Behaviour of gut bacteria upon stress in infants with ileostomy due to Hirschsprung disease

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfilment of the Requirement for the Degree of Masters of Science in Biotechnology

Submitted by

Umama khan
Student ID- 14176005
Session: Spring-2014

Department of Mathematics and Natural Sciences

BRAC University

Mohakhali, Dhaka-1212

Bangladesh

June, 2017

Declaration

This is to declare that the research work embodying the results reported in this thesis entitled "**Behaviour of gut bacteria upon stress in infants with ileostomy due to Hirschsprung disease**" submitted by Umama Khan, has been carried out under the joint supervision and guidance of Professor Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka and Professor Dr. Mahboob Hossain, Microbiology Programme, Department of Mathematics and Natural Sciences, BRAC University in partial fulfilment of MS. in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

Candidate

Umama Khan

Certified:

Professor Dr. Chowdhury Rafiqul Ahsan

Supervisor

Department of Microbiology

University of Dhaka

Dhaka, Bangladesh

Professor Dr. Mahboob Hossain

Supervisor

Department of Mathematics and Natural Sciences

BRAC University

Dhaka-1212, Bangladesh

Dedication

To
My Beloved
Family

Acknowledgement

First and foremost, I articulate my gratitude to the Almighty who facilitated me to perform this research work and submit this dissertation.

My grateful thanks to Prof. A. A. Ziauddin Ahmad, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka, for his kind permission to continue my research work at BRAC University microbiology lab.

I am keen to express my respect and cordial thanks to Prof. Naiyyum Choudhury, Chairman, Bangladesh Atomic Energy Regulatory Authority (BAERA) and Ex-Coordinator of Biotechnology Programme, BRAC University, Dhaka for providing me the chance to work at the department of microbiology under supervision of Dr. Chowdhury Rafiqul Ahsan.

I am much beholden to my supervisor Dr. Chowdhury Rafiqul Ahsan, Professor, Department of Microbiology, University of Dhaka and would like to express my profound appreciation for his encouragement and supervision to enable me to complete the research.

I like to express earnest grateful thanks to my another supervisor Dr. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences BRAC University, Mohakhali, Dhaka, for his kind cooperation and active support throughout my research.

My heartfelt thanks to Dr. Aparna Islam, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka, for her counselling, amiable support and valuable suggestions regarding the work.

I like to express grateful thanks to the Ethical committee of Dhaka Shishu (Children) Hospital, Dhaka, for giving me the permission to collect sample throughout my research.

I would also like to thank my respected Lab officers, Asma Binte Afzal, Shahana Chowdhury and Shamim Akhter Chowdhury, Department of MNS, BRAC University who assisted me in solving many problems during the research work.

My keen gratitude to Shashwata Biswas, Md. Hassan-uz-Zaman, S.M. Tanjil Shah, Somen kumar Mistri, Department of Microbiology, University of Dhaka and Arup Kumar, Department of Microbiology, Noakhali Science and Technology, Noakhali, for their cordial and amiable support during the research work.

My special thanks to Maria Kibtia, Salman Khan, Nahreen Mirza, Md. Sajjad Hussain and Sadia Afsana, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, for their constant help and suggestions in daily work.

My heartfelt appreciation is extended to the staffs of the lab and department, who helped me even beyond their duty hours to continue my research work.

Last but not the least, I owe to my family, for their prolonged patience and nourishment towards my achievement.

Umama Khan,
June, 2017

Contents

Continued.

Continued.

List of Tables

List of Figures

List of Figures (Continued.)

Abbreviation

Abbreviation	Elaboration
HD	Hirschsprung disease
HAEC	Hirschsprung disease associated enterocolitis
M	Molar
N	Normolar
vol.	Volume
v/v	Volume/volume
%	Percentage
g	gravity
spp.	Species
U	Unit
UV	Ultra Violet
^{0}C	Degree Celsius
temp.	Temperature
V	Volt
et al.	and others
lb	Pound
IU	International unit
MW	Molecular weight
OD	Optical density
nm	Nano metre
µm	Micro molar
NA	Nutrient Agar
MRS Agar	De Man Rogosa Sharpe agar
NB	Nutrient broth
TCBS Agar	Thisulphate Citrate Bile salts Sucrose agar
EMB Agar	Eosin methylene blue agar

Continued.

Abbreviation	Elaboration
EDTA	Ethylene di amine tetra acetic acid
PBS	Phosphate buffer saline
TE	Tris EDTA
TBE	Tris Borate EDTA
PCR	Polymerase chain reaction
bp	Base pairs
c-section	Caesarean section
BMI	Body mass index

Abstract

Hirschsprung disease (HD) is a congenital one where the distal part of intestine lacks nerve ganglions, resulting in failure of defecation through anus. As treatment purpose, a stoma is created temporarily in the abdomen. Again, commensal gut bacteria play important roles for maintaining gut homeostasis and loss of harmony in these bacteria may lead to different diseases and may cause complications in already diseased individuals. Considering the stress level on gut bacteria in HD infants and the ability of commensals to endure strenuous conditions, in this study, we investigated the behavioural changes, if any, of the gut bacteria, specifically *Lactobacillus* spp. in HD infants.

Suspected *Lactobacillus* spp. were isolated from stool samples collected from stoma of HD infants and control *Lactobacillus* spp. were isolated from the stool samples of normal infants. After morphological evaluation and biochemical tests, the isolates were further confirmed by PCR with primers based on 16s rRNA. Representative isolates from both the groups were grown under different stress conditions, like pH (pH 2.5-8.5), NaCl (1%-10%) and bile (0.1%-0.3%). Also production of organic acids, antibiotic sensitivity and aggregation properties were checked. Effect of the isolates towards the growth of *Escherichia coli* and *Vibrio cholerae* were also evaluated using *in vitro* culture media and *in vivo* mouse model system.

Bacterial samples isolated from the stool of HD infant and normal infant survived under given stress conditions but optimal growth conditions varied. Again, the isolates from both infants produced acids during fermentations. Regarding antibiotic sensitivity, isolates from HD infant were found to be more prone to resistance. The auto-aggregation percentages were approximately within 70%-80% and co-aggregation percentages with other bacteria were found to be more for the normal one. Both *in vitro* and *in vivo* experiments demonstrated that the isolates from HD infant, were more capable of inhibiting the growth of *E. coli* and *V. cholerae*, when grown together. All these results indicate that, stress may cause dysbiosis of gut microbes and may lead towards different complications in HD babies.

Chapter 1:
Introduction...

1.0 Introduction

Human gut microbial community consists of different types of organisms which reside there through symbiotic activity (Hooper and Gordon, 2001). The alteration of this symbiosis can cause alteration of gut microbial structure which may lead towards various disease conditions (Murri et al., 2013; Pierre et al., 2014). On the other hand, commensal strains with probiotic effect help to maintain the homeostasis of human gut (Liévin-Le Moal and Servin, 2014). Among these commensal gut microbes, *Lactobacillus* spp. is one with probiotic properties, as it has high level of tolerance towards stressed conditions (De Angelis and Gobbetti, 2004) .

As for Hirschsprung disease (HD), it is a condition of aganglionosis of intestine which causes functional obstruction in the intestine and the infant fails to pass stool through anus (Whitehouse and Kernohan, 1948). Sometimes overgrowth of the bacterium stuck there may lead towards several complications, such as enterocolitis (Teitelbaum et al., 1989). So, stressed conditions due to aganglionosis and other factors are evident here for gut bacteria of HD babies (Ward et al., 2012).

In most of the cases of HD patients, the infant has to go through surgical procedures as treatment purpose and in first stage of surgery, a stoma is created in the abdomen to pass stool (Langer et al., 1996). Considering these factors, we have designed our study to compare stressed effects in *Lactobacillus* spp. isolated from stool, which are collected from stoma in HD infant and from the anus in normal infant.

Here, in this part, at first, we are to discuss several points about Hirschsprung disease, gut bacteria in early period of life and in HD infants, *Lactobacillus* spp. as gut bacteria and factors which influence the structure of human intestinal microbes.

1.1 Hirschsprung disease

Hirschsprung disease, a congenital anomaly, also known as congenital aganglionic megacolon, is characterised by the absence of enteric parasympathetic ganglion cells in the submucosal and myenteric plexuses, respectively Auerbach's and Meissner's plexuses along a variable length of the intestine (Shen et al., 2009; Whitehouse and Kernohan, 1948).

Here, the fact is, muscle contraction in the gut helps the digested foods and liquids to pass through the intestine. This wave like propelling movement of gut is called peristalsis which involves different factors as well as the nerves or ganglionic cells between the muscle layers (Shier et al., 1996).

In Hirschsprung disease, the nerves or ganglionic cells are absent from a part of the intestine causing functional obstruction as the neural crest-derived cells failed to form distal enteric nervous system (Heanue and Pachnis, 2007). Moreover, Hirschsprung disease, if untreated or after treatment, can be associated with enterocolitis, a serious complication and a major cause of morbidity and mortality (Teitelbaum et al., 1989). Furthermore, a rare but fatal consequence of this Hirschsprung disease can be unexplained perforation of caecum or appendix leading towards the death of the infant (Amiel et al., 2008).

1.1.1 Epidemiology

The epidemiological study for Hirschsprung disease had been carried out several times after its identification. At early twentieth century, it was estimated that Hirschsprung disease occurred at every 1/10,000 live births in England (Bodian and Carter, 1963). In Denmark, the rate is 0.140 per 1000 live births (Russell et al., 1994). Again in a study done by Spouge and Baird (1985) at British Columbia from 1964-1982, the incident rate found was 1 in 4,417 live birth. In Maryland, USA, the incident rate was 18.6 per 10,0000 live births during 1969-1977 (Goldberg, 1984). However, for Asian region, a study in Yangoon hospital, demonstrated about 44.3% laparotomy for Hirschsprung diseased baby

less than 6 months indicating its high incidence rate (Hlaing and Maung, 1989). Regarding male female ratio, most of these studies described about the higher incidence rate in male than female (Goldberg, 1984; Russell et al., 1994).

1.1.2 Associated anomaly

Many of the infants with Hirschsprung disease are associated with other congenital anomalies. According to Ryan et al. (1992), about 22% of these children and as per Spouge and Baird (1985), 29.8% of cases are associated with one or more different other abnormalities.

Among these anomalies, the most frequent one is Down's syndrome (Goldberg, 1984). After that the common anomalies are Fallots tetrology, cardiac septal defects, Dandy-Walker syndrome etc (Ryan et al., 1992). It also includes deafness, microcephaly, polydactyly, cleft palate, rectal and/or anal stenosis, imperforate anus, hydronephrosis, megalocystis, undescended testes etc (Amiel et al., 2008; Russell et al., 1994; Ryan et al., 1992; Spouge and Baird, 1985).

1.1.3 Clinical features and diagnosis

In most of the cases, Hirschsprung disease becomes a consideration at neonatal period, while the newborn fails to pass meconium within 24-48 hours of birth, which leads towards abdominal distension and can be relieved by rectal stimulation or enemas. Other sign symptoms include green bilious vomiting, poor feeding, jaundice etc. Sometimes it is associated with neonatal enterocolitis as complication (Amiel et al., 2008).

Several other reasons can be there for failure to pass faeces by newborn, as cystic fibrosis associated meconium ileus, meconium plug syndrome, ileal and/or rectal atresia, imperforate anus, anal fistula etc (Amiel et al., 2008; Kessmann, 2006). To diagnose if it is Hirschsprung disease or not, at first the plain x-ray abdomen then contrast enema can

give the view (Kessmann, 2006). But confirmation can be done after histopathological test, which proves the aganglionosis and hypertrophied nerve trunks (Khan et al., 2003).

1.1.4 Treatment and prognosis

As preliminary treatment purpose, temporary enema can be given to pass out the faeces from bowel. But the proper treatment is to go through surgical procedure (Amiel et al., 2008). After recognition of the disease, several types of surgical procedures have been practiced by the paediatric surgeons over time (Swenson, 1996). Among them, the most approached technique was described by Soave (1964), where primary pull through procedure of surgery came to light. With time, there is modification, where two stage surgery is to perform by creating temporary stoma at abdomen and then to close it a few months later up on recovery. A stoma in abdomen after ileostomy/colostomy is shown in figure 1.1. Several studies to check the short and long term outcome for this surgical treatment have been conducted and yet to discover more (Langer et al., 1996; Teitelbaum et al., 2000).

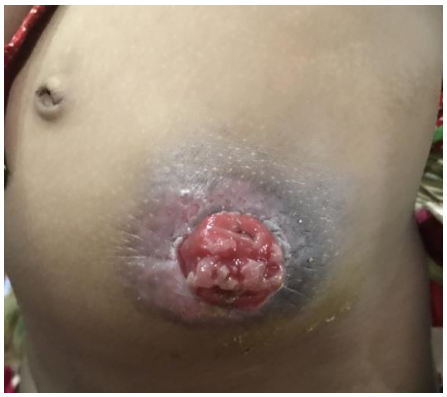


Figure 1.1: **A stoma in abdomen after ileostomy/colostomy in infant with Hirschsprung disease.**

1.2 Gut bacteria

Human gastrointestinal tract harbours different types of organisms, which co exists with each other. However, it is well established that there are symbiotic contribution of commensal microbes (Adlerberth et al., 1991). They help host by enhancing digestion, developing immune system and other host activities (Hooper and Gordon, 2001).

Accordingly, microbiota is a term, used for the microorganisms that colonize in the human gut (Salminen et al., 2006). For past two decades, gut microbiota especially gut bacteria became the hotcake for researchers and different information came to light through research. Starting from the regular important functions (Gill et al., 2006), it is way to unleash the effects of our intestinal flora towards obesity (Turnbaugh et al., 2006), diseases like type 1 diabetes (Murri et al., 2013), even cancer (Tjalsma et al., 2012). Moreover, it may have effects on our nervous system, which includes conditions like autism (Mulle et al., 2013), Hirschsprung disease (Pierre et al., 2014) and so on.

Regarding the diversity of gut bacteria, they usually change with age and different conditions like diet, genetics, bacterial infections, antibiotic treatment etc (Odamaki et al., 2016; Rodríguez et al., 2015). Here, in this part, we are to discuss the diversity of gut bacteria and the factors that influence the diversity, focusing on early life of human.

1.2.1 Gut bacteria in early life/ in neonates

It is evident that bacteria, residing in human intestinal tract, changes with age. But at same stage of life, it may vary due to several other reasons (Rodríguez et al., 2015). Though it was considered previously that the foetal gastrointestinal tract been sterile (Mackie et al., 1999). On the other hand, according to Collado et al. (2016), there are resemblance in bacteria of placenta and amniotic fluid to that of infant meconium, which leads us towards the microbial transfer at ‘foeto-maternal interface’.

Moreover, mode of delivery of the baby is an important factor for diversity of gut microbes in infants (Bettelheim et al., 1974). It has been also affected by the surrounding

environment as well as handling by parents and nurses where the skin microbes can go to the gut and cause change (Lindberg et al., 2004).

In continuation with 'foeto-maternal interface' and delivery mode of the baby, it has also been described that after 3-4 days of birth, the gut bacteria have similarity with the bacterial species isolated from mother's breast milk (Collado et al., 2016). As per Urbaniak et al. (2014), there are bacteria duels in human mammary gland tissue which play roles in modulating the gut bacteria in infant. Accordingly, feeding pattern may be a fact also, as there are differences in gut bacteria between the breast fed and formula fed infants (Lee et al., 2015). With ages, the differentiation becomes evident in the ecosystem of gut bacteria in early life and adult hood. However, it is the microbes that colonize in early life leads toward the microbiome of adulthood (Houghteling and Walker, 2015).

1.2.2 The bacterial composition in infancy and childhood

In infancy and childhood, the variations of gut bacteria are pretty different from that of adulthood. The conditions of the gut are also different. In foetal life, there are similarity of bacteria found in maternal placenta and amniotic fluid (Collado et al., 2016). After birth, *E. coli* and *Streptococcus* spp. have been identified abundantly in newborns' meconium (Mata et al., 1971). Again, in some other studies, Firmicutes had been found as dominating phylum in meconium and *Streptococcus mitis* and *Lactobacillus plantarum* had high prevalence rate (Rodríguez et al., 2015). *Staphylococcus* spp. as well as *S. epidermidis* and *S. aureus,* found in meconium, were much more than the other species (Moles et al., 2013).

Bifidobacterium spp. are another one that prevails in faeces of neonates during their first couple of days following delivery (Mitsou et al., 2008; Tanaka et al., 2009). Among the Bifidus group, the *Bifidobacterium longum* and *Bifidobacterium breve* were detected as dominant species by vaginally delivered, breast-fed infants in first several days of life (Mitsou et al., 2008). Furthermore, *B. longum, B. breve, B. adolescentis,* and *B. bifidum* had been found in faeces of both breast fed and bottle fed infants at first week of age after birth (Benno et al., 1984; Mevissen-Verhage et al., 1987).

Again, aerotolerants and facultative anaerobes prevail as dominant groups of microbes in neonatal gut (Rotimi and Duerden, 1981). They create environment so that strict anaerobes can colonize (Rodríguez et al., 2015). Within first weeks of life other than *Bifidobacterium* spp. strict anaerobe *Bacteroides, Clostridium* spp. have been found in faeces (Rodríguez et al., 2015). As per Benno et al. (1984), *Clostridium paraputrificum, C. perfringens, C. clostridiiforme, C. difficile, C. tertium* had been also found in infant gut.

Enterococcus spp. has been also found as one of the predominant bacterial group in faeces of neonates (Rotimi and Duerden, 1981). Among the *Enterobacteriaceae* members *E. coli* is the predominant one (Nowrouzian et al., 2003). As per Mata et al. (1971), *E. coli* were found in newborns within first hours after birth. Other than *E. coli; Klebsiella* spp. and *Enterobacter* spp. are common *Enterobacteriaceae* members reside as the gut bacteria of neonates (Adlerberth et al., 1991). Again, *Bacteroides fragilis* and *Bacteroides vulgatus* are common among the *Bacteroides* group in infant faeces (Mevissen-Verhage et al., 1987). Another one that had been in considerable amount in infant gut is γ-*Proteobacteria* (Adlerberth et al., 2007; Palmer et al., 2007). Moreover, *Aeromonas* spp., *Pseudomonas* spp. and *Acinetobacter* spp. are other bacteria that are found in neonatal faeces during the first weeks of life (Benno et al., 1984).

Several studies have demonstrated that, the variety of bacteria found in first few hours to first few days are much less than that of later in infancy or childhood (Palmer et al., 2007; Songjinda et al., 2005). Usually, around the age of six months, the colonization rate of bacteria changes from the previous condition. Because, it is the transition period of feeding pattern from milk to solid food known as weaning. Weaning is an important factor regarding colonization pattern of bacteria. *Clostridium coccoides* group and *Bacteroides* groups were more than that of *Bifidobacterium* spp. at six month of age after weaning (Fallani et al., 2011).

1.2.3 *Lactobacillus* spp. as gut bacteria

Lactobacillus spp. are Gram-positive, non-spore forming rods, some of which also show coccobacillary forms (Felis and Dellaglio, 2007; Tannock, 1992). These catalase negative, microaerophilic *Lactobacillus* spp. belong to the group of lactic acid bacteria (Ouoba et al., 2008), which forms lactic acid as carbohydrate end product (Felis and Dellaglio, 2007).

Lactobacillus spp. have been identified from numerous sources. It is present in dairy products like raw milk (Rodríguez et al., 2000), cheese (Joosten and Northolt, 1989), yogurt (Hoque et al., 2010) etc. Isolation of *Lactobacillus* spp. had been also done from fruits, vegetables and soil (Chen et al., 2005; Vitali et al., 2012). Apart from these sources, they are commonly found in human. Starting from oral cavity (Caufield et al., 2006), mammary glands and tissues (Urbaniak et al., 2014), breast milk (Diaz-Ropero et al., 2007), female vagina (Antonio et al., 1999), eventually human faeces harbours *Lactobacillus* spp. indicating its presence in gut (Ahrné et al., 2005). Name of some *Lactobacillus* spp. are demonstrated in Table 1.1.

Lactobacillus spp. are the group of bacteria that are most common among the gut bacteria of human as well as other animals' e.g. pigs, mice, rats, calves, chicken etc (Dec et al., 2016; Mitsuoka, 1992; Takino et al., 2017; Tannock, 1992). Inside the gut of the animals like pigs, mice as well as rats the *Lactobacillus* spp. are colonized along with the lining epithelium, which is non-glandular, stratified squamous epithelium, forming a bacterial biofilm (Fuller et al., 1978; Savage et al., 1968). In contrast to animal gut, the human gut shows a different picture. Here, the lining epithelium is simple columnar epithelium with rich mucous secreting cells (Walter, 2008). According to van der Waaij et al. (2005), the commensal bacteria in human gut have less contact with epithelial cells as they prefer to be in suspension in the gut. However, there are various factors responsible for adherence of *Lactobacillus* spp. in human gut, such as mucin, present in mucous (Tassell and Miler, 2011, Nishiyama et al., 2016). Another important factor is protein which acts as mediator for binding of different *Lactobacillus* spp. with gut e.g. sortase dependent protein, surface layer protein etc (Coconnier et al., 1992; Vélez et al., 2007).

Table 1.1: Name of some *Lactobacillus* species isolated from different sources. (e.g. Food and dairy sources: milk, cheese, yogurt, fruits, vegetables; human source: oral cavity, mammary gland and tissues, breast milk, female vagina, faeces; animal sources: faeces of rats, mice, pigs, calves, chicken; environmental source: soil etc.)

Name of *Lactobacillus* spp.		
L. acetotolerans	*L. delbrueckii subsp. lactis*	*L. oris*
L. agilis	*L.faecis*	*L. parabuchneri*
L. algidus	*L. fructivorans*	*L. paracasei subsp. tolerans*
L. amylolyticus	*L. fermentum*	*L. panis*
L. amylovorus	*L. fructosus*	*L. parakefiri*
L. acidophilus	*L.gastricus*	*L. paracasei subsp. paracasei*
L. alimentarius	*L. gallinarum*	*L. paraplantarum*
L. amylophilus	*L. graminis*	*L. plantarum*
L. animalis	*L. gasseri*	*L. pentosus*
L. antri	*L. helveticus*	*L. pontis*
L. apodemi	*L. homohiochii*	*L. rhamnosus*
L. aviarius subsp. aviaries	*L. hamsteri*	*L. ruminis*
L. aviarius subsp. araffinosus	*L. hilgardii*	*L. reuteri*
L. bifermentans	*L. intestinalis*	*L. rogosae*
L. buchneri	*L. iners*	*L. senioris*
L. bulgericus	*L. johnsonii*	*L. saniviri*
L. brevis	*L. jensenii*	*L. sakei subsp. sakei*
L. casei	*L. kalixensis*	*L. sakei subsp. carnosus*
L. carnis	*L. kefiranofaciens*	*L. salivarius subsp. salicinius*
L. catenaforme	*L. kefirgranum*	*L. salivarius subsp. salivarius*
L. camelliae	*L. kunkeei*	*L. sanfranciscensis*
L. collinoides	*L. kandleri*	*L. sharpeae*
L. cellobiosus	*L. lindneri*	*L. suebicus*
L. coryniformis subsp. coryniformis	*L. mali*	*L. ultunensis*
L. crispatus	*L. manihotivorans*	*L. vaccinostercus*
L. curvatus subsp. melibiosus	*L. malefermentans*	*L. vitulinus*
L. coryniformis subsp. torquens	*L. maltaromicus*	*L. uli*
L. curvatus subsp. curvatus	*L. murinus*	*L. vaginalis*
L. delbrueckii subsp. delbrueckii	*L. nasuensis*	*L. zeae*

References : (Cai et al., 2012; Endo et al., 2013; Kato et al., 2000; Oki et al., 2012; Osawa et al., 2006; Roos et al., 2005; Tabasco et al., 2007; Tannock, 1999)

Being a resident microbe and having established probiotic properties, *Lactobacillus* spp. play beneficial roles in gut. It plays a vital role to decrease the frequency in diarrhoea originating from different causes such as diarrhoea caused by infection through microorganisms like *Clostridium difficile*, rota virus and antibiotic associated diarrhoea, radiation therapy induced diarrhoea and so on (Eser et al., 2012; Kelly and LaMont, 1998; Visich and Yeo, 2010; Zhang et al., 2013).

