5. DISCUSSION

5.1. Discussion

The intestinal gut microbiota which is about 100 trillion microbial cells are harbour in the gut and played an important role in human physiology, metabolism, nutrition and immune function. Disruption of the gut microbiota causes many diseases. The intestinal microbiota of healthy individuals is known to confer a number of health benefits such as pathogen protection, nutrition, host metabolism and immune modulation (O’Hara and Shanahan, 2006; Sekirov et al., 2010). It is thought that the effective therapies for many disease conditions might be based on working with gut microbiota. Probiotics could be life-saving and life-prolonging therapy. Probiotic therapy is considered as a potential therapeutic agents for over many decades but recently medical science has attempted to use probiotic for the dietary management of many diseases and research interest in the field of probiotic gain its momentum recently.The recognition of LAB as a part of the human microbial ecosystem and the understanding of various interconnected influences of that system is an important step for their application as healthy functional food. (Tannock, 2004; Vaughan et al., 2005). Researchers have focused their attention on the isolation and characterisation of novel potential probiotic strains from different sources, primarily the gastrointestinal tracts of animals and human subjects, human milk and less frequently, fruits and fermented foods (Baruzzi et al., 2011; Cho et.al., 2009; Martí‘n et al., 2003; Prins et al., 2010; Verdenelli et al., 2009)

A probiotic strain survived better to exert its function in the same environment from where it was isolated and GIT of infant is the rich source of such preparation. There are few reports on the probiotic characterisation of lactic acid bacteria from the infant faeces. Therefore, it is preferable to isolate LAB from the faeces of the infant since the microorganisms that isolated from the healthy infant faeces would be more likely to colonize in the human intestine. Therefore the objective of this experiment was to select the strain of human origin which possesses probiotic properties.

In this investigation, the search for novel probiotic was attempted by isolating 70 LAB isolates from the 14 faecal samples of infant who are in the age group of 1-1.8
years. The identification of LAB at the species level by conventional methods accurately is a difficult task. However due to large number of samples, conventional method of identification still acts as a useful tool in the identification of the bacteria. The preliminary identification of the isolates showed that they belong to LAB as they show specification of the LAB, except one isolate SW8 which is doubtful as it was catalase positive. *Lactobacillus* is the main genus with rod in shape, while *Lactococcus, Streptococcus, Enterococcus* and *Leuconostoc* are cocci (Axelsson, 1998). In our present investigation, the genus *Lactobacillus* was isolated in maximum number followed by *Enterococcus* and *Leuconostoc*.

In our study, *L. acidophilous* was the most recovered species from infant faecal sample which is in agreement with Khalil et al. (2007) who reported that *L. acidophilous* was the most recovered species from the infant faecal sample. In our phenotypic identification, three isolates of *Lactobacilli* could not be identified up to species as it is difficult for the definite identification of *Lactobacilli* by phenotypic methods only. The isolates SW8 could not be identified. Previous finding showed that obligate homofermentative lactobacilli which are typical of the GIT of human host consist of genetically close species, such as strains of *L. acidophilus*, *L. gasseri*, *L. crispatus*, *L. johnsonii* and *L. delbrukeii* which might also share fermentation profile (Arici et al., 2004; Vandamme et al., 1996; Morelli, 2007).

It had been reported that *L. acidophilus*, *L. plantarum* and *L. fermentum* were commonly found in the faeces of humans (Finegold et al., 1977). Other recovered species in our study were *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. paracasei*, *L. pentosus* and *Leuconostoc paramesenteroid*. The predominance of *L. plantarum* in our study is also correlated to introduction of solid foods in the diet of the infant. The predominance of *L.rhamnosus* was also reported in the faecal sample of infant under 2 years old (Arici et al., 2004). *L. plantarum* was isolated from the faeces of 5-6 months infants (Khalil et al., 2007). They were also isolated from the Estonian and Swedish infants who were in the age group of 1-2 years (Mikelsaar et al., 1993). The Enterococcus identified in our study were *E. faecalis* and *E. faecium*, they were also reported to be isolated from the infant faeces (Khalil et al., 2007).

