indicate successful colitis induction and the significant decrease was observed in male and female rat indicating the recovery effect of the probiotic formulation one treatment. Formulation 2 exhibited non-
significant decrease in nitric oxide level. Formulation 3 exhibited significant decrease in nitric oxide level. Formulation 1 and 3 treatment found to be more promising in male and female as compared to the standard drug treated animal groups. Formulation 4 displayed non-significant decrease in male and non-significant decrease in females. Formulation 1 found best in nitric oxide reduction followed by Formulation 3 they reduced nitric oxide level as similar to normal control and reduction at par with standard drug in case of male and female animals. Formulation 2 and Formulation 4 found to be less effective in reduction of nitric oxide level in male and female both. Catalase level and lipid peroxidase LPO level is also a measure of the oxidative stress due to inflammation in the tissue. Study performed by the Rana et al. (2014) in role of oxidative stress and antioxidant level in erythrocytes in UC patients found to be with increased levels of LPO and decreased level of catalase. In present study, catalase level found to be decreased in intestine of the DC male and female rats indicating TNBS induced colitis in SD rats and increased catalase were observed in Formulation 1 treated male and female animal rats at par with the standard drug. LPO level was found to be significantly increased in intestine of diseased control male and female rats due to colitis induction. LPO level was found to be significantly decreased in Formulation 1 administered male and female rats compared to the diseased control group animals and antioxidant production activity is at par with the standard drug as LPO level found to be far less than the animals treated with standard drug. Formulation 2 also lowered the LPO level in male and female both but decrease was not statistically significant. Formulation 3 also reduced the LPO level results were more significant in female as compared to male rats. The results obtained in vivo supports the ability of the antioxidant production done in vitro with the same isolates. Formulation four also exhibited statistically non-significant decrease in male and female rats. Along with other molecules the antioxidant synthesized by all the five probiotic cultures selected for probiotic formulation preparation reduced the LPO level in the rats. Formulation 1 and Formulation 3 exhibited maximum antioxidant production ability as compared to the formulation two and four. Serum myeloperoxidase level (MPO) act as a key marker for endothelial dysfunction and inflammation in the colon tissues (Odobasic et al., 2014; Kim et al., 2012). In the present study statistically non-significant decrease in MPO level was observed in Formulation 1 treated animals and statistically significant decrease was observed in DC female animals as compared to control and Formulation 1 treated animals compared to DC female indicating anti-inflammatory effect of probiotic formulation one which is at par with standard drug. Formulation 2, 3 and 4 exhibited decrease more
significant in females as compared to male rats. In conclusion, all probiotic formulations found to be effective in decreasing MPO level. Similar results were observed in female SD rats on MPO level in TNBS induced colonic model by Wan Yue-Meng; on product Peifeikang (PFK) a probiotic compound (Yue-meng et al., 2011).

Similarly to our experiment other recent studies demonstrated the positive effect of probiotic. Probiotic beverage composed of *Enterococcus faecium* CRL 183 and *Bifidobacterium longum* ATCC 15707 showed positive effect in rats with chemically induced colitis (Celiberto et al., 2017)

As Formulation 1 and 2 was mixture of *Lactobacillus* and *Enterococcus* ;another study using *Bifidobacterium*, *Lactobacillus* and *Enterococcus* on TNBS induced SD rats effectively ameliorate colitis with serum levels of cytokines (Wan et al., 2010). Formulation 3 and 4 was with *Lactobacillus* similar results were obtained in study with mixed probiotic formulation *B. lactis*, *L. casei*, *L. plantarum* and *L. rhamnosus* done by Yeon Suk Cha exhibited protective effect by supressing serum nitric oxide level in TNBS induced ICR mice (Cha et al., 2014). Another probiotic product Lacteol fort probiotic product mixture of *L. delbrueckii* and *L. fermentum* was significantly decreased in the MPO activity and found promising in UC patients (Hegazy et al., 2010).

One of the probiotic product, VSL# 3, is a cocktail containing *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum* and *Streptococcus salivarius* proved in the treatment of ulcerative colitis (UC) (Tursi et al., 2010). Eight week treatment of this reduces the clinical symptoms and endoscopic appearance of colonic mucosa in UC compared to placebo. In line to this probiotic formulation from the study found effective in reduction of the inflammation in colon compared with control group.

Danone product mixture of three LABLAB*Streptococcus thermophilus* DN-001 147, *Streptococcus thermophilus* DN-001 339 and *Lactobacillus bulgaricus* DN-100 182 used as starter for preparation of fermented food for antidiarrhoeal products. Bacteria found rapidly grower in milk producing lactic acid. Yoghurt prepared found effective in rotavirus diarrhea (Bouley et al., 1995) similar to this strains from this study carries the antibacterial activity against common human pathogen with fermentation ability with additional potentials can be for preparation of commercial product.

Yakult one of the most commercialy demanded product contains *Lactobacillus casei* strain shirotta (LcS) claims for intestinal health (Wong, 2013) in comparison to this formulation prepared in this study carries potential to overall intestinal health including
antipathogenic, antioxidant, anti-inflammatory property. Most important is formulation prepared with indigenous Lactic acid bacterial strains must be more beneficial for the Indian population.

Dairy industries are in demand of the rapid and natural coagulation processes for production of different dairy products. To study other commercial applicability of the probiotic isolates milk curdling ability was tested. All the *Lactobacilli* were able to initiate the milk curdling after 24 h but within 36 h of inoculation indicating their basic fermentative attribute indicating it’s commercial applicability.

Several physical and chemical techniques are routinely used for preserving foodstuffs, agricultural products, and pharmaceuticals etc. Biological materials, however, can be irreversibly damaged during these treatments. Therefore, it is essential to design protective agents to preserve food nutritional quality and texture. LAB being natural flora on vegetables; their prospective application for prevention of vegetable spoilage explores a new upcoming interest in the bio-preservation technology. In the present study *Lactobacillus plantarum* AG40V was tested for *in vivo* applicability in preservation. This isolate successfully reduced the growth of *Pseudomonas* sp., Yeast and moulds during the storage at room temperature on fresh salads (tomato and lettuce) and sprouts (matki).

Similar results were reported by Yang (2012); when tested *Enterococcus faecium, Streptococcus thermophilus* and *Lactobacillus casei* on fresh-cut yellow onions processed at commercial facility. These strains together successfully reduced *Pseudomonas* sp. and Lac+ Enterobacteriaceae during storage at 5°C for 12 days. Another study by Sharpe (2009) with *Lactococcus lactis* and *Enterococcus faecium* on fresh-cut salads remarkable reduced the growth of *Pseudomonas* sp., yeasts and total coliforms. The usefulness of the LAB in preventing the spoilage of fruits, vegetables and other food products was also explained by Sathe (2007) and Trias (2008). This study indicates the applicability of the *Lactobacillus plantarum* AG40V to increase the shelf-life of ready-to-eat cut salads and sprouts besides conferring possible probiotic benefits as demonstrated in this study. A step further is the use of probiotic edible films prepared with LAB strains and/or with its metabolites which offer dual benefits of increasing shelf-life and enhancing the functional value of the food products. Entrapment of the probiotic LAB in the films prevents their growth on the food product (Soukoulis et al., 2014). This extends the application of the probiotic bacteria in different areas of food technology.