Not only diarrhoea, *Lactobacillus* spp. also works against constipation and other gastrointestinal pathologies such as inflammatory bowel disease, ulcerative colitis etc (Indrio et al., 2014; Linskens et al., 2001; Zocco et al., 2006). Other than antibacterial and anti-microbial activity, it also acts against inflammation and muscular atrophy by decreasing the markers responsible (Bindels et al., 2012; Liévin-Le Moal and Servin, 2014). Moreover, *Lactobacillus* spp. also shown immunomodulatory activities, anti-tumour, anti-toxic activities as well as antioxidant activities by preventing lipid peroxidation (Amaretti et al., 2013; Iwabuchi et al., 2012; McIntosh et al., 1999; Mechoud et al., 2012).

With different activities, *Lactobacillus* spp. as resident gut bacteria, fights against the complications and risk factors of cancer, shows cholesterol lowering efficacy in hypercholesteraemic patients, exerts suppressing activities against *Helicobacter pylori* infection in gastric mucosa and so on (Jones et al., 2012; Rafter et al., 2007; Sakamoto et al., 2001; Zhang et al., 2012).

Though there are so many positive roles of *Lactobacillus* spp., the altered scenario also exists. It may cause infection in immunocompetent patients (Fradiani et al., 2010). Several studies demonstrated that this probiotic strain may also cause bacteraemia leading towards septicaemia and death (Arpi et al., 2003; Farina et al., 2001; Robin et al., 2010).

1.2.4 Gut bacteria of Hirschsprung diseased baby

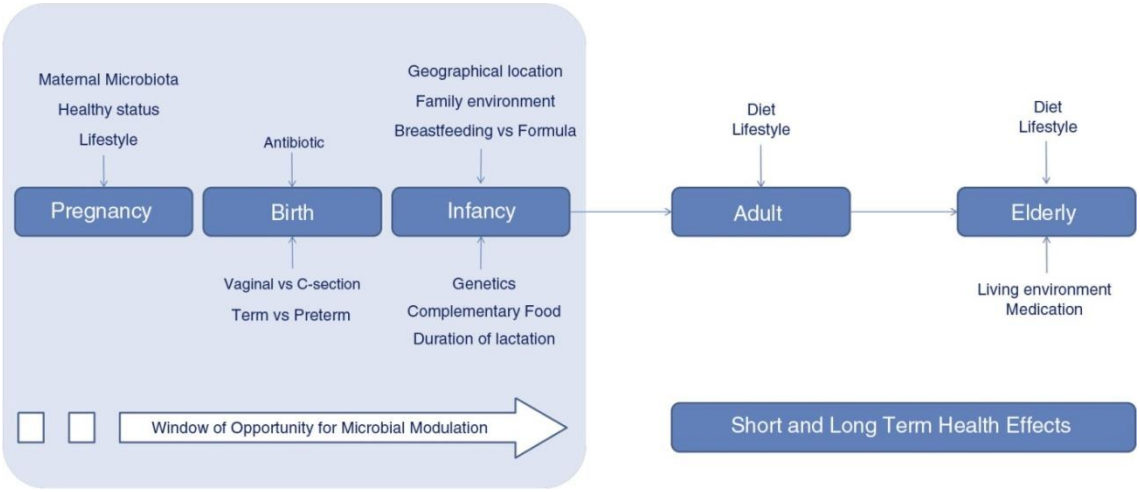
Several studies had been carried out to characterize the gut bacteria in Hirschsprung diseased (HD) babies. For this, various factors have been considered under different ecological niche. A study conducted by Yan et al. (2014), considered the microbial community of HD babies with or without enterocolitis along with segmental variation in intestine. Their study demonstrated that *Bacteroides* spp. and then Proteobacteria were found more in HD babies without enterocolitis, whereas, Proteobacteria and then Firmicutes were more in HD babies with enterocolitis. Similar study also demonstrated that, at genus level, percentages of *Bacteroides* spp. followed by *Enterobacteriaceae*, *Fusobacterium* are more in HD babies without enterocolitis and *Enterobacteriaceae* followed by *Enterococcus* spp., *Acinetobacter* spp. then *Eukaryota* in other group of babies with enterocolitis. It was supported by a study of Frykman et al. (2015), where percentages of *Bacteroides* spp. and Proteobacteria are more than Firmicutes and Verrucomicrobia in both HD groups of babies with or without enterocolitis. The percentage of *Clostridium difficile*, found in HD babies with enterocolitis, suggesting it to be a causal factor for this complication (Teitelbaum et al., 1989).

Regarding *Lactobacillus* spp. and *Bifidobacterium* spp., they were found more in normal babies in comparison with HD babies with or without enterocolitis (Shen et al., 2009). Again, as gut bacteria *Lactobacillus* spp. and *Bifidobacterium* spp. have different probiotic properties. Shen et al. (2009) also suggested that their decreased level may have influence for causing enterocolitis, a fatal complication of Hirschsprung diseased babies.

1.3 Factors influencing gut bacteria

There are several factors that influence the community structure of gut bacteria throughout life. Figure 1.2 depicts various factors responsible from foetal life to adulthood. Some of these factors are discussed here.

Figure 1.2: Factors influencing gut microbial community from pregnancy to elderly phase (Citation: Microbial Ecology in Health & Disease 2015, 26: 26050 - http://dx.doi.org/10.3402/mehd.v26.26050).



1.3.1 Effect of maternal factors

Though it was formerly believed that the foetal gut is a sterile one (Mackie et al., 1999), however, several studies showed that the microbes found in maternal placenta, amniotic fluid are pretty much similar with foetal meconium suggesting vertical transmission before birth (Collado et al., 2016; Koleva et al., 2015).

As per Collado et al. (2016), *Enterobacter* spp., *Escherichia* spp., *Shigella* spp. among Proteobacteria genera and also *Propionibacterium* spp,, *Streptococcus* spp., *Staphylococcus* spp., *Lactobacillus* spp. are some of the examples that are found both in maternal placenta, amniotic fluid and foetal meconium just after birth. Other than maternal placenta and amniotic fluid, microbes in colostrum and maternal mammary gland are another factor that can modulate neonatal gut microbes (Collado et al., 2016; Urbaniak et al., 2014).

Transfer of antibiotic resistance gene is another factor for shaping infant gut bacteria (Gosalbes et al., 2016). Study done by Zhang et al. (2011) suggests that, vertical

transmission may also be responsible for transfer of antibiotic resistance gene like *tet*(M), *ermB, sul2* as well as bla_{TEM} through the bacteria group *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Streptococcus* spp., *Escherichia coli* and *Shigella* spp.

1.3.2 Effect of mode of delivery

Mode of delivery is an important factor that influences the gut microbes of infant (Rodríguez et al., 2015). According to Dominguez-Bello et al. (2010), maternal vaginal and faecal bacteria had more exposure towards vaginally delivered infants than that with infants those are delivered through caesarean section (c-section). Same study also stated that, *Lactobacillus* spp. are the predominant group, transmit from maternal vagina and detected in neonatal gut of vaginally borne infant. As per Matsumiya et al. (2002), a dominant species of maternal vagina, *Lactobacillus crispatus*, was detected in neonatal gut at age of day 5 suggesting the transmission of bacteria during parturition. Apart from *Lactobacillus* spp., the dominant bacteria are *Prevotella* (Bacteroidetes), *Atopobium* (Actinobacteria), *Sneathia* spp. (Fusobacteria), *Enterobacteriaceae* prevail in vaginally delivered babies (de Muinck et al., 2011; Dominguez-Bello et al., 2010). In another study, it showed *Bacteroides* spp. are present more in gut of vaginally delivered infant than that of c-section delivered infant (Fallani et al., 2011).

It most of the cases, infants born via c-section had more exposure with the microbes from skin through handling by parents, nurses and also from hospital environment (Lindberg et al., 2004; Morelli, 2008). Delayed colonization of *Bacteroides* spp. and less diversified bacteria in gut of c-section babies suggest the deviated normal colonization pattern of gut microflora (Jakobsson et al., 2014). This deviation of gut bacteria, depending on mode of delivery, may continue even at the age of seven (Salminen et al., 2004).

1.3.3 Effect of diet

1.3.3.1 Pre-weaning period

After birth, the diet of the new born usually begins with colostrum and then with breast milk, which fight against infection, inflammation and influence the immunoregulatory functions (Morrow and Rangel, 2004). This breast milk contains antibody (IgA), oligosaccharides, glycoconjugates, lactoferrin, leukocytes, cytokines, and other agents (Kverka et al., 2007; Morrow and Rangel, 2004; Morrow et al., 2005). Recent studies have shown that human breast milk also contains microbes (Fernández et al., 2013), including *Lactobacillus* spp. (Diaz-Ropero et al., 2007), *Lactococus* spp. (Beasley and Saris, 2004), *Bifidobacterium* spp. (Makino et al., 2013), *Staphylococcus* spp. as well as *Enterococcus* spp.(Jimenez et al., 2008), *Clostridia* (Jost et al., 2013) and much more which plays role to shape the infant gut bacteria.

The gut bacteria of formula fed infant can be a little different from breast fed infants. According to Balmer and Wharton (1989), *Bifidobacterium* spp. and *Staphylococcus* spp. found more in breast fed infants gut in contrast to formula fed infants, whereas enterococci, coliforms, and clostridia were the prevailing bacteria in bottle fed one. Similar result was found in another study done by Bezirtzoglou et al. (2011), where *Bifidobacterium* spp. were more common in breast fed infant than formula fed one. Altered scenario is also there, as several other studies found that in gut bacteria, both breast fed and formula fed infants harbour very little differences (Adlerberth and Wold, 2009; Mackie et al., 1999).

1.3.3.2 Post weaning period

Introduction of solid food for the first time in infant signifies the upcoming change towards the gut bacteria, which is going to be more ‘adult like’ as well (Fallani et al., 2011). But this change does not occur rapidly. As per the study done by Amarri et al. (2006), the count of *Bifidobacterium* spp. in babies intestine did not change much after 5 months of weaning. Again, Fallani et al. (2011) demonstrate that, though for some period,

Bifidobacterium spp. in infants gut remained predominant, but the count lowers over time. Other than *Bifidobacterium* spp., *Bacteroides* spp., *Enterobacter* spp., *Enterococcus* spp., clostridia (*C. coccoides, C. leptum*) prevail and eventually their number increases over time after commencing complementary food (Fallani et al., 2011; Hopkins et al., 2005; Koenig et al., 2011). However, controversy is there, as with increasing count of clostridia the count of *Enterobacteriaceae* spp. lowers in infant gut at post weaning period (Wang et al., 2004). Not only *Enterobacteriaceae* spp. but also *Enterococcus faecalis* count lowers with increasing period of weaning and with increasing number of Bacteroides-Porphyromonas-Prevotella group, *C. coccoides* group and *Faecalibacterium prausnitzii* subgroup (Dore et al., 1998; Hopkins et al., 2005).

As for *Lactobacillus* spp., it is stated by Ahrné et al. (2005) that *Lactobacillus* spp. colony count increases with age of infant but slightly decreases during the transition period from milk to solid food. The same study of Ahrné et al. (2005) also demonstrated that, the type of *Lactobacillus* spp. changes and other *Lactobacillus* spp., those are predominant in food, takes the charge; e.g. *L. rhamnosus*, *L. gasseri* found more in breast fed infant before weaning whereas *L. paracasei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* found more at later life after weaning, around the age of 12 months to 18 months. According to Amarri et al. (2006), at post weaning period Vancomycin-insensitive *Lactobacillus* spp. count increases at first, followed by declination.

1.3.4 Effect due to antibiotic treatment

Among the factors that influence composition of neonatal gut bacteria, antibiotic treatment is more likely the induced one. Not only neonatal one, antibiotic treatment shapes the human gut microbiota of all ages (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011).

In most of the cases, the diversity of the infant gut flora on both genus and species basis are affected by antibiotic treatment and the microbial community changes more rapidly than those with no or less antibiotic treatment (Yassour et al., 2016). The changes of gut

bacteria caused by antibiotic treatment may persist long after its withdrawal and get back to its previous state may not be even a possible one (De La Cochetière et al., 2005).

A study done by Fouhy et al. (2012) showed that, count of *Proteobacteria* were more and count of *Actinobacteria, Bifidobacterium* spp., *Lactobacillus* spp. were less after 4 weeks of antibiotic treatment, in the treated infants gut than that with control infant having no antibiotic treatment. Though a level of recovery found on *Actinobacteria, Bifidobacterium* spp., *Lactobacillus* spp. count with some differences were found after 8 weeks.

Different other consequence may also occur. Increase of antibiotic resistance gene among gut bacteria is not uncommon after antibiotic treatment (Yassour et al., 2016). Alteration of gene expression and gut barrier function can be an important factor for impact on immunity due to antibiotic exposure (Schumann et al., 2005; Ubeda and Pamer, 2012). Immune disorder like asthma and other allergic diseases can be an aftermath if there are exposures to antibiotics in early life (Droste et al., 2000).

1.3.5 Environmental factor

Different environmental factors also affect the infant gut bacteria. Surrounding environment of the country, in which the baby borne, may be the first environmental factor to consider (Fallani et al., 2011). Again, environmental factor varying in between developing and developed countries or urban and rural areas may also have effect (Adlerberth et al., 1991; Adlerberth et al., 1998).

Enterobacteriaceae spp. found more in gut bacteria of babies at crowded condition in developing countries than developed one (Adlerberth et al., 1991). Other than these factors, skin microbes form hospital stuff as nurse, care giver, parents can enter the gut during nursing and modulate gut microbes (Lindberg et al., 2004). Having elder sibling is another environmental factor to consider. According to Penders et al. (2006), *Bifidobacterium* spp., found more in infants with elder sibling than those who do not.

1.4 Effect of stress towards bacteria

Bacteria found in our surrounding environment are exposed to various strenuous conditions (Aertsen and Michiels, 2004). Similar fact goes for the microbes reside inside human intestine. Starting from acidic condition in stomach, gut bacteria has to survive through several stress factors (Begley et al., 2005; Gray and Shiner, 1967). Even, lack of ganglion cells in part of intestine also serves as stress factor for dysbiosis of gut bacteria (Ward et al., 2012). However, harmony of gut bacteria depends on the stress response delivered by them (Hooper and Gordon, 2001).

For survival and to adjust with the unfavourable conditions, bacteria undertake several adaptive mechanisms. Alteration of bacterial cell membrane, changing the pattern of gene expression and formation of stress proteins are adaptive responses exerted by bacteria towards adversities (Marles-Wright and Lewis, 2007; Russell et al., 1995).

Again, exposure to single stress makes the bacterium adapted for another stress with adaptation mechanisms (Bunning et al., 1990; Jenkins et al., 1990). As for *Lactobacillus* spp., they respond to various ways in response to adversities. According to Kubota et al. (2008), *Lactobacillus* spp. may form biofilm to protect themselves from stress. However, *Lactobacillus* spp. are highly capable to survive and to adapt the hostile conditions in human gut using several adaptive mechanisms which is one of the causes to choose it as probiotic (Kim et al., 2001; Liévin-Le Moal and Servin, 2014).

1.5 Objectives of the study

Recent studies showed that microbial community reside in human intestine, carries importance for aetiology of different diseases. Among these gut bacteria *Lactobacillus* spp. holds an important place as it is a commensal with probiotic properties to withstand the adverse situation and to exert defence mechanism (De Angelis and Gobbetti, 2004; Liévin-Le Moal and Servin, 2014). Again, in Hirschsprung disease the infant gut bacteria had to go through several adverse conditions for survival and to maintain the proper ecological niche in intestine (Frykman et al., 2015; Shen et al., 2009). Keeping this in

consideration, we have designed our study to isolate, check and compare various stress factors on *Lactobacillus* spp. from faeces of infants suffering from Hirschsprung disease and that of normal infant without the disease.

Specific objectives of this study are:

- To isolate *Lactobacillus* spp. from faeces of infants with and without Hirschsprung disease.
- Confirmation of isolated *Lactobacillus* spp. up to genus level by PCR amplification.
- Comparison of stress tolerance ability of the isolated strains for acidic and alkaline conditions, halophilic condition and tolerance to bile.
- Antibiotic sensitivity of the isolates.
- To check cell adherence and aggregation properties.
- Evaluation of effect of isolated *Lactobacillus* spp. towards other gut bacteria both *in vitro* and *in vivo*.

Chapter 2: Materials and Methods...

2.0 Materials and Methods

2.1 Subjects

In this study, the infants from whom the samples were collected had been admitted at Dhaka Shishu (Children) hospital at time of sample collection. Fully informed written consent to collect sample was taken at first from the hospital ethical committee and then from the parents of the babies both Hirschsprung diseased one and normal one. Here, only the stool samples were collected from the infants and no invasive procedure were carried out at time of sample collection.

'Infant A' was a term infant who had abdominal distension 2 days after his birth with immobility of gut. The provisional diagnosis was ano-rectal malformation. Medical management of the condition was done by creation of a stoma in the sigmoid colon. The histopathological test of sample taken from colon during the surgery showed the aganglionosis which leads towards the diagnosis of Hirschsprung disease. 'Infant B' was a term infant who did not have Hirschsprung disease or any other ano-rectal malformation which may lead towards creation of stoma in ileum or colon.

Faecal sample was taken from 'Infant A' at 48 weeks of age and from 'Infant B' at 40 weeks of age. As per the history collected from the parents of the infants, both infants were exclusively breast fed for around first 3 months of life. Then they were both breast fed and formula fed. It was also stated by the parents that, both of them were vaccinated as per 'Expanded programme of immunization' (EPI) available in Bangladesh. None of the infants received probiotics in their diet. We have no information regarding whether or not the mother received probiotics. Both infants were treated with antibiotics as treatment purpose at some period of life.

2.2 Sample collection and bacterial isolation

With all aseptic precaution stool sample was collected from stoma (Infant A), anus (Infant B) through sterile cotton swab. Samples were stored at 4^0C and within 4 hours of sample collection it was transferred to laboratory for further processing.

After proper dilution with physiological normal saline, culture was done on nutrient agar (NA) media then sub cultured in de Man Rogosa and Sharpe agar (MRS agar) to pre-select *Lactobacillus* spp. (De Man et al., 1960). Incubation period was 48 hours at 37^0C in anaerobic condition (anaerobic jars with gas pack). This type of sampling did not allow quantitative assessment through colony count of bacteria but did allow the isolation of bacterial strains present, from the samples, which is a qualitative assessment. Bacteria isolated were stored in medium containing 10% glycerol with skim milk, tryptone and glucose.

2.3 Identification of *Lactobacillus* spp.

At first the isolated single colony was identified by morphological characteristics. Primarily isolated colonies from MRS agar were taken and then Gram staining was done. Gram positive rods have been selected. Then further biochemical tests were done.

2.3.1 Biochemical tests

Biochemical tests of the Gram positive rods were done as per the methods described in Microbiology Laboratory Manual (Cappuccino and Sherman, 2008). The tests include oxidase test, catalase test, nitrates reduction, motility indole urease (MIU) test and carbohydrate fermentation test.

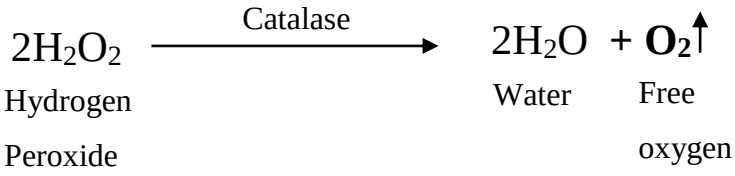
2.3.1.1 Oxidase test

Production of cytochrome oxidase by a bacterium can be tested with *p*-aminodimethylaniline oxalate. With electron donation, colour change occurs and indicates positive result suggesting presence of oxidase enzyme which aids in aerobic respiration of bacterium. Absence of colour change means absence of enzyme, so it's a negative result (Cappuccino and Sherman, 2008).

To test this, selected isolated bacteria colony was taken with sterile loop and smeared on filter paper. Then one drop *p*-aminodimethylaniline oxalate reagent was added and observed if there is any colour change or not for about 30 seconds.

2.3.1.2 Catalase test

Hydrogen peroxide is produced by microbes during aerobic respiration. But its breakdown is necessary because the accumulation of this per oxide can cause the death of the microbes. Catalase enzyme is the one that degrades this hydrogen peroxide and produce free oxygen. The reaction is shown here,

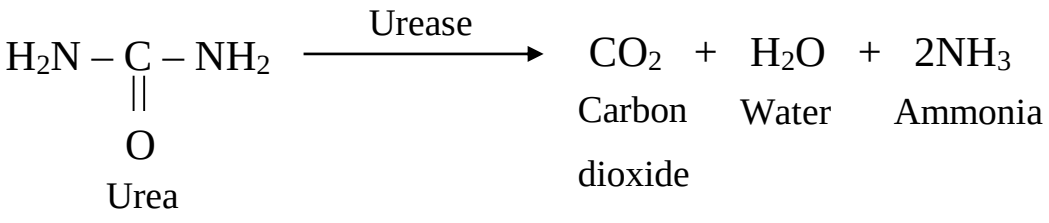


But the organisms that do not have this catalase enzyme fail to degrade this per oxide and thus cannot grow in presence of oxygen. With the addition of H_2O_2 we can check if the bacteria have catalase enzyme or not. If catalase enzyme is present it will break down Hydrogen per oxide and form free oxygen as bubbles. Otherwise it is absent in microbes (Cappuccino and Sherman, 2008).