*In vitro* studies only partially mimic the gut ecosystem but act as a useful screening tool for the selection of LAB for further *in vivo* testing. As reported in some
previous cases, none of the LAB strains grew at pH 2.5 and only very few were reasonably acid tolerant (Jacobsen et al., 1999). The finding of Xanthopoulos et al. (2000) showed that Lactobacillus paracasei and Lactobacillus rhamnosus survived better at pH 3 than the other tested strain. Gupta et al. (1996) observed that only two out of seven Lactobacillus acidophilus strains tested showed growth at pH 3. In our finding the survivability of the isolates at pH 5 were insignificant as all the isolates grew well whereas there were variability of growth of the isolates at pH 2.5. As compared to earlier reports, our findings were interesting as the isolates were able to grow at pH 2.5 after 24 hour of incubation. Conway et al. (1987) observed better survival rate of L. acidophilus when compared to L. bulgaricus and Streptococcus thermophilus at low pH values. According to Erkkila and Petaja (2000), the decrease in the survival rate of probiotic strains under conditions that simulate the transit through the gastrointestinal tract depends on the strain used, and thus a proper selection of strains in the development of dairy probiotic products is vital. At low pH undissociated lipophilic acid molecules of weak acids inhibit microorganisms by entering the cells and dissociating to hydrogen ion within the cells which causes a decrease of the internal pH (Adam and Moss, 1995; Girgis et al., 2003). Acid tolerance response (ATR) may be responsible for the ability of the LAB isolates to grow at low pH. ATR had been observed in Leuconostoc mesenteroides and Lactobacillus plantarum (McDonald et al., 1990), Lactococcus lactis (Hartke et al., 1996) and Enteroccus hirae (Belli and Marquis, 1991). The ability of bacteria to regulate their cytoplasmic or intracellular pH is the most important physiological requirements of the cells for growing under low pH. Proton-translocating ATPase is protein transporter in LAB that maintains pH homeostasis by means of pumping H+ out of cells (Hutkins and Nannen, 1993). Bacterial cells unable to maintain a near neutral intracellular pH during growth or storage at low extracellular pH may lose viability and cellular activity. Moreover, acid resistant bacteria showed a greater resistance to the membrane’s damage due to the decline in extracellular pH compared to non acid-resistant bacteria (Hutkins and Nannen, 1993).

Bile acids are amphipathic molecules with antimicrobial potential that act as a detergent and interfere with biological membrane (Lebeer et al., 2008). Bile salts can disorganize the structures of the cell membrane and cause dissociation of integral membrane protein, resulting in cell content leakage and cell death (Begley et al., 2005). In order to act as a probiotic, the microbes should be able to resist the stress
caused by bile and grow in the intestinal tract. Bile tolerance is more important than
tolerance to low pH as bile salt is more detrimental than the effects of low pH and
encapsulation can protect the bacteria from the harmful effects of low pH. The most
bile-resistant cultures which also possess other desirable characteristics should be
selected as a dietary adjunct (Gilliland and Walker, 1990). In the human GI-tract, the
mean bile salt concentration is believed to be 3000 ppm (0.3%), which is considered
as critical and high enough to screen for resistant strains (Gilliland et al., 1984; Goldin
and Gorbach, 1992). Isolate that showed 0.3% tolerance to bile could be used as
probiotic for swine (Pancheniak and Soccol, 2005). It is important to select a probiotic
strain with a high degree of bile resistance because strains with high tolerance to bile
develop better in the upper small intestine than strains with a low bile tolerance
(Gilliland et al., 1984). Many authors investigated the effect of bile on survival of LAB.
The differentiated tolerance of lactobacilli in the presence of bile salts was also
reported by Gardiner et al. (2002), which evaluated the tolerance of L. fermentum and
L. rhamnosus in different oxgall concentrations. The bile resistance of some strains
vary a lot among the lactic acid bacteria species and between strains themselves
(Xanthopoulos et al., 1997). Kim et al. (1999) examined the effect of bile concentration
in the range of 0 - 0.4% on the Lb. lactis survival and they reported inhibiting effect of
bile at concentration over 0.04%. Xanthopoulos et al (2000) reported survival of 10.3%
to 57.4% of human origin Lactobacillus acidophilus, Lactobacillus gasseri,
Lactobacillus rhamnosus and Lactobacillus reuteri after treatment at 0.15% bile salts.
Khalil et al. (2007) reported that Lb.plantarum (P1, P16 and P167) and Lb fermentum
(P193, P10) were the most bile resistant strain at 0.4% bile treatment for 3 hours and
poor growth was observed in Lb plantarum, Lb paracasei and Enterococcus cultures.

In our present investigation of bile tolerance the isolates L.acidophilus (ST8, SH10, SN2, SW7, ST10), L. fermentum (SH1, SH2, ST6), L. plantarum (SN8, SH), L.
rhamnosus (C4, SC6) E. faecium (SH9, SC10, SA1, SW5, S16), E. faecalis (SW9),
Leuconostoc paramesenteroides (ST9) and SW8 were the most bile resistant strains at
0.5% and 1% bile concentration. Liong and Shah, (2005) reported that most strains of
Lb. acidophilus showed greater acid tolerance, in addition to growth in the presence of
bile salts that was ascribed to the high levels of secreted bile salt hydrolase. In our
study for bile tolerance, most strains of Lb. acidophilus and E. faecium showed bile
tolerance. The ability to resist the bile salt is due to the production of bile salt
hydrolase enzyme. Bile salt activity has been detected in Lactobacillus and
Enterococcus (Begley et al., 2006). Bile salt hydrolase enzyme is able to deconjugate bile salt to amino acids and cholesterols which leads to the reduction of the toxicity of bile acid on bacteria (De Smet et al., 1995). Previous studies have shown that there are two kinds of bile salt hydrolase enzyme i.e. taurodeoxycholic acid hydrolase and taurocholic acid hydrolase, the ability of these two enzymes in hydrolyzing bile salts is different (Moser and Savage, 2001). Therefore, the variation in the response of our tested isolates can be attributed to the differences in the activity of their bile salt hydrolase enzyme. However, other factors such as membrane characteristics and variation in surface properties may have influenced the bile tolerance of strains (Schär-Zammaretti and Ubbink, 2003; Begley et al., 2005). Among the 48 commercial and wild strains of LAB tested, Lb acidophilus was the most interesting species, because it showed high values of resistance to gastric juice and bile salts (Vinderola and Reinheimer 2003). Mituoka (1992) also reported that Lb. acidophilus is the most active strain in the small intestine of humans.