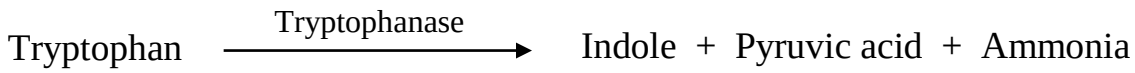
Selected bacterial isolate was taken with a sterile loop and placed in a glass slide. Then a drop of 3% Hydrogen per oxide is to add and to check if there is any bubble formed or not.

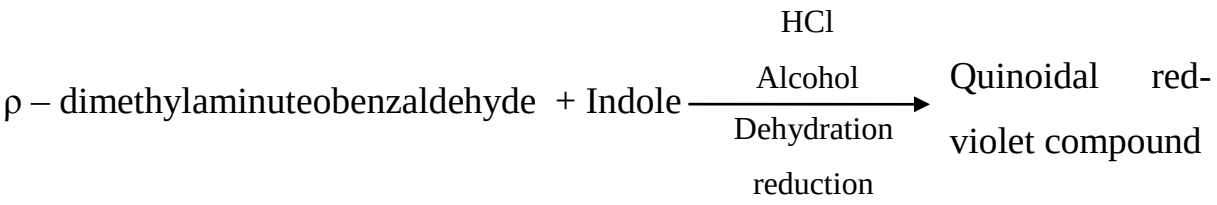
2.3.1.3 Motility Indole Urease (MIU) test

Motility of the bacteria usually indicated in media through diffused growth or turbidity away from inoculation line, where as non-motile one does not grow like that. The presence of urease enzyme can be detected with the colour change of phenol red present in the media. This urease enzyme if present in organism reacts with urea and forms ammonia. This ammonia changes the pH and colour change occurs. The reaction is shown here,



Indole test is done to check if the organism can degrade tryptophan, an essential amino acid. Tryptophanase enzyme is responsible for degradation of tryptophan to indole, pyruvic acid and ammonia. The reaction is shown here,

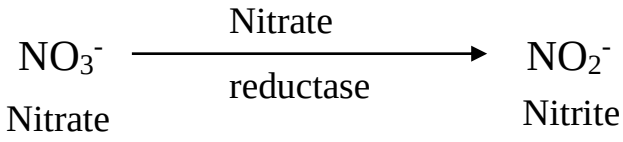


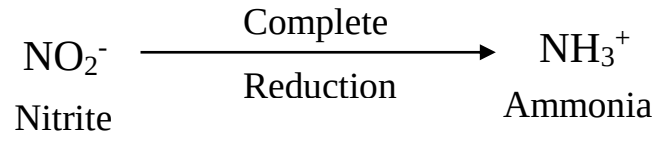


To detect presence of indole, Kovac's reagent is added which contains *p*-dimethylaminobenzaldehyde, butanol and hydrochloric acid. If indole is present it shows cherry red colour, of not then no colour change occurs. Here, in this study, selected bacterial isolates were stabbed into MIU medium and incubated for 24 hours at 37^0C. Motility and urease were checked first. Indole production was tested after addition of kovac's reagent.

2.3.1.4 Nitrate reduction

This biochemical test determines if the microbes can reduce nitrates (NO_3^-) to nitrites (NO_2^-) or further. In absence of oxygen during anaerobic respiration cell uses nitrates (NO_3^-) or sulfates (SO_4^{2-}). Again, some organism goes further and produces ammonia (NH_3^+) or nitrogen (N_2) by reducing nitrites.





Or,

$$2NO_3^- + 12H^+ + 10e^- \longrightarrow N_2 + 6H_2O$$

Nitrate — Molecular nitrogen

In this study, nitrate broth was prepared with 0.1% potassium nitrate (KNO_3) supplementation for this test. After being incubated in the broth, nitrate reduction checked with two solution naming Solution A (Sulfanilic acid), then Solution B (α-naphthylamine). Production of cherry red colour, right after the addition of two solutions indicates positive result, whereas no colour change shows apparent negative result. But there can be a possibility that nitrates being reduced further to nitrites then to ammonia or nitrogen. Then it will show no colour change though the result is a positive one. Addition of a small amount of zinc powder in the nitrate broth with solution A and B will change the colour indicating no nitrate reduction occurred, where as being colour less will indicate positive result.

2.3.1.5 Carbohydrate fermentation

Carbohydrate is used by different organism for energy purpose. Both aerobic and anaerobic bacteria can utilize carbohydrate. Facultative anaerobes can use both pathways. In fermentation, production of organic acid occurs as lactic, formic or acetic acid with or without production of gases naming hydrogen or carbon dioxide gas. This acid production causes the change in pH which is indicated by colour change of broth suggesting positive result. Moreover, inverted Durham tube is there to check if gas production occurred or not. For determination of carbohydrate fermentation along with nutrient broth (NB) specific carbohydrate is to add to serve as substrate and phenol red as pH indicator.

Here, selected bacterial isolate was inoculated in the broth with specific carbohydrate and after 24 hours of incubation at 37^0C, the colour change and gas production observed. In this study, carbohydrates used are,

Glucose, Arabinose, Arginine, Fructose, Galactose, Glycine, Lactose, L-lysine, Maltose, Mannitol, Rhamnose, Starch, Sucrose, Trehalose and Xylose.

2.3.2 Identification through Polymerase chain reaction, PCR

Amplification of specific DNA is now done by Polymerase chain reaction naming PCR (Mullis et al., 1986). Here, the template DNA that is a oligonucleotide sequence, is synthesised over and over again through the presence of PCR polymerase buffers with salt, four deoxyribonucleoside triphosphates and also oligonucleotide primer set (Hill and Stewart, 1992; Mullis et al., 1986). Three different temperature controlled steps naming denaturation, annealing and extension carried out on thermal cycler to achieve the desired result.

2.3.2.1 DNA extraction and PCR amplification

An isolated Gram positive bacterium suggestive of *Lactobacillus* spp. through biochemical tests was then prepared for confirmation by PCR amplification. Preparing template DNA of the bacterium for PCR amplification was done by using Qiagen DNA Mini kit. Purified DNA extracts were then stored in -20^0C.

2.3.2.2 Preparation of master mix

For PCR, reagents used along with the primers are Taq polymerase 5 µl/ml (Invitrogen, India), 10x PCR buffer (Invitrogen, India), deoxynucleoside triphosphates (dNTPs) 2.5 mM, $Mgcl_2$ 50 mM and nuclease free water as required. The reaction volume was 25 µl. After preparing the master mix and addition of template DNA the thermal cycler was used and the cycle was repeated for 40 times. Then the PCR tubes with PCR products were stored at -20˚C for further analysis.

Here, in this current study, the oligonucleotide sequences used as forward and reverse primers set, amount of reagent used as master mix for PCR and the programme that was used for thermal cycler are depicted respectively in Table 2.1, Table 2.2 and Table 2.3.

Table 2.1: The oligonucleotide primers set as forward and reverse one.

Oligonucleotide sequence (5′ - 3’)	Amplicon size (bp)	Reference
Fwd: GAGGCAGCAGTAGGGAATCTTC **Rev: GGCCAGTTACTACCTCTATCCTTCTTC**	126	(Delroisse et al., 2008)

Table 2.2: Amount of reagents used for PCR

Name of the Components	Amount
Nuclease free water	13.875 µl
10x reaction buffer	2.5 µl
$Mgcl_2$ (50mM)	1 µl
dNTP (2.5mM each)	2 µl
Forward primer (25 µM)	0.25 µl
Reverse primer (25 µM)	0.25 µl
Taq polymerase (5U/µl)	0.125 µl
DNA template	5 µl
Sub total	25 µl

Table 2.3: Programme set for thermal cycler

Steps	Temperature	Time
Initial denaturation	94^0C	5 minute
Denaturation	94^0C	30 sec
Annealing	58^0C	45 sec
Extension	72^0C	1 minute 30 sec
Final extension	72^0C	7 minute

2.3.2.3 Agarose gel electrophoresis

The PCR products were further analysed through agarose gel electrophoresis. Here, 2% agarose gel in TE buffer was used. Ethidium bromide was used for gel staining. The PCR products were loaded into gel with 6x loading dye. To estimate size of template DNA, 100 bp DNA ladder was also loaded into the gel. Here, horizontal gel electrophoresis apparatus was used with TBE buffer as running buffer at 80V. The results were observed using a UV transilluminator.

2.4 Analysis of stress tolerance

2.4.1 Assay for pH tolerance

To check the pH tolerance of the isolated *Lactobacillus* spp. of 'Infant A' and 'Infant B', MRS broth with different range of pH 2.5-8.5 were prepared. This pH range covered both acidity and alkalinity. The pH adjustments were done with 99% Acetic acid and 5N NaOH. Then 1% (v/v) overnight culture of the isolates were inoculated in those MRS broths and incubated in 37^0C anaerobically for 24-48 hours. After that the growth were measured by a UV spectrophotometer at 560 nm (OD_{560}) (Hoque et al., 2010).

2.4.2 Assay of Bile tolerance

To check the bile tolerances of the isolated bacteria, protocols described by Spencer and Ragout de Spencer (2001), have been followed with little modification. Here, MRS broth and MRS broth with 0.1%, 0.2% and 0.3% ox-gall bile have been prepared. Isolated *Lactobacillus* spp. from infant's faeces were inoculated in MRS broth and incubated over night at 37^0C temperature. After that the cells were harvested by centrifugation at 5000g for 10 minutes. Then the pellets were washed by and were resuspended to the original volume with PBS through vortex mixing. Then MRS broth with 0.1%, 0.2% and 0.3% ox-gall bile were inoculated by 0.5% v/v bacterial suspension. Then it was incubated again at 37^0C temperature. Optical density at 560 nm (OD_{560}) was measured by using UV spectrophotometer at every 1 hour interval for first 8 hours and then after 24 hours against the blank, which is the uninoculated broth.

2.4.3 Assay of NaCl tolerance

NaCl tolerance was tested for the *Lactobacillus* spp. isolates of both infant, ranging from 1-10% (Hoque et al., 2010). 10 different test tubes containing MRS broth with 1 - 10% NaCl were taken and inoculated with 1% (v/v) culture of the isolates. Within 24 to 48 hour, growth was determined by visual observation through presence or absence of turbidity (Davis, 1955).

2.5 Production of organic acid

Production of organic acid is a characteristic of *Lactobacillus* spp. To quantify the production of organic acid, the selected isolated strain from the faeces of 'Infant A' and 'Infant B' were inoculated with 1% (v/v) overnight culture in 10% skim milk and the initial pH was measured. Prior to inoculation the skim milk was reconstituted and sterilized through autoclaving at 121^0C and 15 lb for 1 minute. Then it was incubated in at 37^0C for 72 hours. At every 24 hours, 48 hours, and 72 hours' samples were collected

and the pH was measured with a digital pH meter. Then titration was done with 0.1 N NaOH to quantify organic acid (Diba et al., 2013; Hoque et al., 2010).

The equation used is,
TTA% = V NaOH x 0.1N x 0.009 x100%

Here, V is volume of NaOH required to neutralize the acid. The value 0.009 comes as 1 ml of 0.1N lactic acid contains 0.009 grammes of lactic acid. Titrable acidity is expressed as % lactic acid, (CH_3-CHOH-COOH, MW=90).

2.6 Assay of antibiotic sensitivity

The antibiotic susceptibility test was done by disc diffusion method as described by Rojo-Bezares et al. (2006), with little modification. To assay the antibiotic sensitivity of the isolated *Lactobacillus* spp., they were grown overnight on MRS agar plate at 37^0C. Then from the growth, suspension was prepared on sterile physiological saline (0.9% NaCl) and standardized with Mcfarland solution 1. After that, from the suspension, lawn culture was done with sterile cotton swab at MRS agar and standard antibiotic discs of different antibiotic were used. The inhibition zone was measured after 24 hours of incubation at 37^0C.

2.7 Aggregation properties

Aggregation property is an important one in probiotic strains. It is of two types naming auto aggregation and co aggregation. When same bacterial strain aggregates with each other it is auto aggregation. Again, when the similar clumping occurs in between different bacterial strain then it is called co-aggregation (Janković et al., 2012).

By co-aggregating with different other bacterial strain, bacterium with this property scavenges out pathogenic strains from the gut and provides protection (Boris et al., 1997; Reid et al., 1988). Multiple factors as epithelial cells, proteins, teichoic acids etc are responsible for these aggregation properties (Reniero et al., 1992). However, as an

established probiotic strain, the *Lactobacillus* spp. shows both auto aggregation and co aggregation properties (Janković et al., 2012; Reid et al., 1988).

2.7.1 Auto-aggregation

In this study, the isolated *Lactobacillus* spp. from 'Infant A' and 'Infant B' were grown on MRS broth overnight at 37^0C. The cells were centrifuged for 15 minutes at 5000g and washed with PBS. Then they were re-suspended with PBS and adjusted to OD 1 with help of UV spectrophotometer at 620 nm. After that the suspension was incubated for 60 minutes at room temperature. Then it was centrifuged at 300g for 2 minutes at 20^0C. OD was taken through UV spectrophotometer of the supernatant at 620 nm (Sadrani et al., 2014).

Calculation, % of auto-aggregation,

$[(At_0 – At_{60})/At_0] \times 100$

Here, At_0 = Initial OD at 620 nm.

At_{60} = OD of supernatant at 620 nm after 60 minutes of incubation.

2.7.2 Co – aggregation

At first the isolated *Lactobacillus* spp. from both infant were grown anaerobically in 10 ml MRS broth at 37^0C for 24 hours. Again, some selected pathogenic strains against which the co-aggregation capability of the *Lactobacillus* spp. to be tested, were grown in 10 ml Nutrient broth at 37^0C for 24 hours. Then the cells were centrifuged at 5000g for 15 minutes. After the cells been harvested, washed and resuspended in sterile PBS, OD was adjusted to 1 at 620 nm for both *Lactobacillus* spp. and the pathogenic strains. 1 ml of each *Lactobacillus* spp. was transferred to the 1 ml suspension of each pathogenic strain. Then OD was taken at 620 nm with spectrophotometer immediately after the relevant strains had been paired. After incubation at room temperature for 60 minutes, the paired suspension was centrifuged at 300g for 2 minutes at 20^0C. Then OD of the supernatant was taken again at 620 nm (Sadrani et al., 2014).

Calculation, % of co-aggregation,

[(Atot – As)/Atot] x 100

Here, Atot = Initial OD at 620 nm taken immediately after relevant strains been paired.
As = OD of supernatant at 620 nm after 60 minutes of incubation.

The strains here to check co-aggregation of *Lactobacillus* spp. used are,

Gram positive

- *Enterococcus faecalis*
- *Staphylococcus aureus* (ATCC 25923)
- *Streptococcus pneumoniae* (ATCC 42619)
- *Bacillus subtilis*
- *Bacillus cererus*

Gram negative

- *Eschereschia coli* (ATCC 25922)
- *Eschereschia coli* (0157: H7)
- *Pseudomonas aeruginosa* (ATCC 27853)
- *Vibrio cholerae*
- *Shigella dysenteriae*

2.7.3 Salt aggregation test

It determines the cell surface hydrophobicity of bacteria (Lindahl et al., 1981). It is an *in vitro* property to evaluate the potentially probiotic strains. As a probiotic strain, cell surface structures and its secretary agents of *Lactobacillus* spp. makes it a more potent one (Lebeer et al., 2008). This adhesion can also determine the colonization capability of a microorganism. Through adhesion ability and colonization of tissues, probiotic

microorganisms can prevent pathogen access interactions, immune response modulation or specific activation on cell receptors (Collado et al., 2005; Sherman et al., 2009).

To do this test, at first isolated gut bacteria that is *Lactobacillus* spp. were grown overnight at 37^0C on MRS broth. Then they were centrifuged at 5000g for 15 minutes and washed twice with phosphate buffer saline (PBS). Again, Ammonium sulphate [$(NH_4)_2SO_4$; pH 6.8] of different molarities (0.02M- 4M) were prepared. Then 10µl aliquot of fresh cell suspension and 10µl Ammonium sulphate [$(NH_4)_2SO_4$; pH 6.8] (0.02M-4M) were mixed on glass slide. After 1 minute, formation of cell aggregation was checked and reading was taken by visual observation. The lowest concentration of Ammonium sulphate [$(NH_4)_2SO_4$; pH 6.8] giving visible aggregation scored at SAT hydrophobicity value (Ljungh et al., 1985; Sadrani et al., 2014).

2.8 Effect towards other gut bacteria

Co-existence of different bacteria is a common habitual fact. But during this co-existence, bacteria exerts several characteristics like co-aggregation, anti-microbial effect etc especially the probiotic strains (Lee and Salminen, 1995). Here, effect on growth along with two different strain naming, *Vibrio cholerae* and *Escherichia coli* have been seen through colony count for the isolated *Lactobacillus* spp. both *in vitro* and *in vivo*.

2.8.1 Effects towards other gut bacteria *in vitro*

At first fresh culture of the all four isolated *Lactobacillus* spp. from 'Infant A' and 'Infant B' were taken. Then suspension was prepared on sterile physiological saline (0.9% NaCl). The similar suspensions were prepared for both *Vibrio cholerae* and *Escherichia coli* (ATCC 25922). All of them were standardized with Mcfarland 4 (1.2×10^9 cfu/ml). After that 1% (v/v) of *Lactobacillus* spp. was added to the 100 ml of Nutrient broth prepared earlier. Then, 1% (v/v) of both *V. cholerae* and *E. coli* suspensions were added to the broth and incubated at 37^0C for 24 hours. Again, 100 ml Nutrient broth was inoculated only with 1% (v/v) *V. cholerae* and *E. coli*. No *Lactobacillus* spp. was added and it served as control. It was also incubated at 37^0C for 24 hours. After 24 hours the samples were

taken and colony count were done by using selective media for each strain, with proper serial dilution. Here, TCBS, EMB and MRS agar media were used as selective media respectively for colony count of *V. cholerae*, *E. coli* and *Lactobacillus* spp. The results were then compared with each other.

2.8.2 Effects towards other gut bacteria *in vivo*

To check the effect of isolated *Lactobacillus* spp. on *V. cholerae* and *E. coli in vivo*, 8 weeks old Swiss albino mouse was used. Like *in vitro*, suspension with fresh culture of the *Lactobacillus* spp., *V. cholerae* and *E. coli* were prepared on sterile physiological saline (0.9% NaCl) and standardized with Mcfarland solution 4 (1.2×10^9 cfu/ml). Here, randomly two isolated strain were selected. However, 50µl of each suspension was given to the mouse as oral gavage. Two mice were given gastric gavage of *Lactobacillus* spp., *V. cholerae* and *E. coli*. However, another mouse was given oral gavage of only *V. cholerae* and *E. coli*, this served as control as well. Stool of all those mice was collected after 24 hours and 48 hours. After weighing the faeces of mice, it was diluted in similar amount of physiological saline (0.9% NaCl).

In continuation of it, the colony of *Lactobacillus* spp., *V. cholerae* and *E. coli* were counted using selective media respectively TCBS, EMB and MRS agar for each strain with proper serial dilution. Hence, the results were further compared with each other.

Chapter 3:

Results...

3.0 Results

3.1 Identification of *Lactobacillus* spp.

At first the bacteria isolated from collected stool samples were identified phenotypically as *Lactobacillus* spp. On MRS agar plate the isolated colony of the bacteria was round elevated sized (0.5-1) mm and was creamy to whitish coloured. After selecting the isolates, they were collected for Gram staining.

Then Gram positive rods were selected for further biochemical tests as *Lactobacillus* spp. hold such characteristics. Four isolates were selected. Among them two isolates were from Infant A (A-1 and A-2) that is diseased one and two isolates were from Infant B (B-1 and B-2) that is apparently healthy one.

3.1.1 Biochemical tests

The results of the biochemical tests are compiled in Table: 3.1. Again, Figure 3.1(a,b) shows the test tubes of MIU test.

Table 3.1: Results showing biochemical tests of 4 *Lactobacillus* spp. isolates

Biochemical Test	A-1	A-2	B-1	B-2
Oxidase	-	-	-	-
Catalase	-	-	-	-
Motility	-	-	-	-
Indole production	-	-	-	-
Urease production	-	-	-	-
Nitrates reduction	-	-	-	-
Gas from glucose	-	-	-	-

(Here, "-" indicates negative result; no positive result has been found and A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

a) 

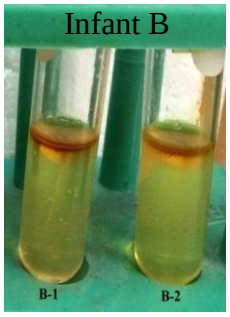
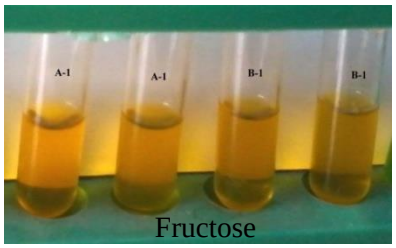
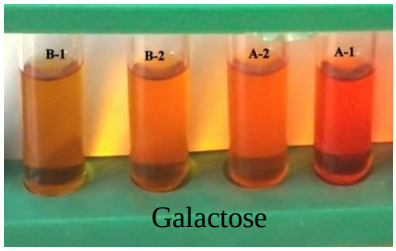
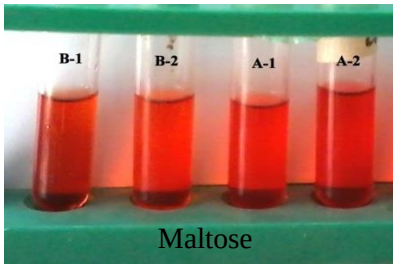
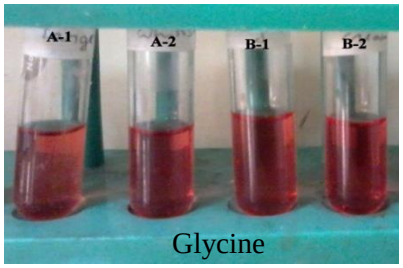
b) 

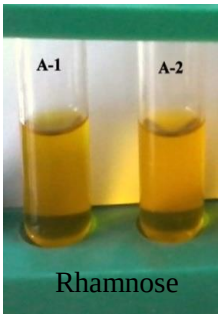
Figure 3.1 (a,b): Test tubes showing Motility Indole Urease (MIU) test result of diseased one 'Infant A' (A-1 and A-2) and normal one 'Infant B' (B-1 and B-2)

a) 

b) 

c) 

d) 

e) 

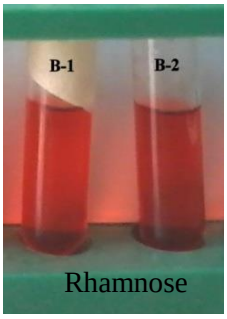
f) 

Figure 3.2 (a,b,c,d,e,f): Test tubes showing carbohydrate fermentation test results of Fructose, Galactose, Maltose, Glycine and Rhamnose for diseased one 'Infant A' (A-1, A-2) and normal one 'Infant B' (B-1, B-2)

3.1.2 Carbohydrate fermentation

Fermentation capability of different carbohydrates is an important characteristic feature of *Lactobacillus* spp. Here, carbohydrate fermentation of the isolates was done for 15 carbohydrates. The results are compiled in Table 3.2 and Figure 3.2 (a,b,c,d,e,f) showing test tubes with some of the carbohydrate fermentation test result of isolates from 'Infant A' and 'Infant B'.

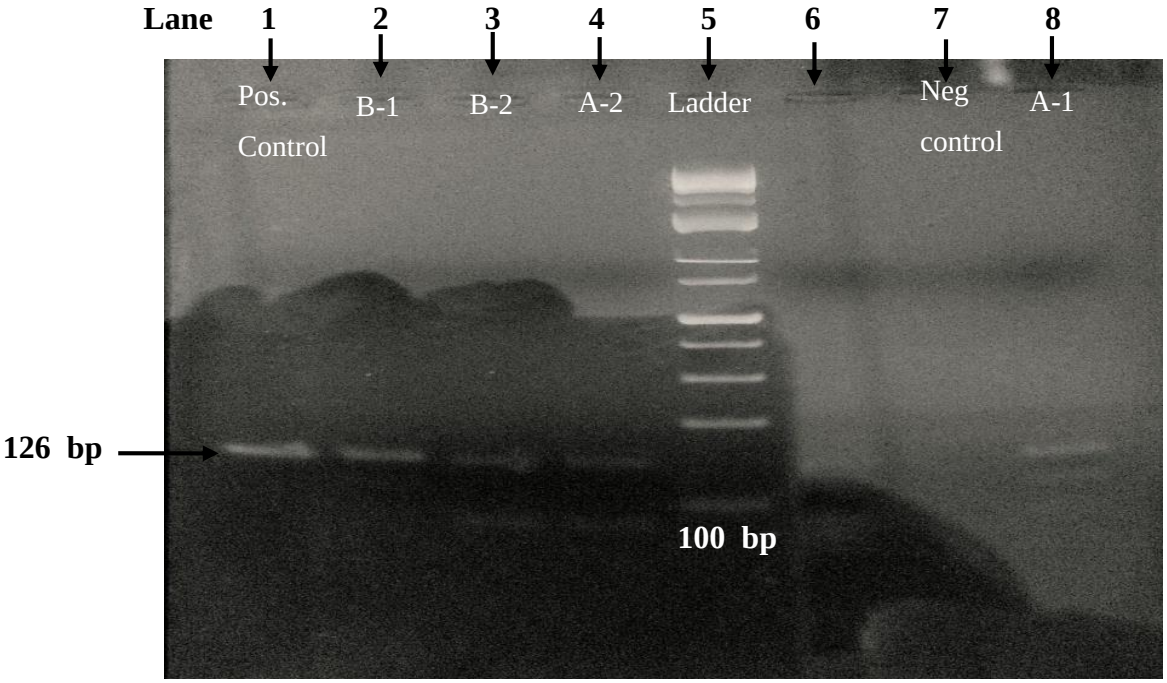
Table 3.2: Results showing carbohydrate fermentation test of 4 *Lactobacillus* spp. isolates

Carbohydrate	A-1	A-2	B-1	B-2
Glucose	+	+	-	-
Fructose	+	+	+	+
Sucrose	+	-	-	-
Arabinose	-	+	-	-
L-arginine	-	-	-	-
Galactose	-	+	+	+
Glycine	-	-	-	-
Lactose	-	-	+	+
L-lysine	-	-	-	-
Maltose	-	-	-	-
Mannitol	-	+	-	-
Rhamnose	+	+	-	-
Starch	-	-	-	-
Xylose	+	+	-	-
Trehalose	-	+	-	-

(Here, "+" indicates fermentation occurred and "-" indicates fermentation did not occur and A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.2 Identification through PCR

The template DNA was amplified through PCR then was checked by agarose gel electrophoresis. The band observed with help of UV transilluminator showed amplicon size 126bp which resembles with the amplicon size of the primer set used. This apparently confirms the identification of the 4 selected strain as *Lactobacillus* spp. genotypically. Figure 3.3 shows the image of agarose gel containing bands of DNA ladder and desired bands of the amplified template DNA.



Lane 1 – Positive control

Lane 2 – Isolate B-1 (normal infant)

Lane 3 – Isolate B-2 (normal infant)

Lane 4 – Isolate A-2 (HD infant)

Lane 5 – DNA Ladder (1kb)

Lane 6 – Blank

Lane 7 – Negative control

Lane 8 – Isolate A-1 (HD infant)

Figure 3.3: Agarose gel containing bands of DNA ladder and desired bands of the PCR amplified template DNA, extracted from isolates of diseased one 'Infant A' (A-1, A-2) and from normal one 'Infant B' (B-1, B-2) with positive and negative control.

3.3 Analysis of stress tolerance

3.3.1 Assay of pH tolerance

The 4 isolated *Lactobacillus* spp. were checked for pH tolerance ranging pH 2.5-8.5. It showed the ability to survive on both acidic and alkaline environments by those strains. The result of the assay is demonstrated here with graphical representation. Figure 3.4, Figure 3.5, Figure 3.6 and Figure 3.7 depicts the results of pH tolerance assay for the isolates respectively A-1, A-2 (from diseased one 'Infant A') and B-1, B-2 (from normal one 'Infant B').

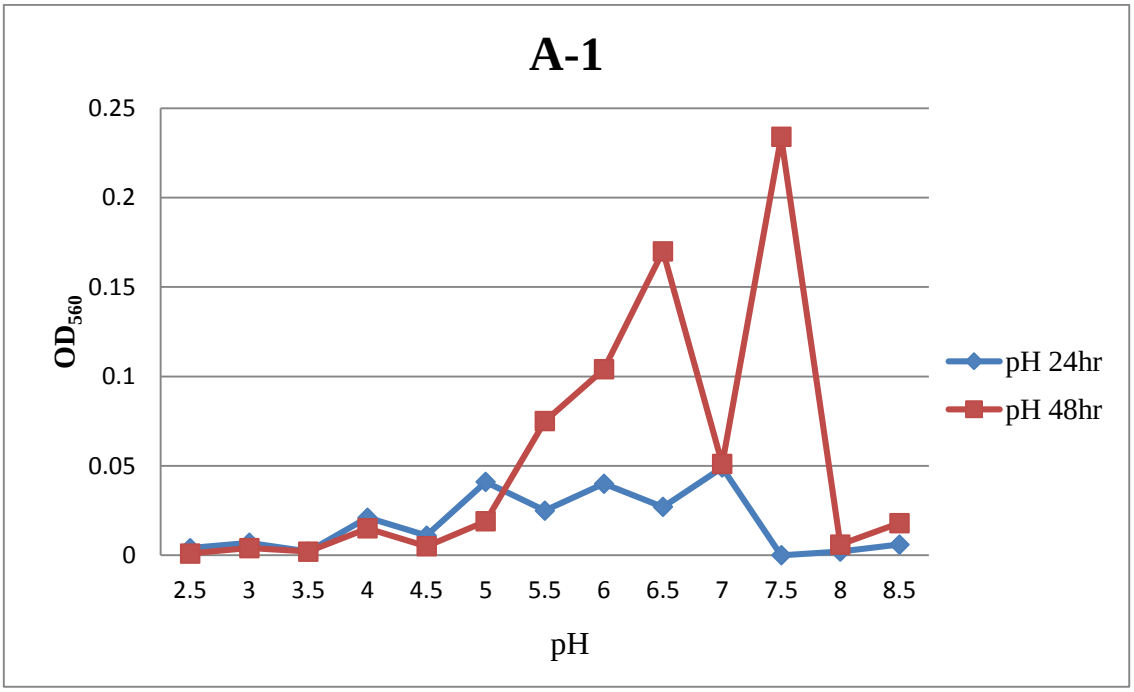


Figure 3.4: Assay of pH tolerance (pH 2.5-8.5) measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, for isolate A-1, isolated from diseased one 'Infant A'.

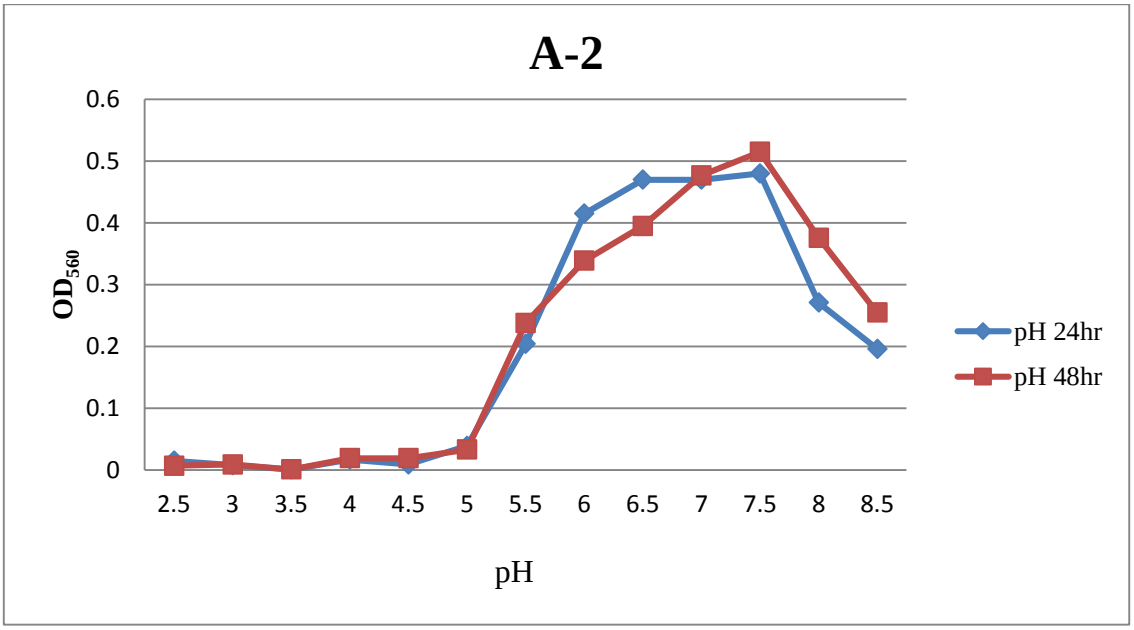


Figure 3.5: Assay of pH tolerance (pH 2.5-8.5) measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate A-2 isolated from diseased one 'Infant A'.

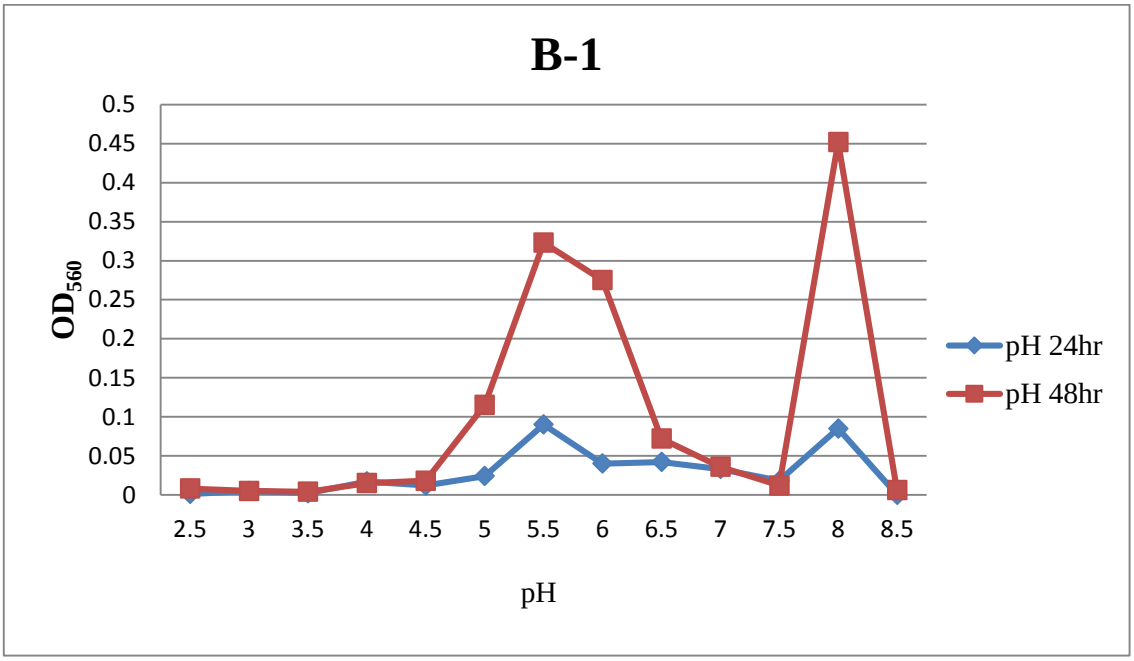


Figure 3.6: Assay of pH tolerance (pH 2.5-8.5) measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate B-1isolated from normal one 'Infant B'.

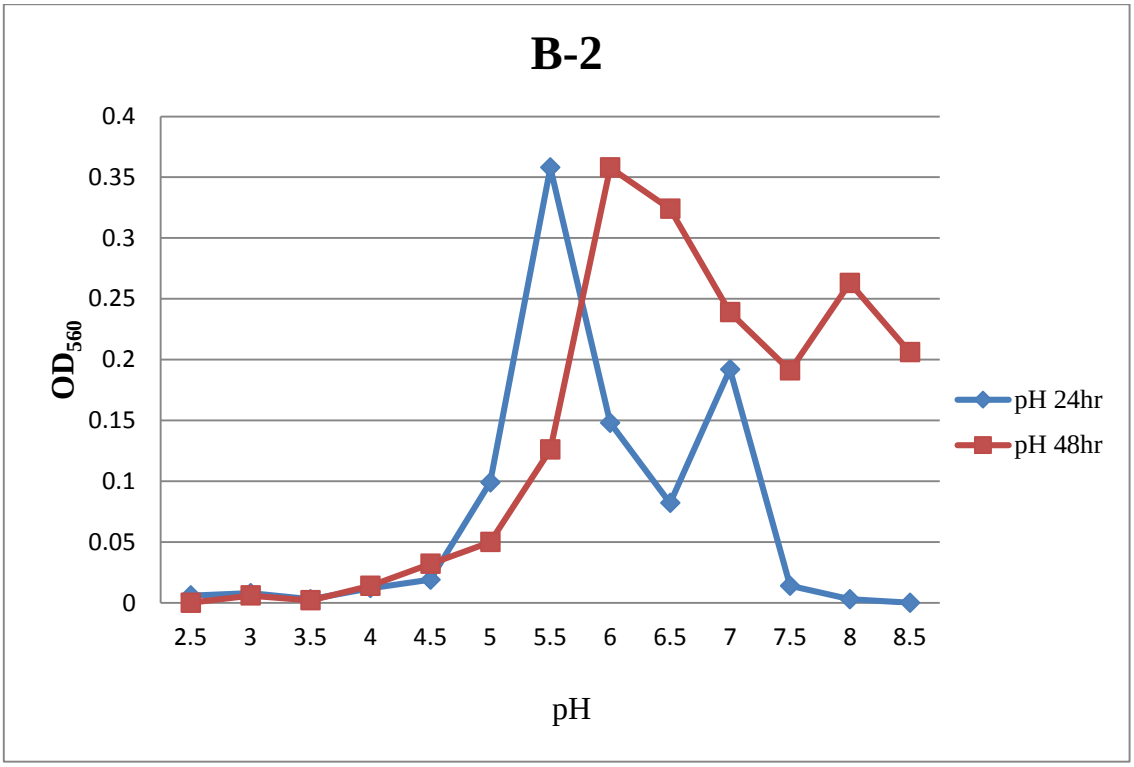


Figure 3.7: Assay of pH tolerance (pH 2.5-8.5) measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate B-1 isolated from normal one 'Infant B'

3.3.2 Assay of bile tolerance

As human gut consist a percentage of bile, so survival ability for any gut microbes in presence of bile is an important factor. Here, in this study four isolated *Lactobacillus* spp. have been checked for their survival ability in presence of different concentration of bile ranging 0.1%-0.3% which is similar to the concentration present in human gut (Spencer and Ragout de Spencer, 2001). Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.11 depicts the results of bile tolerance assay for the isolates respectively A-1, A-2 (from diseased one 'Infant A') and B-1, B-2 (from normal one 'Infant B').

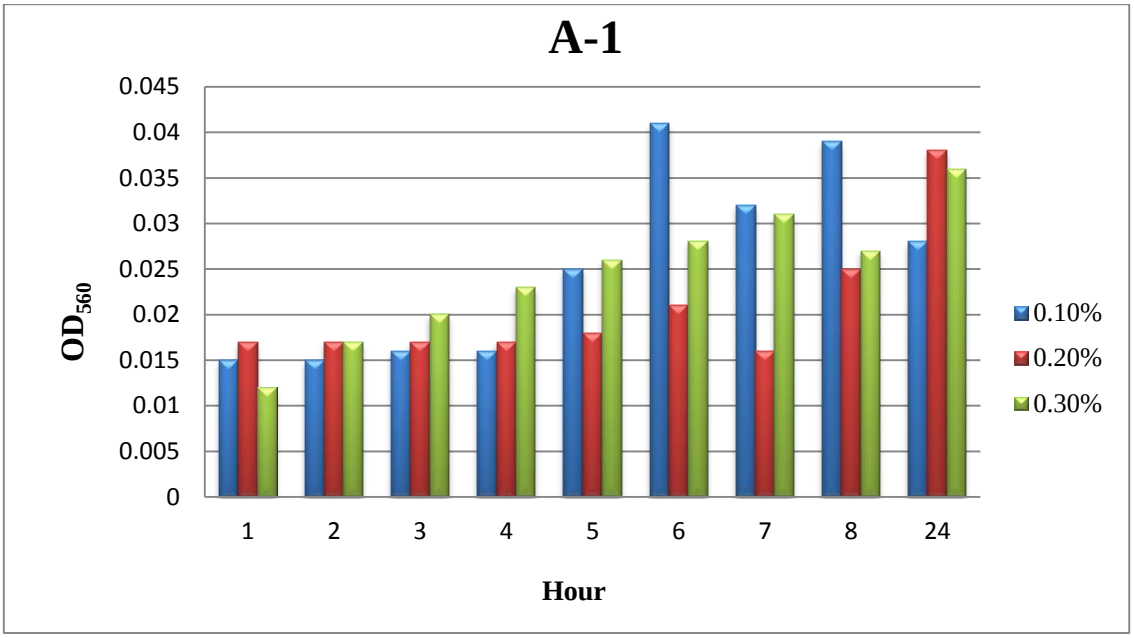


Figure 3.8: Assay of bile (0.1%-0.3%) tolerance measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate A-1 isolated from diseased one 'Infant A'

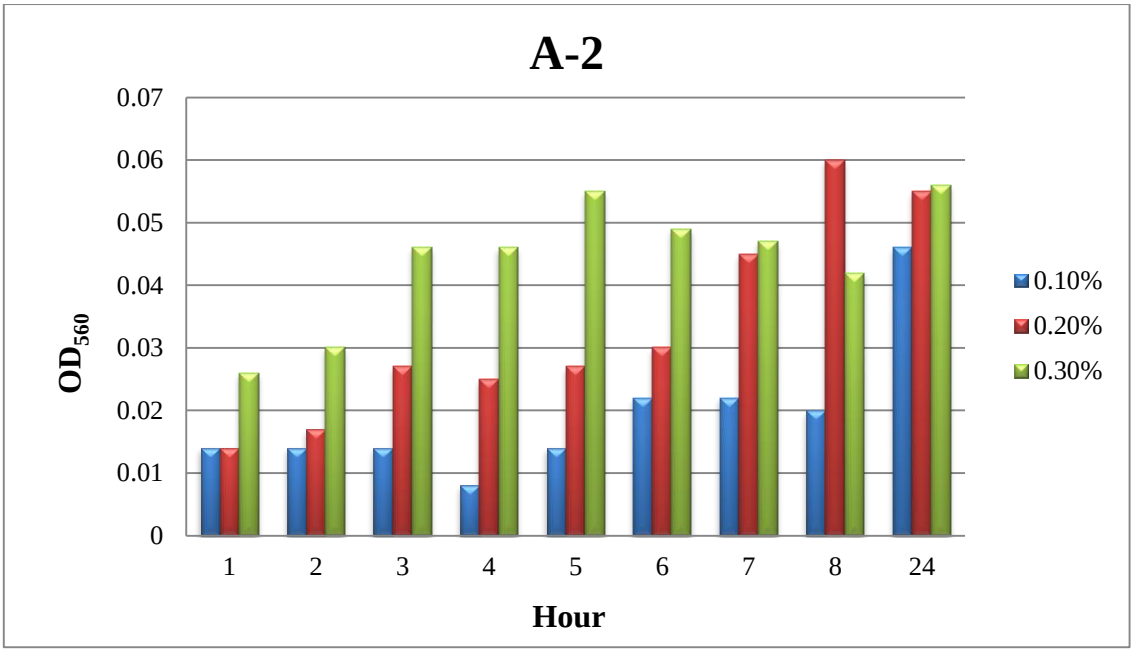


Figure 3.9: Assay of bile (0.1%-0.3%) tolerance measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate A-2, isolated from diseased one 'Infant A'

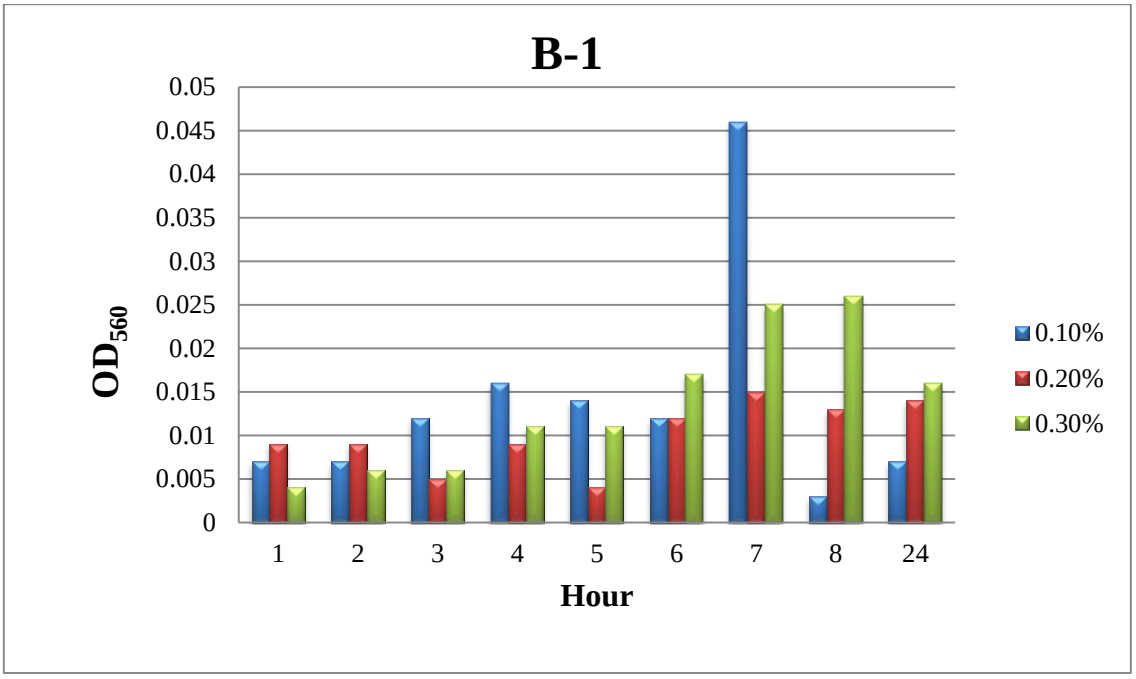


Figure 3.10: Assay of bile (0.1%-0.3%) tolerance measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate B-1, isolated from normal one 'Infant B'