Biogenic amine (BA) are organic compound found in food such as yogurts, cheese, wine, beer, dry sausages and other fermented food (Cosansu, 2009). Food intoxication by biogenic amine causes a number of symptoms of increasing complexity such as headache, migraine, nausea, high blood pressure and even allergic reaction of strong intensity (Giraffa, 2002). LAB have the ability to decarboxylate free amino acids with their enzyme decarboxylase and it is strain specific. Therefore the potential probiotic candidate should be screen for the production of biogenic amine. Potential for BA formation has been reported for several groups of LAB (Joosten and Northold, 1989; Maijala, 1993; Pereira et al., 2001). Biogenic amines are considered as one of the undesirable biogenic metabolites of the starter or probiotic bacteria, need to be used as a criterion for selecting probiotics strains (Marine-Font et al., 1995; Pereira et al., 2001). Potential for BA formation has been reported for several groups of LAB (Joosten and Northold, 1989; Maijala, 1993; Pereira et al., 2001). Most of the qualitative screening procedures to determine the BA potential of micro-organisms involve the use of differential medium containing a pH indicator. Screening medium based on this concept have been reported and used to detect the BA forming bacterial strains (Joosten and Northold, 1989; Choudhury et al., 1990; Maijala, 1993; Bover-Cid and Holzapfel, 1999). The BA-positive result on the decarboxylase medium (DCM) is indicated by the change of colour in response to the indicator to a pH shift. The change in pH is dependent on the production of alkaline
amine from the amino acids in the medium. These alkaline amines are produced by microbial decarboxylation of amino acids. In our assay, positive reaction on decarboxylase broth was recorded when a purple colour occurred and negative reaction was recorded when yellow colour occurred and weak negative was recorded when slightly yellow colour occurred.

One of the most important properties of probiotic is protection against pathogen in the intestinal tract of the host (Suskovic et al., 2010). The production of antimicrobials is considered to be a pathogen-inhibiting mechanism which is exhibited by probiotic bacteria. In order to have an impact on the colonic flora it is important for probiotic strains to show antagonism against pathogenic bacteria via antimicrobial substance production (Saarela et al., 2000). The probiotic strain should have antagonism to at least one of the common pathogen which causes enteric diseases. The *E. Coli* is a common pathogenic bacterium which causes intestinal infection. It is the common cause of diarrhoea, urinary tract infection, pyogenic infection and septicemia. In our present investigation, *E. Coli* was used as an indicator organism for the antibacterial activity. The finding of Xanthopoulos et al (2000) showed that *L. paracasei* ssp. paracasei and *L. acidophilus* from the infant faeces had weak antibacterial activity (0.5mm and 1mm) on *E. coli* and *Y. enterocolitica*. In this assay of antibacterial activity, our finding were found to be higher as compare to the finding of Xanthopoulos et al (2000). The zones of inhibition of the isolates which have antibacterial activities were in the range of 1-5mm (exclusive of disc). The antimicrobial activity of the isolates may be due to the production of organic acids (lactic acid, acetic acid, formic acid), ethanol, diacetyl hydrogen peroxide or bacteriocins. Ehrmann et al. (2002) reported organic acid as the major factor of the antimicrobial multifactorial mechanism of LAB against harmful intestinal bacteria.