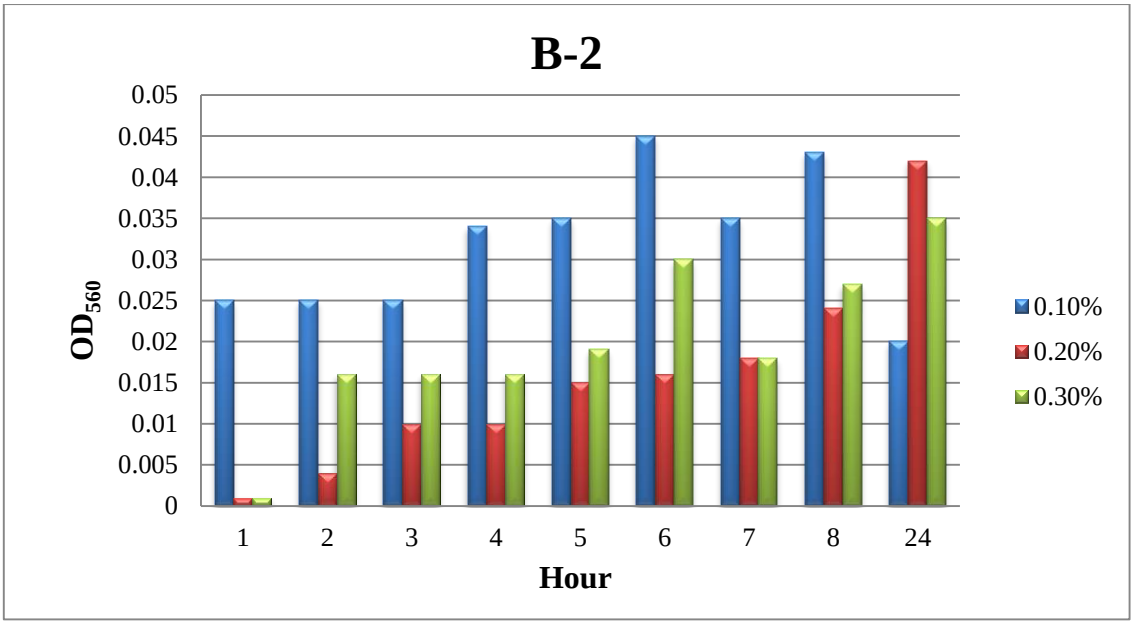


Figure 3.11: Assay of bile (0.1%-0.3%) tolerance measured through UV spectrophotometer (OD_{560}) after 24 hours and 48 hours' time, of isolate B-2, isolated from normal one 'Infant B'

3.3.3 Assay of NaCl tolerance

Survival ability in halophilic condition by the *Lactobacillus* spp. is another factor to investigate. To check this ability by the four isolated *Lactobacillus* spp. from the faeces of the infants on salt, different concentration of NaCl were added with MRS broth ranging 1% - 10%. The result of the NaCl tolerance assay is demonstrated in Table 3.3.

Table 3.3: Assay of NaCl tolerance by isolated *Lactobacillus* spp.

NaCl concentration	A-1	A-2	B-1	B-2
1%	++	++	++	++
2%	++	++	++	++
3%	++	++	++	++
4%	++	++	++	++
5%	++	++	++	++
6%	++	++	++	++
7%	++	++	++	++
8%	++	++	++	++
9%	++	++	+	+
10%	+	+	+	+

(Here, "++" demonstrates good growth and "+" demonstrates moderate growth and A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.4 Production of Organic acid

The concentrations of organic acid, produced by the four isolated *Lactobacillus* spp. were calculated. Percentages of total titrable acidity were also measured and then comparisons in between two infants were done. Table 3.4 demonstrates initial pH of skim milk and pH

after 24 hours, 48 hours and 72 hours' fermentation along with acid concentration and percentage of total titrable acidity of the isolated *Lactobacillus* spp.

Table 3.4: Relationship between pH and Titrable acidity by the isolated *Lactobacillus* spp. after 24 hours, 48 hours and 72 hours of fermentations

Sample	Temp.	Hour	Initial pH of skim milk	pH	Acid conc. (N)	% of TTA
A-1	37^0C	24	6.09	5.2	0.0001	0.9
		48		4.5	0.00045	5.4
		72		4.1	0.0014	7.2
A-2	37^0C	24	6.09	5.6	0.0001	0.9
		48		4.7	0.0006	4.05
		72		4.1	0.0008	12.6
B-1	37^0C	24	6.09	5.4	0.0001	0.9
		48		5.1	0.0006	5.4
		72		5.1	0.0006	5.4
B-2	37^0C	24	6.09	5.8	0.00005	0.45
		48		5.2	0.0003	2.7
		72		5.2	0.0004	3.6

(Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.5 Antibiotic sensitivity assay

To know antibiotic sensitivity or resistance in disc diffusion method several guideline is there. But very few are for fastidious organisms. The Clinical and Laboratory Standards Institute (CLSI) formerly known as National Committee for Clinical Laboratory Standards (NCCLS) had published the first version regarding guideline of antimicrobial susceptibility testing for fastidious organism at 2006 (Jorgensen et al., 2007). To compare the result we have used the known standard described in the CLSI guideline for antimicrobial susceptibility testing (Clinical and Laboratory Standards Institute 2012) similarly in a study by Sharma et al. (2016). In Table 3.5 the range of zone diameter with corresponding sensitivity type. Then Table 3.6 shows the zone diameter of the four isolated *Lactobacillus* spp. found in disc diffusion method for different antibiotics.

Table 3.5: Standard for antibiotic sensitivity assay

Sensitivity type	Zone diameter	Reference
Susceptible	>20 mm	(Sharma et al., 2016)
Intermediate	15-19 mm	
Resistant	≤14 mm	

Table 3.6: Antibiotic sensitivity assay of isolated *Lactobacillus* spp.

Group	Name of antibiotic	Conc.	A-1	A-2	B-1	B-2
β-lactams	Penicillin G	10 units	42 (S)	0 (R)	40 (S)	40 (S)
	Ampicillin	10 µg	30 (S)	26 (S)	40 (S)	45 (S)
	Amoxicillin	10 µg	30 (S)	26 (S)	40 (S)	40 (S)
	Amoxiclav (Amoxicillin/ Clavulenic acid)	20µg/10 µg	35 (S)	30 (S)	42 (S)	48 (S)
	Cloxacillin	5 µg	22 (S)	25 (S)	20 (S)	20 (S)
	Oxacillin	1 µg	0 (R)	0 (R)	15 (I)	22 (S)
Amioglycosides	Kanamycin	30 µg	0 (R)	21 (S)	0 (R)	0 (R)
	Gentamycin	10 µg	0 (R)	20 (S)	14 (R)	15 (I)
	Amikacin	30 µg	0 (R)	0 (R)	22(S)	15 (I)
	Tobramycin	10 µg	0 (R)	20(S)	0 (R)	0 (R)
	Streptomycin	10 µg	0 (R)	0 (R)	0 (R)	0 (R)
	Netilmicin	30 µg	15 (I)	16 (I)	16 (I)	18 (I)
Quinolones	Ciprofloxacin	5 µg	28 (S)	20 (S)	0 (R)	0 (R)
	Norfloxacin	10 µg	20 (S)	18 (I)	0 (R)	0 (R)
	Ofloxacin	5 µg	26 (S)	16 (I)	0 (R)	20 (S)
	Nalidixic acid	30 µg	0 (R)	0 (R)	0 (R)	0 (R)
	Levofloxacin	5 µg	29 (S)	18 (I)	0 (R)	0 (R)
	Moxifloxacin	5 µg	32 (S)	40 (S)	23 (S)	20 (S)
	Pefloxacin	5 µg	18 (I)	16 (I)	0 (R)	0 (R)
Cephalosporins	Ceftriaxone	30 µg	19 (I)	24 (S)	25 (S)	30 (S)
	Cefuroxime	30 µg	34 (S)	0 (R)	25 (S)	32 (S)
	Cefepime	30 µg	32 (S)	0 (R)	30 (S)	30 (S)
	Ceftazidime	30 µg	0 (R)	0 (R)	15 (I)	17 (I)
	Cefexime	5 µg	0 (R)	0 (R)	20 (S)	0 (R)
	Cefoxitin	30 µg	0 (R)	0 (R)	0 (R)	18 (I)

(Continued.)

Table 3.6: Antibiotic sensitivity assay of isolated *Lactobacillus* spp. (Continued.)

Group	Name of antibiotic	Conc.	A-1	A-2	B-1	B-2
	Cephalexin	30 µg	0 (R)	0 (R)	25 (S)	24 (S)
Tetracycline	Tetracycline	30 µg	45 (S)	14 (R)	30 (S)	33 (S)
	Doxycycline	30 µg	47 (S)	20 (S)	30 (S)	35 (S)
	Minocycline	30 µg	50 (S)	20 (S)	30 (S)	35 (S)
	Tigecycline	15 µg	32 (S)	45 (S)	27 (S)	35 (S)
Glycopeptides	Vancomycin	5 µg	0 (R)	26 (S)	0 (R)	0 (R)
Macrolides	Azithromycin	15 µg	30 (S)	0 (R)	25 (S)	25 (S)
	Erythromycin	15 µg	40 (S)	35 (S)	37 (S)	40 (S)
Carbapenem	Imipenem	10 µg	30 (S)	20 (S)	45 (S)	48 (S)
	Meropenem	10 µg	0 (R)	0 (R)	35 (S)	35 (S)
Oxazolidinone	Linezolid	30 µg	44 (S)	36 (S)	35 (S)	36 (S)
Aminocoumarin	Novobiocin	30 µg	30 (S)	21 (S)	18 (I)	19 (I)
Sulfonamides	Cotrimoxazole (trimethoprim /suiphamethoxazole)	85 µg	0 (R)	0 (R)	0 (R)	0 (R)
Azolidione	Nitrofurantoin	300 µg	30 (S)	26 (S)	27 (S)	27 (S)
Lincosamides	Clindamycin	2 µg	32 (S)	14 (R)	32 (S)	35 (S)
Others	Chloramphenicol	30 µg	35 (S)	30 (S)	35 (S)	40 (S)
	Piperacillin/Tazobactam	100µg /10µg	43 (S)	0 (R)	42 (S)	45 (S)
	Rifampicin	5 µg	35 (S)	20 (S)	25 (S)	35 (S)
	Metronidazole	50 µg	0 (R)	0 (R)	0 (R)	0 (R)
	Polymyxin B	300IU	0 (R)	10 (R)	0 (R)	0 (R)

(Here, the unit of measurement for zone diameter is ‘mm’. Again, ‘S’ means susceptible, ‘I’ means intermediate resistant and ‘R’ means resistant. A-1, A-2 are isolates from Hirschsprung diseased infant that is ‘Infant A’ and B-1, B-2 are isolates from normal infant that is ‘Infant B’).

3.6 Aggregation properties

3.6.1 Auto aggregation

It is evident that auto-aggregation that is aggregation between themselves considered as an important characteristic for the commensal bacterium duels in gut. Regarding current study, auto-aggregation percentages showed by the isolated *Lactobacillus* spp. are pretty high. The highest percentage has been found in A-2 (80.6%) whereas the lowest one has been shown by another isolate of same infant 'Infant A', A-1 (70.6%). Table 3.7 contains the auto aggregation percentages of the four isolates from 'Infant A' and 'Infant B'.

Table 3.7: Auto-aggregation percentages of isolated *Lactobacillus* spp.

Sample	Auto-aggregation percentages
A-1	**70.6%**
A-2	**80.6%**
B-1	**74.6%**
B-2	**79.4%**

(Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.6.2 Co-aggregation

The co-aggregation percentages of the *Lactobacillus* spp. in current study are more towards the Gram positive bacteria than that of Gram negative one. Again, in between the isolates of two infants, percentages of co-aggregation for *Lactobacillus* spp. from "Infant A" found less than "Infant B" though the ratio of difference is a minor one.

The highest co-aggregation percentage among the isolates regarding Gram negative strains specifically for *Shigella dysenteriae* was exerted by A-2 that is 61.01%. Accordingly, towards Gram positive bacteria it was for *Bacillus subtilis* shown by B-1 which is 58.33%. Table 3.8 and Table 3.9 depict the co-aggregation percentages of isolates respectively for Gram negative and Gram positive bacteria.

Table 3.8: Co-aggregation percentages for Gram negative bacteria

Gram negative	**A-1**	**A-2**	**B-1**	**B-2**
***E. coli* (ATCC 25922)**	42.87%	18.61%	25.21%	13.36%
***E.coli* (0157: H7)**	17.07%	15.12%	20.97%	15.33%
***P. aeruginosa* (ATCC 27853)**	18.87%	13.97%	47.47%	25.37%
Vibrio cholerae	13.33%	11.99%	14.65%	10.68%
Shigella dysenteriae	26.01%	61.01%	19.73%	12.04%

(Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

Table 3.9: Co-aggregation percentages for Gram positive bacteria

Gram positive	A-1	A-2	B-1	B-2
E. faecalis	**27.5%**	**21.92%**	**20.97%**	**26.64%**
S. aureus **(ATCC 25923)**	**20.79%**	**17.05%**	**25.53%**	**14.22%**
S. pneumoniae **(ATCC 42619)**	**20.18%**	**27.44%**	**25.18%**	**18.99%**
Bacillus subtilis	**32.36%**	**38.2%**	**58.33%**	**8.03%**
Bacillus cereus	**46.58%**	**32.35%**	**48.39%**	**23.4%**

(Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.6.3 Salt aggregation test

Along with auto aggregation and co-aggregation salt aggregation test (SAT) has been done with Ammonium sulphate of different molarity ranging 0.01M-4M by the isolated *Lactobacillus* spp. The visible level of aggregation with the salt was determined and demonstrated as SAT value as described by Sadrani et al. (2014). In Table 3.10, results of salt aggregation test for all four isolates are shown.

Table 3.10: Salt aggregation test results (SAT value) by isolated *Lactobacillus* spp.

Isolate	SAT value
A-1	**0.1M**
A-2	**0.5M**
B-1	**2.5M**
B-2	**0.01M**

(Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B')

3.7 Effect towards other gut bacteria

To check the effect of *Lactobacillus* spp. with other gut bacteria during co-existence this was done. Here, *Vibrio cholerae* and *Escherichia coli* were used along with isolated *Lactobacillus* spp. It was done through colony count method both *in vitro* using culture media and *in vivo* using mouse model system. Here, Figure 3.12 shows the image of a Swiss albino mouse used *in vivo* and Figure 3.13 shows test tube containing mouse stool.

Figure 3.12: Swiss albino mouse, used to check effect towards other bacteria *in vivo*

Figure 3.13: Faeces collected from Swiss albino mouse

3.7.1 Effect towards other gut bacteria *in vitro*

In this study, in vitro effects of the four *Lactobacillus* spp. towards *V. cholerae* and *E. coli* have been demonstrated through graphical representation. Here, the overall growth of the bacteria is less after addition of *Lactobacillus* spp. than the control without *Lactobacillus* spp. Figure 3.14 demonstrates the graphical representation of *in vitro* effect towards other bacteria by all four isolate after 24 hours through colony count.

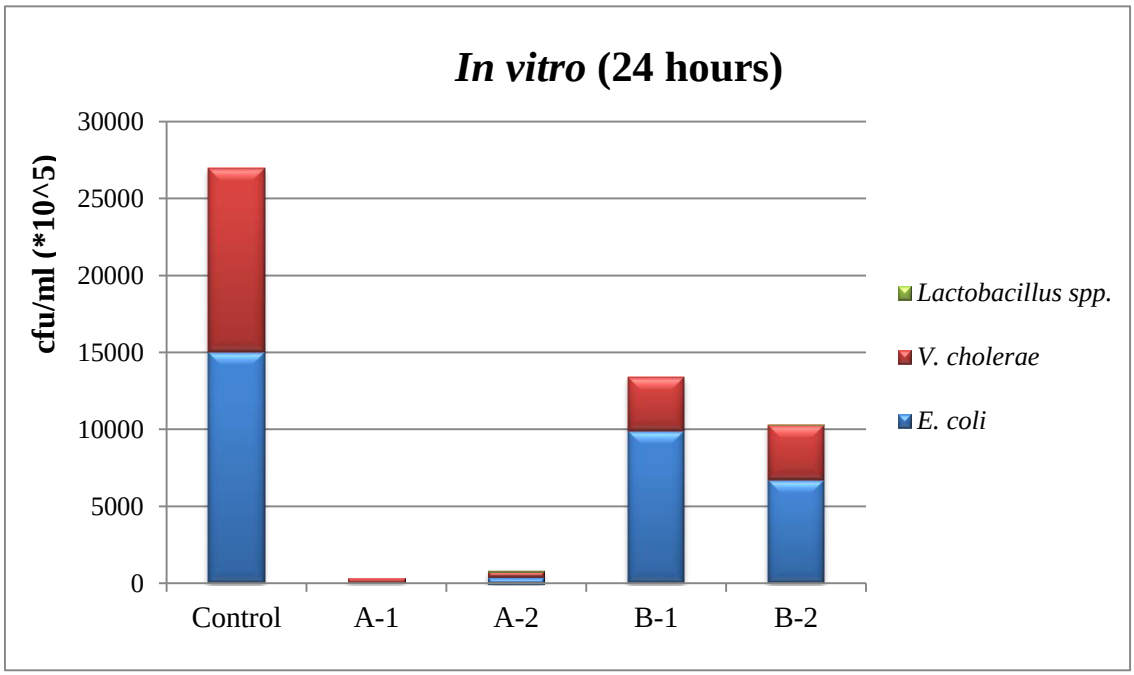


Figure 3.14: Effect towards *E. coli* and *V. cholerae in vitro* by isolates after 24 hours through colony count. Here, A-1, A-2 are isolates from Hirschsprung diseased infant that is 'Infant A' and B-1, B-2 are isolates from normal infant that is 'Infant B'

3.7.2 Effect towards other gut bacteria *in vivo*

Faeces of mouse have been used here to check the effect of isolated *Lactobacillus* spp. towards *V. cholerae* and *E. coli in vivo*. The graph here shows lesser growth of all bacteria in test mice than the control used after 24 hours. But, after 48 hours increased growth of *Lactobacillus* spp. have been seen in both test mice. Figure 3.15 and Figure 3.16 demonstrates the graphical representation of *in vivo* effect towards other bacteria by isolated *Lactobacillus* spp. through colony count respectively after 24 hours and 48 hours.

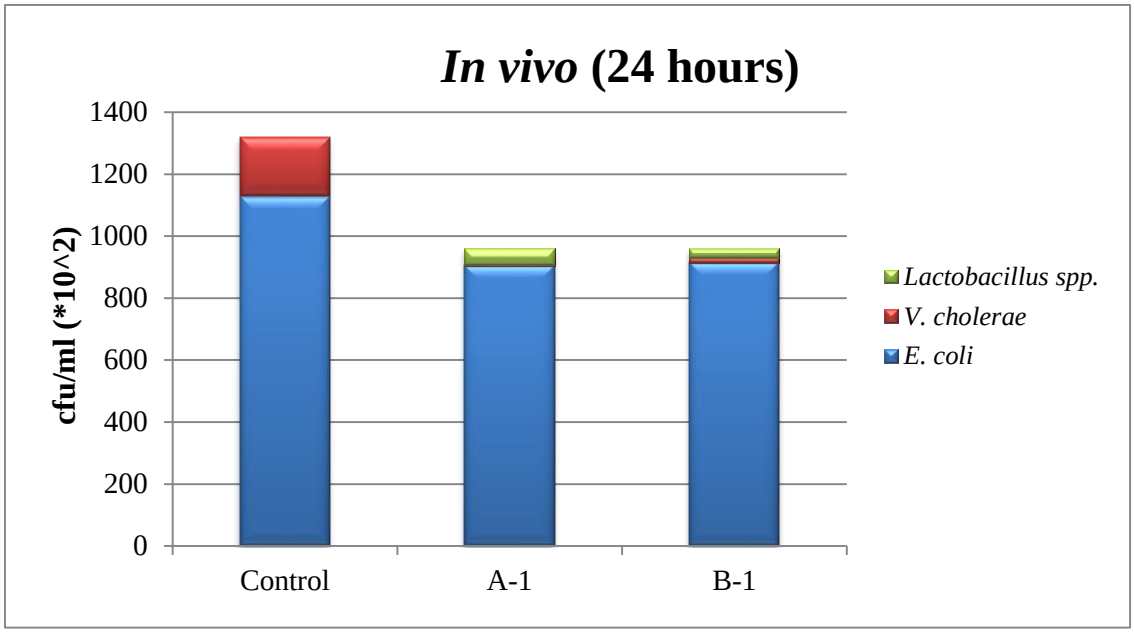


Figure 3.15: Effect towards *E. coli* and *V. cholerae in vivo* by isolates after 24 hours through colony count. Here, A-1 is isolate from Hirschsprung diseased infant that is 'Infant A' and B-1 is isolate from normal infant that is 'Infant B'

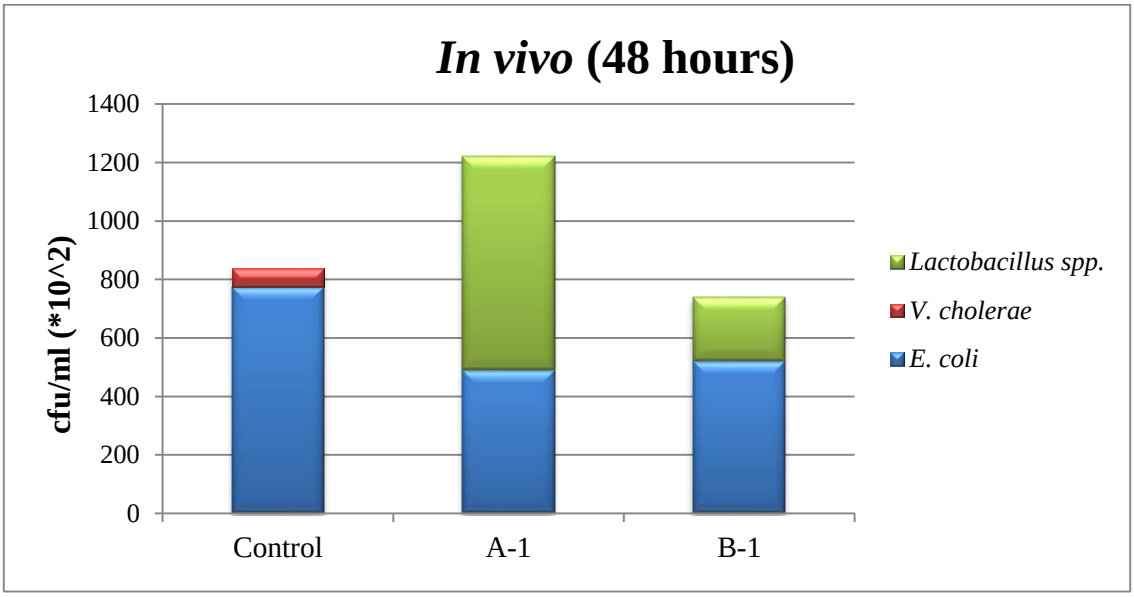


Figure 3.16: Effect towards *E. coli* and *V. cholerae in vivo* by isolates after 48 hours through colony count. Here, A-1 is isolate from Hirschsprung diseased infant that is 'Infant A' and B-1 is isolate from normal infant that is 'Infant B'

Chapter 4: Discussion...

4.0 Discussion

A commensal gut microbe helps to maintain homeostatic condition of gut through its symbiotic effect (Kernbauer et al., 2014). But different disease conditions may disrupt this homeostasis of gut. As for Hirschsprung disease, a study by Shen et al. (2009), showed the lower number of *Lactobacillus* spp. and *Bifidobacterium* spp. in babies with Hirschsprung disease associated enterocolitis (HAEC) than those who did not develop enterocolitis, suggesting probable bacterial relevance towards formation of enterocolitis in Hirschsprung diseased infants. Similar result was found for Firmicutes of infant with Hirschsprung disease and with HAEC infants as has been described by Ward et al. (2012) and suggests that this dysbiosis may be due to the absence of nerve ganglions in distal portion of gut. So it is hypothesised here that along with aganglionosis, stress may be an effect for dysbiosis in Hirschsprung diseased babies. And to check the stressed effects, *Lactobacillus* spp. have been chosen here to represent gut bacteria, because, as a gut bacteria, *Lactobacillus* spp. work not only through symbiosis, but they have different probiotic effect too (Lebeer et al., 2008). For comparison of the stressed effect on gut bacteria, specifically *Lactobacillus* spp., a healthy baby without Hirschsprung disease and colostomy was chosen as control.

In this study, after morphological identification of *Lactobacillus* spp., several biochemical tests were done. Then carbohydrate fermentation for 15 sugars had been done and differentiation was found. Four isolates from two infants, that are from diseased one 'Infant A' (A-1, A-2) and from normal one 'Infant B' (B-1, B-2), were selected randomly. All four isolates fermented fructose. As for glucose, strains from "Infant A" fermented glucose but strain from "Infant B" did not. It has been seen that fermentation capability is more from strains of "Infant A" than from "Infant B". Strains from "Infant A" fermented 9 sugars out of 15, whereas strains from other one fermented only 3 sugars. Again, in between the two strains of "Infant A", isolate A-2 fermented arabinose, galactose, mannitol, trehalose which were not fermented by isolate A-1 and the vice versa has been occurred in case of sucrose. Though the strains of 'Infant B' showed lesser capability of fermentation but in between themselves they showed similar pattern of fermentation.

Usually, differentiation on fermentation is used to identify bacteria group as well as species (Delost, 2014), but this can also be the outcome of stress. On the other hand, confirmation of bacterial strain up to genus level has been done here through PCR amplification. Accordingly, the primers used were described by Delroisse et al. (2008) designed on basis of genus specific 16S rRNA. The amplification was done on thermal cycler. The preferred band found as per amplicon size 126 bp on agarose gel electrophoresis confirmed the four isolated strains as *Lactobacillus* spp. Thus from each infant, two *Lactobacillus* spp. were selected and comparative study was carried out.

After identification as *Lactobacillus* spp., the survival ability of the strains on acidic and alkaline environment were carried out using medium with varying range of pH (pH 2.5-8.5). As for acidic condition, the gastric pH of human had been recorded as pH 1.0-2.5, which is highly acidic one (Evans et al., 1988). Here, in this study, we checked the survival ability of the isolated *Lactobacillus* spp. up to pH 2.5 for about 48 hours. Among the four isolates, B-2 did not survive on pH 2.5 after 48 hours, but survived up to 24 hours. Other three isolates survived the whole period though none of them showed much growth. So, all the isolates were highly tolerable to acidic condition as gastric emptying time is around 2-4 hours irrespective of sex, age and Body Mass Index (BMI) (Hellmig et al., 2006).

Coming out from acidic condition of stomach, the pH of proximal ileum of human was determined as 6.6; distal ileum 7.5, where as in caecum it was found as 6.4 (Evans et al., 1988). So while checking tolerance of pH resembling different parts of the gastrointestinal tract in human, optimal growth condition was also determined for all four isolates. Other than isolate B-1 from normal infant, all three isolate survived up to pH 8.5, whereas B-1 survived up to pH 8.

For isolate A-1 from diseased infant, optimal growth was found at pH 7 after 24 hours and good growth had been found at pH 6.5 at 48 hours' period and optimal growth is in pH 7.5. Variation has been found in between 24 hours and 48 hours' growth period. For isolate A-2 from same infant, it was pH 7.5 after both 24 hours and 48 hours. It showed fairly good growth from pH 6 to pH 8 at both time periods. Again, the isolate B-1 from normal infant, showed optimal growth at pH 8, but pH 5.5 also found better for growth

than other pH level at both 24 hours and 48 hours' time period. Again, pH 5.5 is the optimal one at 24 hours for isolate B-2 and good growth was found in pH 7. At 48 hours' time period pH 6-6.5 showed optimal to good growth.

Usually the good growing condition of different isolated *Lactobacillus* spp. found within pH 5.5-6.5 (De Man et al., 1960; Hoque et al., 2010). A deviation of growth condition regarding pH has been found in all four isolates here. Isolate B-1 and B-2 from normal infant showed a bit similarity with those previous studies.

Tolerance to inhibitory substances like bile is an important characteristic to bacteria with probiotic properties (Lee and Salminen, 1995). Human intestinal tract contains bile secreted into duodenum from gall bladder after been synthesised in liver which can be up to 0.3% (Hofmann et al., 1983; Spencer and Ragout de Spencer, 2001). So it is necessary to have resistance effect towards bile by the bacterium to pass the proximal part of intestine and survive, not to multiply (Pancheniak and Soccol, 2005).

Here in this study, all four isolates survived and even multiplied in 0.1%, 0.2% and 0.3% bile after 24 hours of incubation. Initially the multiplication rate was slow, in between 6 to 8 hour the multiplication rate increased. Regarding the concentration of bile, variability in tolerance has been seen. Isolate A-2, from diseased infant, is highly tolerable to 0.3% bile whereas isolate B-2, from normal infant, showed high growth at 0.1% bile. Previous study by Floch et al. (1972) and Kurdi et al. (2006), suggest that the presence of bile acid has negative impact on growth of different intestinal microflora including *Lactobacillus* spp. Despite this inhibitory effect, *Lactobacillus* spp. grow in presence of bile, suggesting adaptation of stress factors by the bacteria (Sahadeva et al., 2011). Though several other factors as activity of bile salt hydrolase helps to grow *Lactobacillus* spp. in the presence of bile (Šušković et al., 2000).

The survival ability in halophilic condition of the isolates was also checked. It was found that all four isolates survived on medium supplemented with 10% NaCl. The strain A-1 and A-2, from Hirschsprung diseased one 'Infant A', showed good growth at 1%-9% NaCl and moderate growth on 10% NaCl. Again, strain B-1 and B-2, from normal one 'Infant B', showed good growth towards 1%-8% and moderate growth on 9% and 10%.

This result matches with the study done by Korkeala et al. (1992), where with increasing percentages of NaCl, the percentage of growth decreases. However, the survival capability of the strains found more here than the strain studied by Hoque et al. (2010) and Rahman et al. (2016) for probiotic properties.

In case of organic acid production, the results given by all four isolates were pretty much similar. Like several other studies by Hoque et al. (2010) and Diba et al. (2013), all of them produced acid during milk fermentation. The pH value lowered with time indicating increasing concentration of acid. The concentration of acid found most among the strain isolated from diseased infant 'Infant A' (A-1) at 72 hours.

Checking out antibiotic resistance pattern of commensal gut microbe, specially that have probiotic effect, is relatively a new concept (Jorgensen, 2004). In some cases, commensal bacteria harbours antibiotic resistant genes and sometimes it is contemplated as helpful characteristic. So that the bacteria can sustain at time of antibiotic treatment in intestine and maintain homeostasis there by scavenging out the pathogenic one (Bacha et al., 2010). But adverse effect of this characteristic is also there. Because, this resistant gene usually located in plasmid, can be transferred from probiotic one to pathogenic one leading it towards multi drug resistant bacterium (Devirgiliis et al., 2014; Ouoba et al., 2008).

Here, in this study other than isolate A-2, rest of the three isolates showed high susceptibility to penicillin G. Several other studies done with *Lactobacillus* spp. showed both resistance and susceptibility towards penicillin (Delgado et al., 2005; Temmerman et al., 2003). Among the β-lactams all four isolates were found to be highly susceptible towards ampicillin, amoxicillin, cloxacillin and combination of amoxicillin/clavulenic acid. This corresponds with the study of Sharma et al. (2016) done with *Lactobacillus* spp. other than oxacillin. Oxacillin was found to be resistant by the isolate A-1 and A-2, from diseased baby and intermediate susceptibility shown by B-1 and B-2, from normal baby.

Regarding the protein synthesis inhibitors (aminoglycosides), streptomycin was found highly resistant by *Lactobacillus* spp. isolated from different sources (Katla et al., 2001;

Klare et al., 2007). Similarly, all four strains showed no inhibition zone with streptomycin suggesting it to be resistant. In case of amikacin, both isolates from "Infant A" that is with colostomy, exerts no inhibition zone where as isolates from "Infant B" shown intermediate to high susceptibility. Netilmicin showed moderate susceptibility to all four isolates. High frequencies of resistance were found in case of kanamycin, gentamycin and tobramycin in all four isolates. Similar pattern of resistance was reported earlier in case of *Lactobacillus* spp. in another study by Zhou et al. (2005).

The isolates from Hirschsprung diseased "Infant A" showed less resistance towards quinolones working on bacterial DNA than that of "Infant B". It was reported by Sharma et al. (2016), that most of the strains of *Lactobacillus* spp. were found to be resistant by ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and sparfloxacin. However, in this study, two isolates from normal one "Infant B" B-1 and B-2 showed no zone for ciprofloxacin, norfloxacin, levofloxacin, pefloxacin. But, isolates from "Infant A" showed intermediate to high sensitivity on these antibiotics. As for moxifloxacin, it is highly susceptible for all four isolate. Nalidixic acid was reported to be resistant by different *Lactobacillus* spp. in most of the studies (Sharma et al., 2016; Zhou et al., 2005). In this current study it has been also found that none of the isolates exhibit inhibition zone for nalidixic acid.

Cephalosporin is another group of antibiotics work on bacteria by hampering the synthesis of peptidoglycan layer of cell wall, which is thicker in Gram positive bacteria than that of Gram negative one (Madigan et al., 1997; Sharma et al., 2016). However, in this current study, ceftazidime, cefexime, cefoxitine, cephalexin showed no zone for the *Lactobacillus* spp. isolates of "Infant A". Again for isolates of "Infant B", they showed variability. For cefuroxime and cefepime, only isolate A-2 showed resistance where as other found to be susceptible. All four isolates were found susceptible in case of ceftriaxone. Similar types of sensitivity towards ceftriaxone by *Lactobacillus* spp. were reported previously by Sharma et al. (2016).

Lactobacillus spp. were found to be resistant towards tetracycline in previous studies (Zago et al., 2011). In contrast to this, we found all of the four isolates to be susceptible to tetracycline, doxycycline, tigecycline and minocycline. Accordingly, tetracycline

sensitive *Lactobacillus* spp. were previously reported by Sharma et al. (2016) and D'Aimmo et al. (2007).

In the study done by Sharma et al. (2016) all the *Lactobacillus* spp. were found sensitive to erythromycin and chloramphenicol and a few found resistant to azithromycin. Similarity has been found here as all four isolates are highly sensitive by erythromycin and chloramphenicol. As for azithromycin, isolate A-2 showed no zone of inhibition but other three isolate found susceptible.

Vancomycin resistance was observed in three of four isolated *Lactobacillus* spp. here. Only A-2 was sensitive to vancomycin. Though vancomycin usually works against Gram positive organisms but it was also demonstrated that *Lactobacillus* spp. might have intrinsic resistance against glycopeptides (Johnson et al., 1990). In relevance to this, several studies has reported about vancomycin resistant *Lactobacillus* spp. isolates (Sharma et al., 2016; Temmerman et al., 2003).

Carbapenem group of antibiotics are another important broad spectrum group. In this study, sensitivity was found by all four isolated *Lactobacillus* spp. for imipenem, which is similar with the study done by Swenson et al. (1990). But both isolates from the baby with ileostomy exhibit high resistance towards meropenem, whereas isolates from the baby without ileostomy exhibit sensitivity.

In this study, all the isolates were sensitive to piperacillin/tazobactam combination except A-2, where it is a resistant one. Intermediate to high susceptibility was seen by all four isolates in case of linezolid, novobiocin, clindamycin, nitrofurantoin and rifampicin. Similar results were obtained in different other studies (D'Aimmo et al., 2007; Danielsen and Wind, 2003; Sharma et al., 2016).

Again altered scenario of resistance was seen for cotrimoxazole, metronidazole and polymyxin B where all four strains were highly resistant as like as the results obtained by Sharma et al. (2016) and D'Aimmo et al. (2007).

In comparison of the isolates from diseased one “Infant A” and normal one “Infant B” it was being observed that frequency of resistance is more for the groups of β-lactams,

aminoglycosides, cephalosporins, macrolides, carbapenem in “Infant A”. For “Infant B” they are quinolones and glycopeptides. Similar pattern for both sensitivity and resistance among the infants were found in case of tetracyclines, oxazolidinone, aminocoumarin, sulfonamides, azolidione, lincosamides chloramphenicol, rifampicin, metronidazole and polymyxin B.

It has been suggested that, aggregation properties of any bacterial strain may work by formation of biofilm (Rickard et al., 2003). This characteristic is considered as a positive one for commensal gut bacteria to protect intestinal wall and stimulate immune system by forming physico-chemical barrier and by elimination of non-desirable pathogenic bacteria (Castagliuolo et al., 2005; Ledder et al., 2008; Voltan et al., 2007). Again, studies conducted by Kos et al. (2003) and Jankovic et al. (2003) demonstrates that, characteristics related to adherence with epithelial cells, mucosal surface proteins, gene encoding aggregation promoting factor supplement the aggregation. As both auto-aggregation and co-aggregation properties may be a strain specific one, several studies have been carried out and demonstrated about these properties for *Lactobacillus* spp. (Janković et al., 2012; Sadrani et al., 2014). Cell surface properties as hydrophobicity, surface layer protein, mucous binding protein, lectin-carbohydrate interaction are important for aggregation of *Lactobacillus* spp. in between themselves or with other bacteria (Ekmekci et al., 2009; Ledder et al., 2008).

The isolated *Lactobacillus* spp. from current study showed auto-aggregation percentages approximately in between 70% to 80%. Auto-aggregation percentages, both higher (≥80%) and lower (≤70%) have been observed for *Lactobacillus* spp. in several other studies (Kos et al., 2003; Sadrani et al., 2014). In this study, isolate A-2 showed the highest percentage (80.6%) and isolate A-1 showed the lowest one (70.6%). The difference between the four isolates regarding auto-aggregation percentages is minor here.

The percentages of co-aggregation showed by isolates from normal one “Infant B” were more than that of diseased one “Infant A”. Though the highest percentage were exerted by isolate A-2 against *Shigella dysenteriae* (61.01%). The lowest one is by B-2 (8.03%) with *Bacillus subtilis.* All four isolates showed more aggregation towards Gram positive

organisms than Gram negative one which is similar to the study done by Sadrani et al. (2014). Among the Gram negative microorganisms' co-aggregation percentage ranges from 61.01%-11.99% by isolates from "Infant A". For "Infant B" it ranges 47.47%-10.68%. For Gram positive bacteria, the co-aggregation percentage range is 46.58%-17.05% for "Infant A" isolates and 58.33%-8.03% for "Infant B". The range and variation of range are higher in "Infant A" for Gram negative organisms whereas alternative picture was observed in case of Gram positive organisms. However, the variation seen here in between the isolates of "Infant A" and "Infant B" are relatively less.

In this study, ammonium sulphate was used to evaluate the cell surface hydrophobicity by salt aggregation test (SAT). The isolate from "Infant B", B-2 showed high hydrophobicity on basis of SAT value by aggregating with Ammonium sulphate of lower molarity (0.01M) whereas B-1 showed the lower one with high molarity (2.5M).

In this current study, the effect of isolated *Lactobacillus* spp. towards *Vibrio cholerae* and *Escherichia coli* was observed. After growing in nutrient broth during *in vitro* method the colony count was done. Here, the growth of *V. cholerae* and *E. coli* was much less being test studies after addition of *Lactobacillus* spp. than the control, in which only *V. cholerae* and *E. coli* were present and *Lactobacillus* spp. were absent. Again, in between the isolated *Lactobacillus* spp. the bacteria from diseased one showed more impact than that of normal one as *V. cholerae* and *E. coli* growth were much less with addition of A-1 and A-2 than B-1 and B-2 respectively.

To check the effect towards other bacteria found during *in vitro* method, it was done *in vivo* using mouse model system. Here, it was done using isolate A-1 and B-1. After 24 hours, enumeration of the bacterial colony from mice faeces using selective media revealed less growth of *V. cholerae* and *E. coli* in the mice with *Lactobacillus* spp. oral gavage than control one without *Lactobacillus* spp. oral gavage, similar to the result found *in vitro*. Similar suppressive effects towards *V. cholerae* and *E. coli* were also found after colony count at 48 hours. But *Lactobacillus* spp. count were found more at 48 hours sampling time for both the isolate A-1 and B-1. The percentages of growth of *V. cholerae* and *E. coli* at 24 hours and 48 hours were found similar while using A-1 and B-1.

Again, the growth of *E. coli* was found more at both 24 hours and 48 hours sampling time than *V. cholerae*. It can be due to the fact that, *E. coli* strain are already present in mice gut (Tannock et al., 1988). Similarly, increased count of *Lactobacillus* spp. at 48 hours and presence of it in control mice without *Lactobacillus* spp. gavage may be due to the lactic acid bacteria (LAB) present in mice gut (Dong et al., 1987; Tannock, 1992).

Through the result, it may indicate that, addition of *Lactobacillus* spp. might have effect on growth suppression of other two bacteria. As because, both *in vitro* and *in vivo* test result showed lesser growth of *V. cholerae* and *E. coli* with *Lactobacillus* spp. than control. Variations within the isolates of infant were also found during *in vitro* test. But during *in vivo* variation was minimal.

Concluding remarks

It has been already stated previously that stress or unfavourable conditions may affect the characteristics of the bacteria and several stress protein can be formed due to the adverse effects (De Angelis and Gobbetti, 2004; Marles-Wright and Lewis, 2007). Moreover, due to stress, different factors help the bacteria to adapt the adverse conditions (Foster, 2005; Schmidt and Zink, 2000). Here, in this study, different types of stress effects in the *Lactobacillus* spp. of infants with and without Hirschsprung disease were investigated. Several variations were found here in between the strains. Again, variations of results were also found in between the isolates of the same baby. However, this study is up to genus level of *Lactobacillus* spp. The differentiation found here can be due to the difference in species level. The next stage of this study is to check up to the species level of *Lactobacillus* spp. and then to evaluate the formation of stressed protein. It will lead us to study further regarding dysbiosis of gut microbes and their consequences in Hirschsprung diseased babies.

References...

References

Adlerberth, I., Carlsson, B., de Man, P., Jalil, F., Khan, S.R., Larsson, P., Mellander, L., Svanborg, C., Wold, A.E., and Hanson, L.A. (1991). Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. Acta paediatrica Scandinavica *80*, 602-610.

Adlerberth, I., Jalil, F., Carlsson, B., Mellander, L., Hanson, L.A., Larsson, P., Khalil, K., and Wold, A.E. (1998). High turnover rate of Escherichia coli strains in the intestinal flora of infants in Pakistan. Epidemiology and Infection *121*, 587-598.

Adlerberth, I., Strachan, D.P., Matricardi, P.M., Ahrné, S., Orfei, L., Åberg, N., Perkin, M.R., Tripodi, S., Hesselmar, B., and Saalman, R. (2007). Gut microbiota and development of atopic eczema in 3 European birth cohorts. Journal of Allergy and Clinical Immunology *120*, 343-350.

Adlerberth, I., and Wold, A.E. (2009). Establishment of the gut microbiota in Western infants. Acta paediatrica *98*, 229-238.

Aertsen, A., and Michiels, C.W. (2004). Stress and how bacteria cope with death and survival. Critical reviews in microbiology *30*, 263-273.

Ahrné, S., Lönnermark, E., Wold, A.E., Åberg, N., Hesselmar, B., Saalman, R., Strannegård, I.-L., Molin, G., and Adlerberth, I. (2005). Lactobacilli in the intestinal microbiota of Swedish infants. Microbes and Infection *7*, 1256-1262.

Amaretti, A., di Nunzio, M., Pompei, A., Raimondi, S., Rossi, M., and Bordoni, A. (2013). Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. Applied microbiology and biotechnology *97*, 809-817.

Amarri, S., Benatti, F., Callegari, M.L., Shahkhalili, Y., Chauffard, F., Rochat, F., Acheson, K.J., Hager, C., Benyacoub, J., Galli, E., *et al.* (2006). Changes of gut microbiota and immune markers during the complementary feeding period in healthy breast-fed infants. Journal of pediatric gastroenterology and nutrition *42*, 488-495.

Amiel, J., Sproat-Emison, E., Garcia-Barcelo, M., Lantieri, F., Burzynski, G., Borrego, S., Pelet, A., Arnold, S., Miao, X., and Griseri, P. (2008). Hirschsprung disease, associated syndromes and genetics: a review. Journal of medical genetics *45*, 1-14.

Antonio, M.A., Hawes, S.E., and Hillier, S.L. (1999). The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. Journal of Infectious Diseases *180*, 1950-1956.

Arpi, M., Vancanneyt, M., Swings, J., and Leisner, J.J. (2003). Six cases of Lactobacillus bacteraemia: identification of organisms and antibiotic susceptibility and therapy. Scandinavian journal of infectious diseases *35*, 404-408.