In the recent decade, releasing of antibiotics in biosphere seriously increased which leads to a strong selective pressure for the emergence and persistence of resistant LAB strains. There is concern over the possible spread of antibiotic resistance determinants from bacteria used in probiotic products. Routine testing of antibiotic susceptibility lactic acid bacteria (LAB) and bifidobacteria may be advisable for checking the biosafety of potential probiotic isolates (Florez et al., 2008).
Since LAB are naturally present in fermented food and GI tract and are also added as starter culture or probiotic bacteria in industrial food production, there has been concern about the raise of the antibiotic resistance of these beneficial bacteria. Although the use of LAB has a long and safe history and has acquired the ‘generally regarded as safe’ (GRAS) status, selected strains should be evaluated before use for its used as probiotic. Therefore, the evaluation of antibiotic susceptibility of potential probiotic strains is an important selection criterion due to the increase incidence of antibiotic resistance of LAB. In the present assay of antibiotic susceptibility, 8 antibiotics discs such as penicillin, ampicillin, nalidic acid, norfloxacin, gentamicin, kanamycin, tetracycline and vancomycin were used for the test. A low resistance of some lactobacilli to β-lactam antibiotics including penicillin has been reported (Danielsen and Wind, 2003; Mathur and Singh, 2005). Liasi et al. (2009) also found that lactobacilli isolated from the infant faeces were mostly susceptible to β-lactamase inhibitors. Arici et al (2004) reported that lactobacilli isolated from the infant faeces were susceptible to Penicillin G. In our present investigation, most of the isolates of LAB were found to be susceptible to penicillin and ampicillin. Our finding is in agreement with Liasi et al. (2009) who found that LAB strains were resistant to quinolines group of antibiotics-nalidixic acid and norfloxacin. Previous studies (Arici et al., 2004; Xanthopoulos et al., 2000) also reported that the lactobacilli isolated from the infant faeces were susceptible to tetracycline. In our finding, most of the isolates were susceptible to tetracycline. It has been reported that *Lactobacillus spp.* is generally susceptible to tetracycline (Rojo-Bezares et al., 2006; D'Aimmo et al., 2007). In the present finding, low resistance of isolates to aminoglycosides group of antibiotics were observed, however in earlier finding by Zhou et al., (2005) and Termerman et al., (2003) claimed that most lactobacillus and enterococcus were resistant to aminoglycoside antibiotics. Eight out of twenty one isolates were resistant to vancomycin.

According to the current definition of Schrezenmeir and deVrese (2001) a probiotic strain is expected to transiently persist in the GIT of the host and to reach high numbers of viable cells in the targeted part of the gut. Persistence is thought to be dependent on the mode of administration and on the intrinsic properties of the bacterial strain, including resistance to gastric acid and bile and adhesion to the intestinal epithelium or mucus (Tuomola et al., 2001). The adhesion of the bacteria to intestinal epithelium represents the first step in the colonization process (Tuomola et
al. 2001), and adhesion characteristics are important when selecting probiotics. Lactic acid bacteria can preferentially occupy a space or form a biofilm on the surface of intestinal lining that would otherwise be colonized by a pathogen. Thus LAB induces a competitive environment. Irrespective of the route of administration, the *L. plantarum* strains were detected for longer periods than was *L. salivarius* UCC118 in the feces of mice (Pavan et al., 2003). Vesa et al. (2000) have shown that *L. plantarum* NCIMB8826 reached the human ileum with survival rate of 7%, which was far superior to the survival rate of the other tested LAB (Vesa et al., 2000). In our invivo colonisation study, five strains of LAB were evaluated for their ability to persist in the GITs of mice and only the two LAB isolates SW8 and ST9 were able to transiently persist in the GIT of the mouse since they were detected in the faecal sample of mice during the feeding periods and after the feeding was stop. Goldin et al. (1992) demonstrated that 60-80% of individuals consuming *Lb. rhamnosus* GG excreted this strain for 3-4 days, but only 33% of the population after 7 days.

Although cholesterol is an important basic block for body tissues, elevated blood cholesterol is a well-known major risk factor for coronary heart diseases (Aloglu and Oner, 2006). WHO has predicted that, by 2030, cardiovascular diseases will remain the leading causes of death, affecting approximately 23.6 million people around the world (WHO, Geneva, Switzerland, 2009). Several animal and clinical trials have shown a positive association between cholesterol levels and the risks of coronary heart disease. Supplementation of diet with fermented dairy products or lactic acid bacteria containing dairy products has shown the potential to reduce serum cholesterol levels. Various approaches have been used to alleviate elevated cholesterol level, including the use of probiotics. Pharmacological agents that effectively reduce cholesterol levels are available for the treatment of high cholesterol; however, they are expensive and are known to have severe side effects (Bliznakov, 2002). Consumption of lactic acid bacteria is a natural way to alleviate the cholesterol. In our study, the mice (*n* = 5) were feed with the LAB isolates SW8, SC10, ST9, SH10 and SC2. Out of the 5 mice, the serum cholesterol of the two mice which received the isolates SW8 and ST9 were found to be less than the other treated mice and the control. The two isolates SW8 and ST9 which were able to adhere the intestinal epithelium of the mice have the hypocholesterolemic effect. The hypocholesterolemic effect may be due to the bile salt hydrolase activity which deconjugate the bile which result in the cholesterol breakdown.
Conventional method of identification is difficult and not reliable for the species confirmation. Molecular technique has to be used for the species confirmation. Phylogenetic analysis based on comparisons of 16S ribosomal DNA (rDNA) sequence data is now routinely used in the determination of taxonomic relationships between microorganisms and in the design of taxon-specific probes for use in microbial identification and molecular ecology studies (Amman et al., 1993; Ludwig and Schleifer, 1994; Olsen et al., 1994; Ward et al., 1995).