Bacha, K., Mehari, T., and Ashenafi, M. (2010). Antimicrobial susceptibility patterns of LAB isolated from Wakalim, a traditional ethiopian fermented sausage. Journal of food safety *30*, 213-223.

Balmer, S., and Wharton, B. (1989). Diet and faecal flora in the newborn: breast milk and infant formula. Archives of Disease in Childhood *64*, 1672-1677.

Beasley, S.S., and Saris, P.E.J. (2004). Nisin-Producing Lactococcus lactis Strains Isolated from Human Milk. Applied and Environmental Microbiology *70*, 5051-5053.

Begley, M., Gahan, C.G., and Hill, C. (2005). The interaction between bacteria and bile. FEMS microbiology reviews *29*, 625-651.

Benno, Y., Sawada, K., and Mitsuoka, T. (1984). The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol Immunol *28*, 975-986.

Bettelheim, K., Breadon, A., Faiers, M.C., O'Farrell, S.M., and Shooter, R. (1974). The origin of O serotypes of Escherichia coli in babies after normal delivery. Journal of Hygiene *72*, 67-70.

Bezirtzoglou, E., Tsiotsias, A., and Welling, G.W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe *17*, 478-482.

Bindels, L.B., Beck, R., Schakman, O., Martin, J.C., De Backer, F., Sohet, F.M., Dewulf, E.M., Pachikian, B.D., Neyrinck, A.M., Thissen, J.-P., *et al.* (2012). Restoring Specific Lactobacilli Levels Decreases Inflammation and Muscle Atrophy Markers in an Acute Leukemia Mouse Model. PLoS ONE *7*, e37971.

Bodian, m., and Carter, O. (1963). A family study of Hirschsprung's disease. Annals of human genetics *26*, 261-277.

Boris, S., Suárez, J.E., and Barbés, C. (1997). Characterization of the aggregation promoting factor from Lactobacillus gasseri, avaginal isolate. Journal of applied microbiology *83*, 413-420.

Bunning, V.K., Crawford, R.G., Tierney, J.T., and Peeler, J.T. (1990). Thermotolerance of Listeria monocytogenes and Salmonella typhimurium after sublethal heat shock. Applied and Environmental Microbiology *56*, 3216-3219.

Cai, Y., Pang, H., Kitahara, M., and Ohkuma, M. (2012). Lactobacillus nasuensis sp. nov., a lactic acid bacterium isolated from silage, and emended description of the genus Lactobacillus. International journal of systematic and evolutionary microbiology *62*, 1140-1144.

Cappuccino, J.G., and Sherman, N. (2008). Microbiology: a laboratory manual, Vol 9 (Pearson/Benjamin Cummings Boston, MA).

Castagliuolo, I., Galeazzi, F., Ferrari, S., Elli, M., Brun, P., Cavaggioni, A., Tormen, D., Sturniolo, G.C., Morelli, L., and Palù, G. (2005). Beneficial effect of auto-aggregating Lactobacillus crispatus on experimentally induced colitis in mice. FEMS Immunology & Medical Microbiology *43*, 197-204.

Caufield, P., Li, Y., Dasanayake, A., and Saxena, D. (2006). Diversity of lactobacilli in the oral cavities of young women with dental caries. Caries research *41*, 2-8.

Chen, Y.S., Yanagida, F., and Shinohara, T. (2005). Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. Letters in applied microbiology *40*, 195-200.

Coconnier, M.H., Klaenhammer, T.R., Kernéis, S., Bernet, M.F., and Servin, A.L. (1992). Protein-mediated adhesion of Lactobacillus acidophilus BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Applied and Environmental Microbiology *58*, 2034-2039.

Collado, M.C., Gueimonde, M., Hernandez, M., Sanz, Y., and Salminen, S. (2005). Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. Journal of food protection *68*, 2672-2678.

Collado, M.C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Scientific reports *6*, 23129.

D'Aimmo, M.R., Modesto, M., and Biavati, B. (2007). Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. International journal of food microbiology *115*, 35-42.

Danielsen, M., and Wind, A. (2003). Susceptibility of Lactobacillus spp. to antimicrobial agents. International journal of food microbiology *82*, 1-11.

Davis, G. (1955). The classification of lactobacilli from the human mouth. Microbiology *13*, 481-493.

De Angelis, M., and Gobbetti, M. (2004). Environmental stress responses in Lactobacillus: a review. Proteomics *4*, 106-122.

De La Cochetière, M.F., Durand, T., Lepage, P., Bourreille, A., Galmiche, J.P., and Doré, J. (2005). Resilience of the Dominant Human Fecal Microbiota upon Short-Course Antibiotic Challenge. Journal of Clinical Microbiology *43*, 5588-5592.

De Man, J., Rogosa, d., and Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. Journal of applied Bacteriology *23*, 130-135.

de Muinck, E.J., Oien, T., Storro, O., Johnsen, R., Stenseth, N.C., Ronningen, K.S., and Rudi, K. (2011). Diversity, transmission and persistence of Escherichia coli in a cohort of mothers and their infants. Environmental microbiology reports *3*, 352-359.

Dec, M., Puchalski, A., Nowaczek, A., and Wernicki, A. (2016). Antimicrobial activity of Lactobacillus strains of chicken origin against bacterial pathogenss. International microbiology : the official journal of the Spanish Society for Microbiology *19*, 57-67.

Delgado, S., Flórez, A.B., and Mayo, B. (2005). Antibiotic susceptibility of Lactobacillus and Bifidobacterium species from the human gastrointestinal tract. Current microbiology *50*, 202-207.

Delost, M.D. (2014). Introduction to Diagnostic Microbiology for the Laboratory Sciences (Jones & Bartlett Publishers).

Delroisse, J.-M., Boulvin, A.-L., Parmentier, I., Dauphin, R.D., Vandenbol, M., and Portetelle, D. (2008). Quantification of Bifidobacterium spp. and Lactobacillus spp. in rat fecal samples by real-time PCR. Microbiological research *163*, 663-670.

Dethlefsen, L., Huse, S., Sogin, M.L., and Relman, D.A. (2008). The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. PLoS Biology *6*, e280.

Dethlefsen, L., and Relman, D.A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proceedings of the National Academy of Sciences of the United States of America *108*, 4554-4561.

Devirgiliis, C., Zinno, P., Stirpe, M., Barile, S., and Perozzi, G. (2014). Functional screening of antibiotic resistance genes from a representative metagenomic library of food fermenting microbiota. BioMed research international *2014*.

Diaz-Ropero, M.P., Martin, R., Sierra, S., Lara-Villoslada, F., Rodriguez, J.M., Xaus, J., and Olivares, M. (2007). Two Lactobacillus strains, isolated from breast milk, differently modulate the immune response. J Appl Microbiol *102*, 337-343.

Diba, F.S., Hossain, K.M., Azim, M., and Hoque, M. (2013). Isolation, Characterization and Determination of Antimicrobial Properties of Lactic Acid Bacteria from Human Milk. Jordan Journal of Biological Sciences *6*.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences of the United States of America *107*, 11971-11975.

Dong, M.-Y., Chang, T.-W., and Gorbach, S.L. (1987). Effects of feeding Lactobacillus GG on lethal irradiation in mice. Diagnostic microbiology and infectious disease *7*, 1-7.

Dore, J., Sghir, A., Hannequart-Gramet, G., Corthier, G., and Pochart, P. (1998). Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal Bacteroides populations. Syst Appl Microbiol *21*, 65-71.

Droste, J.H.J., Wieringa, M.H., Weyler, J.J., Nelen, V.J., Vermeire, P.A., and Van Bever, H.P. (2000). Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? Clinical & Experimental Allergy *30*, 1548-1553.

Ekmekci, H., Aslim, B., and Ozturk, S. (2009). Characterization of vaginal lactobacilli coaggregation ability with Escherichia coli. Microbiology and immunology *53*, 59-65.

Endo, A., Irisawa, T., Futagawa-Endo, Y., Salminen, S., Ohkuma, M., and Dicks, L. (2013). Lactobacillus faecis sp. nov., isolated from animal faeces. International journal of systematic and evolutionary microbiology *63*, 4502-4507.

Eser, A., Thalhammer, F., Burghuber, F., Hogenauer, C., Stockenhuber, F., Wenisch, C., Widhalm, K., and Reinisch, W. (2012). Probiotics for the prevention of antibiotic-induced diarrhea. Zeitschrift fur Gastroenterologie *50*, 1089-1095.

Evans, D., Pye, G., Bramley, R., Clark, A., Dyson, T., and Hardcastle, J. (1988). Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut *29*, 1035-1041.

Fallani, M., Amarri, S., Uusijarvi, A., Adam, R., Khanna, S., Aguilera, M., Gil, A., Vieites, J.M., Norin, E., Young, D., *et al.* (2011). Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology *157*, 1385-1392.

Farina, C., Arosio, M., Mangia, M., and Moioli, F. (2001). Lactobacillus casei subsp. rhamnosus sepsis in a patient with ulcerative colitis. Journal of clinical gastroenterology *33*, 251-252.

Felis, G.E., and Dellaglio, F. (2007). Taxonomy of Lactobacilli and Bifidobacteria. Current issues in intestinal microbiology *8*, 44-61.

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., and Rodríguez, J.M. (2013). The human milk microbiota: Origin and potential roles in health and disease. Pharmacological Research *69*, 1-10.

Floch, M.H., Binder, H.J., Filburn, B., and Gershengoren, W. (1972). The effect of bile acids on intestinal microflora. The American journal of clinical nutrition *25*, 1418-1426.

Foster, P.L. (2005). Stress responses and genetic variation in bacteria. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis *569*, 3-11.

Fouhy, F., Guinane, C.M., Hussey, S., Wall, R., Ryan, C.A., Dempsey, E.M., Murphy, B., Ross, R.P., Fitzgerald, G.F., Stanton, C., *et al.* (2012). High-Throughput Sequencing Reveals the Incomplete, Short-Term Recovery of Infant Gut Microbiota following Parenteral Antibiotic Treatment with Ampicillin and Gentamicin. Antimicrobial Agents and Chemotherapy *56*, 5811-5820.

Fradiani, P.A., Petrucca, A., Ascenzioni, F., Di Nucci, G., Teggi, A., Bilancini, S., and Cipriani, P. (2010). Endocarditis caused by Lactobacillus jensenii in an immunocompetent patient. Journal of medical microbiology *59*, 607-609.

Frykman, P.K., Nordenskjöld, A., Kawaguchi, A., Hui, T.T., Granström, A.L., Cheng, Z., Tang, J., Underhill, D.M., Iliev, I., and Funari, V.A. (2015). Characterization of bacterial and fungal microbiome in children with Hirschsprung disease with and without a history of enterocolitis: a multicenter study. PloS one *10*, e0124172.

Fuller, R., Barrow, P., and Brooker, B. (1978). Bacteria associated with the gastric epithelium of neonatal pigs. Applied and Environmental Microbiology *35*, 582-591.

Gill, S.R., Pop, M., DeBoy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., and Nelson, K.E. (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. Science (New York, NY) *312*, 1355-1359.

Goldberg, E. (1984). An epidemiological study of Hirschsprung's disease. International journal of epidemiology *13*, 479-485.

Gosalbes, M.J., Valles, Y., Jimenez-Hernandez, N., Balle, C., Riva, P., Miravet-Verde, S., de Vries, L.E., Llop, S., Agerso, Y., Sorensen, S.J., *et al.* (2016). High frequencies of antibiotic resistance genes in infants' meconium and early fecal samples. Journal of developmental origins of health and disease *7*, 35-44.

Gray, J., and Shiner, M. (1967). Influence of gastric pH on gastric and jejunal flora. Gut *8*, 574-581.

Heanue, T.A., and Pachnis, V. (2007). Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nature Reviews Neuroscience *8*, 466-479.

Hellmig, S., Von Schöning, F., Gadow, C., Katsoulis, S., Hedderich, J., Fölsch, U.R., and Stüber, E. (2006). Gastric emptying time of fluids and solids in healthy subjects determined by 13C breath tests: influence of age, sex and body mass index. Journal of gastroenterology and hepatology *21*, 1832-1838.

Hill, P.J., and Stewart, G.S. (1992). The polymerase chain reaction in molecular and micro-biology. Biotechnology and Genetic Engineering Reviews *10*, 343-378.

Hlaing, T., and Maung, M. (1989). An epidemiologic profile of laprotomy cases in Yangon Children's Hospital.

Hofmann, A., Molino, G., Milanese, M., and Belforte, G. (1983). Description and simulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. Cholic acid in healthy man. Journal of Clinical Investigation *71*, 1003.

Hooper, L.V., and Gordon, J.I. (2001). Commensal host-bacterial relationships in the gut. Science *292*, 1115-1118.

Hopkins, M.J., Macfarlane, G.T., Furrie, E., Fite, A., and Macfarlane, S. (2005). Characterisation of intestinal bacteria in infant stools using real-time PCR and northern hybridisation analyses. FEMS Microbiology Ecology *54*, 77-85.

Hoque, M., Akter, F., Hossain, K., Rahman, M., Billah, M., and Islam, K. (2010). Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selective regional yoghurts. World Journal of Dairy & Food Sciences *5*, 39-46.

Houghteling, P.D., and Walker, W.A. (2015). Why is initial bacterial colonization of the intestine important to the infant's and child's health? Journal of pediatric gastroenterology and nutrition *60*, 294-307.

Indrio, F., Di Mauro, A., Riezzo, G., Civardi, E., Intini, C., Corvaglia, L., Ballardini, E., Bisceglia, M., Cinquetti, M., Brazzoduro, E., *et al.* (2014). Prophylactic use of a probiotic in the prevention of colic, regurgitation, and functional constipation: a randomized clinical trial. JAMA pediatrics *168*, 228-233.

Iwabuchi, N., Yonezawa, S., Odamaki, T., Yaeshima, T., Iwatsuki, K., and Xiao, J.Z. (2012). Immunomodulating and anti-infective effects of a novel strain of Lactobacillus paracasei that strongly induces interleukin-12. FEMS immunology and medical microbiology *66*, 230-239.

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C., Bjorksten, B., Engstrand, L., and Andersson, A.F. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut *63*, 559-566.

Jankovic, I., Ventura, M., Meylan, V., Rouvet, M., Elli, M., and Zink, R. (2003). Contribution of aggregation-promoting factor to maintenance of cell shape in Lactobacillus gasseri 4B2. Journal of bacteriology *185*, 3288-3296.

Janković, T., Frece, J., Abram, M., and Gobin, I. (2012). Aggregation ability of potential probiotic Lactobacillus plantarum strains. International Journal of Sanitary Engineering Research *6*, 17-22.

Jenkins, D., Chaisson, S., and Matin, A. (1990). Starvation-induced cross protection against osmotic challenge in Escherichia coli. Journal of bacteriology *172*, 2779-2781.

Jimenez, E., Delgado, S., Fernandez, L., Garcia, N., Albujar, M., Gomez, A., and Rodriguez, J.M. (2008). Assessment of the bacterial diversity of human colostrum and screening of staphylococcal and enterococcal populations for potential virulence factors. Research in microbiology *159*, 595-601.

Johnson, A.P., Uttley, A., Woodford, N., and George, R. (1990). Resistance to vancomycin and teicoplanin: an emerging clinical problem. Clinical Microbiology Reviews *3*, 280-291.

Jones, M.L., Martoni, C.J., Parent, M., and Prakash, S. (2012). Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. The British journal of nutrition *107*, 1505-1513.

Joosten, H., and Northolt, M. (1989). Detection, growth, and amine-producing capacity of lactobacilli in cheese. Applied and Environmental Microbiology *55*, 2356-2359.

Jorgensen, J.H. (2004). Need for susceptibility testing guidelines for fastidious or less-frequently isolated bacteria. Journal of clinical microbiology *42*, 493-496.

Jorgensen, J.H., Hindler, J.F., Reller, L.B., and Weinstein, M.P. (2007). New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. Clinical infectious diseases *44*, 280-286.

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. The British journal of nutrition *110*, 1253-1262.

Katla, A.-K., Kruse, H., Johnsen, G., and Herikstad, H. (2001). Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. International journal of food microbiology *67*, 147-152.

Kato, Y., Sakala, R., Hayashidani, H., Kiuchi, A., Kaneuchi, C., and Ogawa, M. (2000). Lactobacillus algidus sp. nov., a psychrophilic lactic acid bacterium isolated from vacuum-packaged refrigerated beef. International journal of systematic and evolutionary microbiology *50*, 1143-1149.

Kelly, C.P., and LaMont, J.T. (1998). Clostridium difficile infection. Annual review of medicine *49*, 375-390.

Kernbauer, E., Ding, Y., and Cadwell, K. (2014). An enteric virus can replace the beneficial function of commensal bacteria. Nature *516*, 94-98.

Kessmann, J. (2006). Hirschsprung's disease: diagnosis and management. Surgery *100*.

Khan, A., Vujanic, G., and Huddart, S. (2003). The constipated child: how likely is Hirschsprung's disease? Pediatric surgery international *19*, 439-442.

Kim, W.S., Perl, L., Park, J.H., Tandianus, J.E., and Dunn, N.W. (2001). Assessment of stress response of the probiotic Lactobacillus acidophilus. Current microbiology *43*, 346-350.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., and Goossens, H. (2007). Antimicrobial susceptibilities of Lactobacillus, Pediococcus and Lactococcus human isolates and cultures intended for probiotic or nutritional use. Journal of antimicrobial chemotherapy *59*, 900-912.

Koenig, J.E., Spor, A., Scalfone, N., Fricker, A.D., Stombaugh, J., Knight, R., Angenent, L.T., and Ley, R.E. (2011). Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences *108*, 4578-4585.

Koleva, P.T., Kim, J.-S., Scott, J.A., and Kozyrskyj, A.L. (2015). Microbial programming of health and disease starts during fetal life. Birth Defects Research Part C: Embryo Today: Reviews *105*, 265-277.

Korkeala, H., Alanko, T., and Tiusanen, T. (1992). Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta veterinaria Scandinavica *33*, 27.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., and Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. Journal of applied microbiology *94*, 981-987.

Kubota, H., Senda, S., Nomura, N., Tokuda, H., and Uchiyama, H. (2008). Biofilm formation by lactic acid bacteria and resistance to environmental stress. Journal of bioscience and bioengineering *106*, 381-386.

Kurdi, P., Kawanishi, K., Mizutani, K., and Yokota, A. (2006). Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. Journal of bacteriology *188*, 1979-1986.

Kverka, M., Burianova, J., Lodinova-Zadnikova, R., Kocourkova, I., Cinova, J., Tuckova, L., and Tlaskalova-Hogenova, H. (2007). Cytokine profiling in human colostrum and milk by protein array. Clinical chemistry *53*, 955-962.

Langer, J.C., Fitzgerald, P.G., Winthrop, A.L., Srinathan, S.K., Foglia, R.P., Skinner, M.A., Ternberg, J.L., and Lau, G.Y. (1996). One-stage versus two-tage Soave pull-through for Hirschsprung's disease in the first year of life. Journal of pediatric surgery *31*, 33-37.

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S.C. (2008). Genes and molecules of lactobacilli supporting probiotic action. Microbiology and Molecular Biology Reviews *72*, 728-764.

Ledder, R.G., Timperley, A.S., Friswell, M.K., Macfarlane, S., and McBain, A.J. (2008). Coaggregation between and among human intestinal and oral bacteria. FEMS microbiology ecology *66*, 630-636.

Lee, S.A., Lim, J.Y., Kim, B.-S., Cho, S.J., Kim, N.Y., Kim, O.B., and Kim, Y. (2015). Comparison of the gut microbiota profile in breast-fed and formula-fed Korean infants using pyrosequencing. Nutrition Research and Practice *9*, 242-248.

Lee, Y.-K., and Salminen, S. (1995). The coming of age of probiotics. Trends in Food Science & Technology *6*, 241-245.

Liévin-Le Moal, V., and Servin, A.L. (2014). Anti-Infective Activities of Lactobacillus Strains in the Human Intestinal Microbiota: from Probiotics to Gastrointestinal Anti-Infectious Biotherapeutic Agents. Clinical Microbiology Reviews *27*, 167-199.

Lindahl, M., Faris, A., Wadström, T., and Hjerten, S. (1981). A new test based on 'salting out'to measure relative hydrophobicity of bacterial cells. Biochimica et Biophysica Acta (BBA)-General Subjects *677*, 471-476.

Lindberg, E., Adlerberth, I., Hesselmar, B., Saalman, R., Strannegård, I.-L., Åberg, N., and Wold, A.E. (2004). High Rate of Transfer of Staphylococcus aureus from Parental Skin to Infant Gut Flora. Journal of Clinical Microbiology *42*, 530-534.

Linskens, R.K., Huijsdens, X.W., Savelkoul, P.H., Vandenbroucke-Grauls, C.M., and Meuwissen, S.G. (2001). The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. Scandinavian journal of gastroenterology Supplement, 29-40.

Ljungh, A., Hjerten, S., and Wadström, T. (1985). High surface hydrophobicity of autoaggregating Staphylococcus aureus strains isolated from human infections studied with the salt aggregation test. Infection and immunity *47*, 522-526.

Mackie, R.I., Sghir, A., and Gaskins, H.R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr *69*, 1035s-1045s.

Madigan, M.T., Martinko, J.M., and Parker, J. (1997). Brock biology of microorganisms, 11th edition edn.

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T., Oishi, K., Martin, R., Ben-Amor, K., Knol, J., *et al.* (2013). Mother-to-Infant Transmission of Intestinal Bifidobacterial Strains Has an Impact on the Early Development of Vaginally Delivered Infant's Microbiota. PLOS ONE *8*, e78331.

Marles-Wright, J., and Lewis, R.J. (2007). Stress responses of bacteria. Current opinion in structural biology *17*, 755-760.

Mata, L., Urrutia, J., and Lechtig, A. (1971). Infection and nutrition of children of a low socioeconomic rural community. The American journal of clinical nutrition *24*, 249.

Matsumiya, Y., Kato, N., Watanabe, K., and Kato, H. (2002). Molecular epidemiological study of vertical transmission of vaginal Lactobacillus species from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy *8*, 43-49.

McIntosh, G.H., Royle, P.J., and Playne, M.J. (1999). A probiotic strain of L. acidophilus reduces DMH-induced large intestinal tumors in male Sprague-Dawley rats. Nutrition and cancer *35*, 153-159.

Mechoud, M.A., Juarez, G.E., de Valdez, G.F., and Rodriguez, A.V. (2012). Lactobacillus reuteri CRL 1098 and Lactobacillus acidophilus CRL 1014 differently reduce in vitro immunotoxic effect induced by Ochratoxin A. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association *50*, 4310-4315.

Mevissen-Verhage, E.A., Marcelis, J.H., de Vos, M.N., Harmsen-van Amerongen, W.C., and Verhoef, J. (1987). Bifidobacterium, Bacteroides, and Clostridium spp. in fecal samples from breast-fed and bottle-fed infants with and without iron supplement. Journal of Clinical Microbiology *25*, 285-289.