The rRNA gene is the most conserved DNA in all cells of the organism. Portions of the rDNA sequence from distantly related organisms are quite similar and sequences from distantly related organisms can be precisely aligned which makes the true differences easy to measure among the related organism. Therefore, the genes that encode the rRNA (rDNA) have been extensively used to determine taxonomy, phylogeny and the estimation of the rates of species divergence among bacteria. Therefore the comparison of 16S rDNA sequence can show evolutionary relationship among the microorganisms. In our present investigation the two LAB isolates SW8 and ST9 were identified by using 16SrDNA sequencing. In the conventional phenotypic identification in our experiment, the isolate ST9 was identified as *Leuconostoc paramesenteroid* and SW8 could not be identified. In the molecular identification, the two isolates ST9 and SW8 were identified as *Weissella paramesenteroides strain FMA204* and *Bacillus coagulans* respectively.

*Bacillus coagulans*, which is one of the most promising lactic acid producing spore-forming organism used as a probiotic agent, was originally described by Hammer in 1915 from the evaporated milk. They are spore forming lactic acid bacteria (SFLAB). SFLAB are a group of Gram-positive bacteria, sharing characteristics common to the genera *Bacillus* (spore forming, motile) and *Lactobacillus* (micro-aerophilic, lactic acid production) (Suzuki and Yamasato, 1994). *Bacillus coagulans* is one of the most promising spore-forming bacteria used as a probiotic agent, due to its ability to form spores endows probiotics with higher resistance to technological stresses during production and storage processes (Hyronimus et al., 2000). Since then the thermo-stability of *Bacillus coagulans* and its probiotic attributes explored by many researchers to develop a useful and stable probiotic culture for use in a tropical country like India (Lopamudra and Gandhi, 2004). Beside the *Lactobacillus* species, the SFLAB have a good resistance to different environmental stress (Hyronimus et al.,
SFLAB are already used as probiotic for human and animal. A number of products containing spores of *B. coagulans* are currently commercialized as probiotics for human use (Hong et al. 2005; La Rosa et al. 2003). Ratna Sudha et al., 2010 had identified a new isolate - *Bacillus coagulans* “Unique IS-2” from the human faeces and found to have promising probiotic properties.

*Weissella paramesenteroides* which is formerly known as *Leuconostoc paramesenteroides* was described by Garvie in 1986, is one of the predominant lactic acid bacteria (LAB) species in fresh vegetables and can even be found in processed meat substrates such as fermented sausages and dry salami (Björkroth et al., 2002). In a previous research study, volatile compounds which are known to be therapeutically important are produced by the *Leuconostoc paramesenteroides*, a Native Laboratory Isolate (Shobha and Agrawal, 2007). A study carried out by Shobharani and Agrawal (2011) isolated *Leuconostoc paramesenteroides* from cheddar cheese and found to posses probiotic characteristic.
6. CONCLUSIONS

6.1. Conclusions

With the increased in changing lifestyle and pollution, man are prone to many kind of infectious diseases and moreover with the increase incidence of antibiotic resistance of bacterial pathogen, antibiotics are less effective in curing the diseases. The solution for the existing problem lies in searching for the alternative therapy which should be effective and should not have any side effects.

Probiotic is the most promising solution for these existing problems. Probiotic which is a relatively new term meaning 'for life' is associated with those microorganisms which are beneficial for maintaining good health in human and animals. The concept of existence of the probiotics dates back to 19th century, when the Russian Scientist Eli Metchnikoff one of the founders of modern immunology stated that the longevity of the Bulgarian people were linked with their consumption of yoghurt. Over the past few decades, there is an increasing interest in the field of probiotics. The interests in probiotics also stems from the growing universal awareness among the consumers regarding the safety aspects related with chemical drugs. The emerging need of safe and natural therapeutics without any adverse effects could be one of the main reasons behind the expanding market of probiotics. Probiotics being endowed with large number of beneficial attributes offer tremendous opportunities for their extensive application in almost all segments including food, pharma and cosmetics. The WHO declared probiotics to be the next most important immune defense system when antibiotics are useless

LAB are the main probiotics groups of microorganisms. They can be isolated from the different sources as they are ubiquitous in nature and generally associated with habitats rich in nutrients such as various food products such as milk, meat, vegetables etc., normal flora of the mouth, intestine and vagina of mammals but for human application they should preferably of human origin. LAB are present in the gastrointestinal tract of the man. They are normal resident in the GIT of man and played a very beneficial role in the well being of the host. GIT of infant is a source of probiotic rich LAB. Limited studies are available on the probiotic characterization of LAB of human origin. From the scientific data, it is also evident that probiotic properties
are strain specific. In order to act as a probiotic the microbes should meet certain criteria such as the microbes should be able to resist the stress factor of low pH of stomach and the bile in the intestine. They should possess antimicrobial property and also should be least resistance to the commonly used antibiotics. They should also be able to adhere to the intestinal epithelium. They should also influence the human metabolic activities (cholesterol assimilation, vitamin production, etc). Therefore taking into account that there are different effects between the probiotic strains and those different strains which belong to the same species function differently with regards to physiological characteristics rendering them different or improved probiotic properties against other bacteria, the identification and characterization of the probiotic strains is very important.

Considering the importance of characterization of probiotics strains of the same species and its limited studies on LAB of human origin for its human application the present research work was focussed with the following objectives:

(i). To isolate and identify the Lactic Acid Bacteria (LAB) from the infant faeces by using conventional methods.

(ii). To determine the tolerance of low pH, bile acid and determine the production of biogenic amines.

(iii). To examine the antipathogenic activities of LAB on certain pathogen like *E. coli* and to study the antibiotic susceptibility of the selected LAB.

(iv). To evaluate the *in vivo* properties of LAB using animal model and quantitative estimation of effects of selected LAB on cholesterol of mice.

(v). To identify the probiotic LAB by using molecular technique.

Isolation and identification of LAB from the infant faeces in the age group of 1-1.8 years was carried out by using conventional phenotypic methods such as microscopic examination, catalase test, gas production from glucose, growth at different temperature and NaCl concentration, arginine hydrolysis test and sugar fermentation test. In this study 66 isolates were identified upto species and 3 isolates could not be identified upto species and one isolates was doubtful of being LAB so could not be identified. The
present research finding was the identification of *L. acidophilus* as the dominant LAB in the faeces of infant which is in consistent with the previous finding.

After the identification of the lactic acid bacteria by using conventional methods, the isolates were subjected to invitro probiotic characterization by applying different tests such as low pH, bile salt, biogenic amine, antimicrobial activity and antibiotic susceptibility. *In vitro* assays can give an indication of probiotic properties and can be the first part of the selection process. The isolates which were able to grow at low pH and bile underwent biogenic amine test. Biogenic amine test is also an important probiotic selection criteria as biogenic amine are harmful to health. Then the isolates which were negative to biogenic amine were tested for the antibacterial activities by using pathogenic *E. coli* as the indicator organism. Probiotic microorganism should possess antibacterial property to at least one of the common pathogen. Antibiotic susceptibility test was also done for the bacterial isolates which showed zone of inhibition in the antibacterial test. In the recent years, there is concerned over the possible spread of antibiotic resistance determinants from bacteria used in probiotic products so, it is important for the testing of the antibiotic susceptibility of the bacterial isolates which are used in the probiotic as a safety measures.

The best five isolates - SW8, ST9, SC10, SH10 and SN2 fulfil all the criteria of *in vitro* probiotic property and they were selected for the *in vivo* test and feeding of the bacterial isolates to the mice took place. Among the five isolates only the two isolates, SW8 and ST9 were able to adhere the mouse’s intestine as these isolates were detected in the faeces of mice which were fed with these isolates. The quantitative estimation of the cholesterol level indicated that the cholesterol level of the mice which fed the isolates SW8 and ST9 were lower than the mice which fed the isolates SC10, SH10, SN2 and the control mice.

Probiotics effects are known to be strain specific. Therefore, strain identity becomes important to associate a specific effect with a particular strain. Therefore, it is very important to perform proper identification in order to associate a specific effect with a particular strain. Molecular identification of the two probiotic strains SW8 and ST9 was done by using 16S rDNA sequence analysis and identified as *Bacillus coagulans* and *Weissella paramesenteroides strain FMA204* respectively.
The present research work isolated and identified a new isolate *Bacillus coagulans* SW8 as a probiotic. Even though the research study focussed on the isolation of LAB, the probiotic isolates SW8 was identified as lactic acid forming *Bacillus coagulans* by molecular characterisation. Nearest homolog of SW8 was found to be *Lactobacillus thermophilus* (GeneBank Accession Number: M58832.1 LBARR16SAD).

In the phenotypic identification, SW8 could not be identified as it is difficult to identify *Bacillus coagulans* by only phenotypic identification. *Bacillus coagulans* was originally known as *Lactobacillus. sporogenes*. The *Lactobacillus sporogenes* or as it should be classified, *Bacillus coagulans*, represents the archetypal misidentified probiotics and its annoveration among lactobacilli has often been matter of debate. In fact, since this bacterium shows characteristics of both genera *Lactobacillus* and *Bacillus*, its taxonomic position between the families Lactobacillaceae and Bacillaceae has often been discussed. They are not normal inhabitant of GIT. Being a spore former this strain could be of great commercial value because of their survivability in acidic, alkaline, heated and cold preserved foods and beverages as well and could open a new horizon in the field of biotherapy. *Bacillus coagulans* is found in several probiotic supplements currently available in the market. It is considered to be a superior probiotic than the other probiotic supplements due to its spore forming capacity, it has higher resistance to technological stresses during production and storage processes. The other probiotic strain (ST9) which was identified was *Weissella paramesenteroides* which is formerly known as *Leuconostoc paramesenteroides*. The other close homologs which were 0.99 alignment were *Weissella sp.* (GeneBank Accession Number: JX193634.1). They are generally found in plants and in dairy products and not the normal resident of GIT. They are transit microorganisms. This microbe was present in the GIT of infant due to the consumption of dairy products by the infant. At the best of my knowledge, this is the first research finding in India in which the probiotic strain *Weissella paramesenteroides* from the infant faeces was isolated and characterised as probiotic.