Mitsou, E., Kirtzalidou, E., Oikonomou, I., Liosis, G., and Kyriacou, A. (2008). Fecal microflora of Greek healthy neonates. Anaerobe *14*, 94.

Mitsuoka, E.T. (1992). The human gastrointestinal tract. In The Lactic Acid Bacteria Volume 1 (Springer), pp. 69-114.

Moles, L., Gómez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., Fernández, L., Rodríguez, J.M., and Jiménez, E. (2013). Bacterial Diversity in Meconium of Preterm Neonates and Evolution of Their Fecal Microbiota during the First Month of Life. PLoS ONE *8*, e66986.

Morelli, L. (2008). Postnatal development of intestinal microflora as influenced by infant nutrition. The Journal of nutrition *138*, 1791S-1795S.

Morrow, A.L., and Rangel, J.M. (2004). Human milk protection against infectious diarrhea: implications for prevention and clinical care. Paper presented at: Seminars in pediatric infectious diseases (Elsevier).

Morrow, A.L., Ruiz-Palacios, G.M., Jiang, X., and Newburg, D.S. (2005). Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. The Journal of nutrition *135*, 1304-1307.

Mulle, J.G., Sharp, W.G., and Cubells, J.F. (2013). The Gut Microbiome: A New Frontier in Autism Research. Current psychiatry reports *15*, 337-337.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Paper presented at: Cold Spring Harbor symposia on quantitative biology (Cold Spring Harbor Laboratory Press).

Murri, M., Leiva, I., Gomez-Zumaquero, J.M., Tinahones, F.J., Cardona, F., Soriguer, F., and Queipo-Ortuño, M.I. (2013). Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Medicine *11*, 46-46.

Nowrouzian, F., Hesselmar, B., Saalman, R., Strannegard, I.-L., Aberg, N., Wold, A.E., and Adlerberth, I. (2003). Escherichia coli in Infants' Intestinal Microflora: Colonization Rate, Strain Turnover, and Virulence Gene Carriage. Pediatr Res *54*, 8-14.

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J.-z., Abe, F., and Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC microbiology *16*, 90.

Oki, K., Kudo, Y., and Watanabe, K. (2012). Lactobacillus saniviri sp. nov. and Lactobacillus senioris sp. nov., isolated from human faeces. International journal of systematic and evolutionary microbiology *62*, 601-607.

Osawa, R., Fujisawa, T., and Pukall, R. (2006). Lactobacillus apodemi sp. nov., a tannase-producing species isolated from wild mouse faeces. International journal of systematic and evolutionary microbiology *56*, 1693-1696.

Ouoba, L.I.I., Lei, V., and Jensen, L.B. (2008). Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. International Journal of Food Microbiology *121*, 217-224.

Palmer, C., Bik, E.M., DiGiulio, D.B., Relman, D.A., and Brown, P.O. (2007). Development of the human infant intestinal microbiota. PLoS biol *5*, e177.

Pancheniak, E.d.F.R., and Soccol, C.R. (2005). Biochemical characterization and identification of probiotic Lactobacillus for swine. Bol Centro Pesqui Process Aliment *23*, 299-310.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den Brandt, P.A., and Stobberingh, E.E. (2006). Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy. Pediatrics *118*, 511-521.

Pierre, J.F., Barlow-Anacker, A.J., Erickson, C.S., Heneghan, A.F., Leverson, G.E., Dowd, S.E., Epstein, M.L., Kudsk, K.A., and Gosain, A. (2014). Intestinal Dysbiosis and Bacterial Enteroinvasion in a Murine Model of Hirschsprung's Disease. Journal of pediatric surgery *49*, 1242-1251.

Rafter, J., Bennett, M., Caderni, G., Clune, Y., Hughes, R., Karlsson, P.C., Klinder, A., O'Riordan, M., O'Sullivan, G.C., Pool-Zobel, B., *et al.* (2007). Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. The American Journal of Clinical Nutrition *85*, 488-496.

Rahman, M.M., Hossain, K.M., and Rahman, S.M. (2016). Isolation, characterization, and properties study of probiotic lactic acid bacteria of selected yoghurt from Bangladesh. African Journal of Microbiology Research *10*, 23-31.

Reid, G., McGroarty, J.A., Angotti, R., and Cook, R.L. (1988). Lactobacillus inhibitor production against Escherichia coli and coaggregation ability with uropathogens. Canadian Journal of Microbiology *34*, 344-351.

Reniero, R., Cocconcelli, P., Bottazzi, V., and Morelli, L. (1992). High frequency of conjugation in Lactobacillus mediated by an aggregation-promoting factor. Microbiology *138*, 763-768.

Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E., and Handley, P.S. (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends in microbiology *11*, 94-100.

Robin, F., Paillard, C., Marchandin, H., Demeocq, F., Bonnet, R., and Hennequin, C. (2010). Lactobacillus rhamnosus meningitis following recurrent episodes of bacteremia in a child undergoing allogeneic hematopoietic stem cell transplantation. J Clin Microbiol *48*, 4317-4319.

Rodríguez, E., González, B., Gaya, P., Nuñez, M., and Medina, M. (2000). Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. International Dairy Journal *10*, 7-15.

Rodríguez, J.M., Murphy, K., Stanton, C., Ross, R.P., Kober, O.I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M.C., *et al.* (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. Microbial Ecology in Health and Disease *26*, 10.3402/mehd.v3426.26050.

Rojo-Bezares, B., Sáenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F., and Torres, C. (2006). Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. International journal of food microbiology *111*, 234-240.

Roos, S., Engstrand, L., and Jonsson, H. (2005). Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis sp. nov., isolated from human stomach mucosa. International journal of systematic and evolutionary microbiology *55*, 77-82.

Rotimi, V.O., and Duerden, B.I. (1981). The development of the bacterial flora in normal neonates. J Med Microbiol *14*, 51-62.

Russell, M.B., Russell, C.A., and Niebuhr, E. (1994). An epidemiological study of Hirschsprung's disease and additional anomalies. Acta Paediatrica *83*, 68-71.

Russell, N., Evans, R., Ter Steeg, P., Hellemons, J., Verheul, A., and Abee, T. (1995). Membranes as a target for stress adaptation. International journal of food microbiology *28*, 255-261.

Ryan, E.T., Ecker, J.L., Christakis, N.A., and Folkman, J. (1992). Hirschsprung's disease: associated abnormalities and demography. Journal of pediatric surgery *27*, 76-81.

Sadrani, H., Dave, J., and Vyas, B. (2014). Screening of potential probiotic Lactobacillus strains isolated from fermented foods, fruits and of human origin. Asian Journal of Pharmaceutical and Clinical Research *7*.

Sahadeva, R., Leong, S., Chua, K., Tan, C., Chan, H., Tong, E., Wong, S., and Chan, H. (2011). Survival of commercial probiotic strains to pH and bile. International Food Research Journal *18*.

Sakamoto, I., Igarashi, M., Kimura, K., Takagi, A., Miwa, T., and Koga, Y. (2001). Suppressive effect of Lactobacillus gasseri OLL 2716 (LG21) on Helicobacter pylori infection in humans. The Journal of antimicrobial chemotherapy *47*, 709-710.

Salminen, S., Benno, Y., and de Vos, W. (2006). Intestinal colonisation, microbiota and future probiotics? Asia Pacific journal of clinical nutrition *15*, 558-562.

Salminen, S., Gibson, G.R., McCartney, A.L., and Isolauri, E. (2004). Influence of mode of delivery on gut microbiota composition in seven year old children. Gut *53*, 1388-1389.

Savage, D.C., Dubos, R., and Schaedler, R.W. (1968). The gastrointestinal epithelium and its autochthonous bacterial flora. Journal of Experimental Medicine *127*, 67-76.

Schmidt, G., and Zink, R. (2000). Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve. International journal of food microbiology *55*, 41-45.

Schumann, A., Nutten, S., Donnicola, D., Comelli, E.M., Mansourian, R., Cherbut, C., Corthesy-Theulaz, I., and Garcia-Rodenas, C. (2005). Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome. Physiological Genomics *23*, 235-245.

Sharma, P., Tomar, S.K., Sangwan, V., Goswami, P., and Singh, R. (2016). Antibiotic resistance of Lactobacillus sp. isolated from commercial probiotic preparations. Journal of Food Safety *36*, 38-51.

Shen, D.-H., Shi, C.-R., Chen, J.-J., Yu, S.-Y., Wu, Y., and Yan, W.-B. (2009). Detection of intestinal bifidobacteria and lactobacilli in patients with Hirschsprung's disease associated enterocolitis. World Journal of Pediatrics *5*, 201-205.

Sherman, P.M., Ossa, J.C., and Johnson-Henry, K. (2009). Unraveling mechanisms of action of probiotics. Nutrition in Clinical Practice *24*, 10-14.

Shier, D., Butler, J., and Lewis, R. (1996). Hole's Human Anatomy (McGraw-Hill).

Soave, F. (1964). Hirschsprung's disease: a new surgical technique. Archives of disease in childhood *39*, 116.

Songjinda, P., Nakayama, J., Kuroki, Y., Tanaka, S., Fukuda, S., Kiyohara, C., Yamamoto, T., Izuchi, K., Shirakawa, T., and Sonomoto, K. (2005). Molecular monitoring of the developmental bacterial community in the gastrointestinal tract of Japanese infants. Bioscience, biotechnology, and biochemistry *69*, 638-641.

Spencer, J.F.T., and Ragout de Spencer, A.L. (2001). Food microbiology protocols (Totowa, NJ: Humana Press).

Spouge, D., and Baird, P. (1985). Hirschsprung disease in a large birth cohort. Teratology *32*, 171-177.

Šušković, J., Kos, B., Matošić, S., and Besendorfer, V. (2000). The effect of bile salts on survival and morphology of a potential probiotic strain Lactobacillus acidophilus M92. World Journal of Microbiology and Biotechnology *16*, 673-678.

Swenson, J., Facklam, R., and Thornsberry, C. (1990). Antimicrobial susceptibility of vancomycin-resistant Leuconostoc, Pediococcus, and Lactobacillus species. Antimicrobial agents and chemotherapy *34*, 543-549.

Swenson, O. (1996). Early history of the therapy of Hirschsprung's disease: facts and personal observations over 50 years. Journal of pediatric surgery *31*, 1003-1008.

Tabasco, R., Paarup, T., Janer, C., Peláez, C., and Requena, T. (2007). Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in fermented milk. International Dairy Journal *17*, 1107-1114.

Takino, T., Kato-Mori, Y., Motooka, D., Nakamura, S., Iida, T., and Hagiwara, K. (2017). Postnatal changes in the relative abundance of intestinal Lactobacillus spp. in newborn calves. Journal of Veterinary Medical Science *advpub*.

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., Shirakawa, T., Sonomoto, K., and Nakayama, J. (2009). Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunology & Medical Microbiology *56*, 80-87.

Tannock, G., Crichton, C., Welling, G., Koopman, J., and Midtvedt, T. (1988). Reconstitution of the gastrointestinal microflora of lactobacillus-free mice. Applied and environmental microbiology *54*, 2971-2975.

Tannock, G.W. (1992). The lactic microflora of pigs, mice and rats. In The Lactic Acid Bacteria Volume 1 (Springer), pp. 21-48.

Tannock, G.W. (1999). Identification of lactobacilli and bifidobacteria. Curr Issues Mol Biol *1*, 53-64.

Teitelbaum, D.H., Caniano, D.A., and Qualman, S.J. (1989). The pathophysiology of Hirschsprung's-associated enterocolitis: importance of histologic correlates. Journal of pediatric surgery *24*, 1271-1277.

Teitelbaum, D.H., Cilley, R.E., Sherman, N.J., Bliss, D., Uitvlugt, N.D., Renaud, E.J., Kirstioglu, I., Bengston, T., and Coran, A.G. (2000). A decade of experience with the primary pull-through for Hirschsprung disease in the newborn period: a multicenter analysis of outcomes. Annals of surgery *232*, 372-380.

Temmerman, R., Pot, B., Huys, G., and Swings, J. (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. International journal of food microbiology *81*, 1-10.

Tjalsma, H., Boleij, A., Marchesi, J.R., and Dutilh, B.E. (2012). A bacterial driver–passenger model for colorectal cancer: beyond the usual suspects. Nat Rev Micro *10*, 575-582.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature *444*, 1027-1131.

Ubeda, C., and Pamer, E.G. (2012). Antibiotics, microbiota, and immune defense. Trends in immunology *33*, 459-466.

Urbaniak, C., Cummins, J., Brackstone, M., Macklaim, J.M., Gloor, G.B., Baban, C.K., Scott, L., O'Hanlon, D.M., Burton, J.P., Francis, K.P., *et al.* (2014). Microbiota of Human Breast Tissue. Applied and Environmental Microbiology *80*, 3007-3014.

van der Waaij, L.A., Harmsen, H.J., Madjipour, M., Kroese, F.G., Zwiers, M., van Dullemen, H.M., de Boer, N.K., Welling, G.W., and Jansen, P.L. (2005). Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. Inflammatory bowel diseases *11*, 865-871.

Vélez, M.P., De Keersmaecker, S.C.J., and Vanderleyden, J. (2007). Adherence factors of Lactobacillus in the human gastrointestinal tract. FEMS Microbiology Letters *276*, 140-148.

Visich, K.L., and Yeo, T.P. (2010). The prophylactic use of probiotics in the prevention of radiation therapy-induced diarrhea. Clinical journal of oncology nursing *14*, 467-473.

Vitali, B., Minervini, G., Rizzello, C.G., Spisni, E., Maccaferri, S., Brigidi, P., Gobbetti, M., and Di Cagno, R. (2012). Novel probiotic candidates for humans isolated from raw fruits and vegetables. Food microbiology *31*, 116-125.

Voltan, S., Castagliuolo, I., Elli, M., Longo, S., Brun, P., D'Incà, R., Porzionato, A., Macchi, V., Palu, G., and Sturniolo, G.C. (2007). Aggregating phenotype in Lactobacillus crispatus determines intestinal colonization and TLR2 and TLR4 modulation in murine colonic mucosa. Clinical and Vaccine Immunology *14*, 1138-1148.

Walter, J. (2008). Ecological Role of Lactobacilli in the Gastrointestinal Tract: Implications for Fundamental and Biomedical Research. Applied and Environmental Microbiology *74*, 4985-4996.

Wang, M., Ahrne, S., Antonsson, M., and Molin, G. (2004). T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. Journal of microbiological methods *59*, 53-69.

Ward, N., Pieretti, A., Dowd, S., Cox, S., and Goldstein, A. (2012). Intestinal aganglionosis is associated with early and sustained disruption of the colonic microbiome. Neurogastroenterology & Motility *24*, 874-e400.

Whitehouse, F.R., and Kernohan, J.W. (1948). Myenteric plexus in congenital megacolon: study of eleven cases. Archives of Internal Medicine *82*, 75-111.

Yan, Z., Poroyko, V., Gu, S., Zhang, Z., Pan, L., Wang, J., Bao, N., and Hong, L. (2014). Characterization of the intestinal microbiome of Hirschsprung's disease with and without enterocolitis. Biochemical and biophysical research communications *445*, 269-274.

Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A.-M., Härkönen, T., Ryhänen, S.J., Franzosa, E.A., Vlamakis, H., Huttenhower, C., Gevers, D., *et al.* (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. Science Translational Medicine *8*, 343ra381-343ra381.

Zago, M., Fornasari, M.E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., Reinheimer, J., and Giraffa, G. (2011). Characterization and probiotic potential of Lactobacillus plantarum strains isolated from cheeses. Food Microbiology *28*, 1033-1040.

Zhang, J.W., Du, P., Gao, J., Yang, B.R., Fang, W.J., and Ying, C.M. (2012). Preoperative probiotics decrease postoperative infectious complications of colorectal cancer. The American journal of the medical sciences *343*, 199-205.

Zhang, L., Kinkelaar, D., Huang, Y., Li, Y., Li, X., and Wang, H.H. (2011). Acquired Antibiotic Resistance: Are We Born with It? Applied and Environmental Microbiology *77*, 7134-7141.

Zhang, Z., Xiang, Y., Li, N., Wang, B., Ai, H., Wang, X., Huang, L., and Zheng, Y. (2013). Protective effects of Lactobacillus rhamnosus GG against human rotavirus-induced diarrhoea in a neonatal mouse model. Pathogens and disease *67*, 184-191.

Zhou, J., Pillidge, C., Gopal, P., and Gill, H. (2005). Antibiotic susceptibility profiles of new probiotic Lactobacillus and Bifidobacterium strains. International journal of food microbiology *98*, 211-217.

Zocco, M.A., dal Verme, L.Z., Cremonini, F., Piscaglia, A.C., Nista, E.C., Candelli, M., Novi, M., Rigante, D., Cazzato, I.A., Ojetti, V., *et al.* (2006). Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. Alimentary pharmacology & therapeutics *23*, 1567-1574.

Appendices...

Appendix-I

Media composition

Name of the media	Composition	
	Name of the ingredients	Amount(gram in litre)
Nutrient Agar (Himedia, India)	Peptone	5.0
	Beef extract	3.0
	Agar	15.0
MacConkey Agar (Himedia, India)	Peptones (meat and casein)	3.0
	Pancreatic digest of gelatine	17.0
	Lactose monohydrate	10.0
	Bile salts	1.50
	Sodium chloride	5.0
	Crystal violet	0.001
	Neutral red	0.03
	Agar	15.0
MRS Agar (Oxoid, UK)	Peptone	10.0
	`Lab-Lemco' powder	8.0
	Yeast extract	4.0
	Glucose	20.0
	Sorbitan mono-oleate	1ml
	Dipotassium hydrogen phosphate	2.0
	Sodium acetate. $3H_2O$	5.0
	Triammonium citrate	2.0
	Magnesium sulphate. $7H_2O$	0.2
	Manganese sulphate. $4H_2O$	0.05
	Agar	10.0
MRS Broth (Oxoid, UK)	Peptone	10.0
	`Lab-Lemco' powder	8.0
	Yeast extract	4.0
	Glucose	20.0
	Sorbitan mono-oleate	1ml
	Dipotassium hydrogen phosphate	2.0
	Sodium acetate. $3H_2O$	5.0
	Triammonium citrate	2.0
	Magnesium sulphate. $7H_2O$	0.2
	Manganese sulphate. $4H_2O$	0.05

Name of the media	Composition	
	Name of the ingredients	Amount(gram in litre)
XLD Agar (Himedia, India)	Lactose	7.5
	Sucrose	7.5
	Sodium Thiosulfate	6.8
	L-Lysine	5.0
	Sodium Chloride	5.0
	Xylose	3.75
	Yeast Extract	3.0
	Sodium Deoxycholate	2.5
	Ferric Ammonium	0.8
	Phenol Red	0.08
	Agar	15.0
Eosine Methylene Blue Agar (Oxoid, UK)	Peptone	10.00
	Dipotassium phosphate	2.00
	Lactose	5.00
	Sucrose	5.00
	Eosine yellow	0.14
	Methylene blue	0.065
	Agar	13.50
Thiosulfate-Citrate-Bile Salts-Sucrose Agar (Difco^{TM}BD)	Proteose Peptone	10.00
	Yeast extract	5.00
	Sodium thiosulphate	10.00
	Sodium citrate	10.00
	Ox gall	8.00
	Sucrose	10.00
	Sodium chloride	10.00
	Ferric citrate	1.00
	Bromo thymol blue	0.04
	Agar	15.00
Nitrate Broth	Peptone	5.0
	Beef extract	3.0
	Potassium nitrate	5.0

Name of the media	Composition	
	Name of the ingredients	Amount(gram in litre)
Motility Indole Urea Agar (Oxoid, UK)	Casein enzymic hydrolysate	10.00
	Dextrose	1.00
	Sodium chloride	5.00
	Phenol red	0.01
	Agar	2.00
	40% Urea Solution	5 ml
Phenol red broth (Carbohydrate fermentation)	Tryptone	10.00
	NaCl	5.0
	Sugar	5.0
	Phenol red	0.2
	Distilled water	1L
	pH	7.3
Skim milk, tryptone, glucose, glycerol transport medium (STGG)	Skim milk powder	20.0
	Tryptone soya broth	30.0
	Glucose	5.0
	Glycerol	100ml
	Distilled H_2O	900ml

Appendix-II

Buffers and reagents

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 litre by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

1M Tris-HCL

1.576 gm Tris-HCL was added into 10ml distilled water for 10 ml Tris-HCL and pH was adjusted to pH 8.0.

10 x TBE (pH 8.3)

54.0 gm of Tris-base, 27.5 gm of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 500 ml. The buffer was stored at room temperature.

TE buffer

For 100 ml TE buffer 1 ml 1M Tris-HCl (pH 8.0) and 0.2 ml 0.5M EDTA (pH 8.0) were taken. Then the volume was adjusted by distilled water

Catalase reagent

35% Hydrogen peroxide

Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminium foil and stored at $4^{o}C$.

Oxidase reagent

100 mg of N, N, N1, N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminium foil. Then the solution was stored at $4^{o}C$.

Nitrate reagent

Solution A: Sulfanilic acid
1gm of sulfanilic acid was dissolved in 125 ml of 5N acetic acid.

Solution B: Alpha-napthylamine
0.625 gm of α-napthaylamine dissolved in 120ml of 5N acetic acid.

Crystal violet

Solution A: 2.0 gm of crystal violet (95% dye content) added into 20 ml of 95% ethyl alcohol.

Solution B: 0.8 gm of ammonium oxalate monohydrate was added into 80.0 ml of distilled water.
*Solution A and B was mixed.

Gram's iodine

1.0 gm of iodine, 2.0 gm of potassium iodide was added into 300 ml of distilled water and stored in amber bottle.

Ethyl alcohol (95%)

95 ml of ethyl alcohol (100%) was added into 5 ml of distilled water.

Safranin

0.25 ml of safranin was dissolved in 10 ml of 95% ethyl alcohol and was added into 100 ml of distilled water.

Appendix- III

Instruments

The important equipment used through the study are listed below

Name	Origin
Autoclave Model no: WAC-47	Korea
Analytical weight balance (Core series)	Adam,UK
Anaerobic gas jar	Oxoid, UK
Centrifuge, Code: 5433000.011	Eppendorf, Germany
Freezer (-20°C)	Siemens, Germany
Refrigerator (4°C), Model: 0636	Samsung
Millipore filter, 045 µm	UK
Incubator, Incucell	MMM group, Germany
Oven, Model:MH6548SR	LG, China
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Laminar airflow cabinet, Model: SLF-V, Vertical	SAARC Group, Bangladesh
Micropipette (2-20µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Micropipette (100-1000µl)	Eppendorf, Germany
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Vortex Mixture	VWR International
Water bath	Korea
Thermal Cycler, Model no: 2720	Applied Biosystems, USA
Horizontal Gel Electrophoresis Unit	Wealtec Corporation, USA
UV Transilluminator	Wealtec Corporation, USA
UV spectrophotometer	Shimadzu, Japan