The characterization of the new probiotic strains *Bacillus coagulans* SW8 and *Weissella paramesenteroides* ST9 in the present research work would open a new horizon in the field of biotherapy. These two novel probiotics would act as a superior probiotics supplement for the human consumption. However, large numbers of controlled clinical trials and technological properties such as strain stability, viability in products are needed for the commercialization of these probiotics.
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### LIST OF CHEMICALS

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</tr>
<tr>
<td>7</td>
<td>Hydrogen Peroxide</td>
<td>025328</td>
</tr>
<tr>
<td>8</td>
<td>Agar</td>
<td>DM 1337</td>
</tr>
<tr>
<td>9</td>
<td>MacConkey Agar</td>
<td>MH081-100G</td>
</tr>
<tr>
<td>10</td>
<td>NaCl</td>
<td>RM853-500G</td>
</tr>
<tr>
<td>11</td>
<td>Peptone</td>
<td>RM001-500G</td>
</tr>
<tr>
<td>12</td>
<td>Phenol red</td>
<td>021030</td>
</tr>
<tr>
<td>13</td>
<td>Arginine(check)</td>
<td>Z00070</td>
</tr>
<tr>
<td>14</td>
<td>Nessler’s reagent</td>
<td>019086</td>
</tr>
<tr>
<td>15</td>
<td>Oxbile</td>
<td>CM1621</td>
</tr>
<tr>
<td>16</td>
<td>L-Histidine Monohydrochloride</td>
<td>037118</td>
</tr>
<tr>
<td>17</td>
<td>L-Ornithine Monohydrochloride</td>
<td>937191</td>
</tr>
<tr>
<td>18</td>
<td>L-Tyrosine</td>
<td>037156</td>
</tr>
<tr>
<td>19</td>
<td>L-Lysine Monohydrochloride</td>
<td>037129</td>
</tr>
<tr>
<td>20</td>
<td>Tryptone</td>
<td>CM1014</td>
</tr>
<tr>
<td>21</td>
<td>Yeast extract</td>
<td>023799</td>
</tr>
<tr>
<td></td>
<td>Ingredient</td>
<td>Code</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>22</td>
<td>Beef extract</td>
<td>033031</td>
</tr>
<tr>
<td>23</td>
<td>Glucose</td>
<td>RM1367-500G</td>
</tr>
<tr>
<td>24</td>
<td>MgSO4</td>
<td>024963</td>
</tr>
<tr>
<td>25</td>
<td>MnSO4</td>
<td>010355</td>
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<tr>
<td>26</td>
<td>FeSO4</td>
<td>028401</td>
</tr>
<tr>
<td>27</td>
<td>Ammonium citrate</td>
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</tr>
<tr>
<td>28</td>
<td>Calcium carbonate</td>
<td>010068</td>
</tr>
<tr>
<td>29</td>
<td>Actidione (Cycloheximide)</td>
<td>CMS5583-1G</td>
</tr>
<tr>
<td>30</td>
<td>Rifampicin</td>
<td>CMS1889-1G</td>
</tr>
</tbody>
</table>
RECIPES FOR CULTURE MEDIA AND BIOCHEMICAL TESTS

Peptone water

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 7.4 and autoclaved at 121°C for 15 minutes.

MRS agar (DM 1641)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10.000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.000</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.000</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.000</td>
</tr>
<tr>
<td>Ammonium citrate</td>
<td>2.000</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.000</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.100</td>
</tr>
</tbody>
</table>
Manganese sulphate 0.050
Dipotassium phosphate 2.000
Agar 12.000
Distilled water 1L

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 6.5 and autoclaved at 121°C for 15 minutes.

**MRS broth  (DM 1369)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10.000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.000</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.000</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.000</td>
</tr>
<tr>
<td>Ammonium citrate</td>
<td>2.000</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.000</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.100</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.050</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2.000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 6.5 and autoclaved at 121°C for 15 minutes.
**Modified MRS broth for growth at 6.5% NaCl**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10.000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.000</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.000</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.000</td>
</tr>
<tr>
<td>Ammonium citrate</td>
<td>2.000</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.000</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.100</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.050</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2.000</td>
</tr>
<tr>
<td>NaCl</td>
<td>65</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 6.5 and autoclaved at 121°C for 15 minutes.

**Phenol Red Glucose broth**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose Peptone</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.018</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1L</td>
</tr>
</tbody>
</table>
All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 7.3 and autoclaved at 121°C for 15 minutes.

**Modified MRS for arginine test**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.000</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.000</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.000</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.100</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.050</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2.000</td>
</tr>
<tr>
<td>NaCl</td>
<td>65</td>
</tr>
<tr>
<td>Arginine</td>
<td>.3</td>
</tr>
<tr>
<td>sodium citrate</td>
<td>2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 6.5 and autoclaved at 121°C for 15 minutes.

**Nessler reagent**

- Mercuric chloride (HgCl₂) 10.0 g
- Potassium iodide (KI) 7.0 g
- Sodium hydroxide (NaOH) 16.0 g
- Water (ammonia free) 100.0 ml
### Modified MRS for bile tolerance test

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10.00</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.00</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium citrate</td>
<td>2.00</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td></td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.10</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2.00</td>
</tr>
<tr>
<td>Oxgall</td>
<td>0.05%, 0.5%, and 1%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water.

### Decarboxylase media for biogenic amine test

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>Meat extract</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>25</td>
</tr>
</tbody>
</table>
Glucose 0.5
Tween 80 1
MgSO\textsubscript{4} 0.2
MnSO\textsubscript{4} 0.05
FeSO\textsubscript{4} 0.04
Ammonium Citrate 2
Calcium Carbonate 0.1
Amino acid 10
Bromocresol purple 0.06
Distilled water 1L

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 5.3 and autoclaved at 121°C for 15 minutes.

**Nutrient broth**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1L</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in distilled water. The pH of the medium was adjusted to 7.2 and autoclaved at 121°C for 15 minutes.
### CARBOHYDRATES USED FOR CARBOHYDRATE FERMENTATION TESTS

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the sugar</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arabinose</td>
<td>DD001-1VL</td>
</tr>
<tr>
<td>2</td>
<td>Cellobiose</td>
<td>DD028-1VL</td>
</tr>
<tr>
<td>3</td>
<td>Lactose</td>
<td>DD004-1VL</td>
</tr>
<tr>
<td>4</td>
<td>Galactose</td>
<td>DD016-1VL</td>
</tr>
<tr>
<td>5</td>
<td>Mannose</td>
<td>DD007-1VL</td>
</tr>
<tr>
<td>6</td>
<td>Rhamnose</td>
<td>DD010-1VL</td>
</tr>
<tr>
<td>7</td>
<td>Raffinose</td>
<td>DD029-1VL</td>
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<tr>
<td>8</td>
<td>Mannitol</td>
<td>DD006-1VL</td>
</tr>
<tr>
<td>9</td>
<td>Sorbitol</td>
<td>DD012-1VL</td>
</tr>
<tr>
<td>10</td>
<td>Trehalose</td>
<td>DD031-1VL</td>
</tr>
<tr>
<td>11</td>
<td>Xylose</td>
<td>DD014-1VL</td>
</tr>
<tr>
<td>12</td>
<td>Melibiose</td>
<td>DD030-1VL</td>
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<tr>
<td>13</td>
<td>Salicin</td>
<td>DD011-1VL</td>
</tr>
<tr>
<td>14</td>
<td>Fructose</td>
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<tr>
<td>15</td>
<td>Maltose</td>
<td>DD005-1VL</td>
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<tr>
<td>16</td>
<td>Sucrose</td>
<td>DD013-1VL</td>
</tr>
<tr>
<td>17</td>
<td>Ribose</td>
<td>RM197-25G</td>
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</table>
## ANTIBIOTIC SUSCEPTIBILITY DISC

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Name of the antibiotics</th>
<th>Levels</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Penicillin G</td>
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<td>SD028-1PK</td>
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<tr>
<td>2</td>
<td>Kanamycin</td>
<td>30 mcg</td>
<td>SD017-1PK</td>
</tr>
<tr>
<td>3</td>
<td>Vancomycin</td>
<td>30 mcg</td>
<td>SD045-1PK</td>
</tr>
<tr>
<td>4</td>
<td>Tetracycline</td>
<td>30 mcg</td>
<td>SD037-1PK</td>
</tr>
<tr>
<td>5</td>
<td>Gentamicin</td>
<td>120 mcg</td>
<td>SD195-1PK</td>
</tr>
<tr>
<td>6</td>
<td>Norfloxacin</td>
<td>10 mcg</td>
<td>SD057-1PK</td>
</tr>
<tr>
<td>7</td>
<td>Ampicillin</td>
<td>10 mcg</td>
<td>SD002-1PK</td>
</tr>
<tr>
<td>8</td>
<td>Nalidixic acid</td>
<td>30 mcg</td>
<td>SD021-1PK</td>
</tr>
</tbody>
</table>
MOLECULAR CHARACTERIZATION RECIPES

DNA Extraction Solution

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Pl. #</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>612104680501730</td>
<td>FC46</td>
<td>DNA Extraction Solution 50 ml, 1 EA</td>
</tr>
</tbody>
</table>

PCR Mixture

Genomic DNA: ~20ng

dNTP mix (2.5mM each): 1.0μl

Forward Primer: 100ng

Reverse Primer: 100ng

Taq Buffer A (10X): 1X

Taq Polymerase enzyme: 3U

Glass distilled water: to make up the volume 50μl

RESTRICTION ENZYMES