FINAL PROJECT REPORT

MAJOR RESEARCH PROJECT

UGC File No. 39-193/2010 (SR) Dated: 27/12/2010

Entitled

"Screening and evaluation of intestinal microbiota in breastfed neonates as potential probiotics"

Principal Investigator

Dr. Mrs. Sapre Vaijayanti Rajiv
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SUBMITTED TO ,

UNIVERSITY GRANTS COMMISSION

BHADUR SHAH JAFAR MARG

NEW DELHI-110002

(APRIL 1,2011-MARCH 31,2014)

Ref No.BVDU/YMC/Micro/ /2015-16 Date:

To,
The Secretary,
University Grants Commission,
Bhadur Shah Jafar Marg,
New Delhi-110002.

Subject: Submission of final report of Major Research Project File No UGC File No. 39-193/2010(SR) dated 27/12/2010 by Dr. Mrs. Sapre Vaijayanti Rajiv, Associate Professor Department of Microbiology, Yashwantrao Mohite College of Arts ,Sciences and Commerce, Erandwane, Pune.,411038

Sir,
We are pleased to forward the final report of the major research project in Microbiology (Science), as refered above, completed by Dr. Mrs. Sapre Vaijayanti Rajiv, Associate Professor Department of Microbiology, Yashwantrao Mohite College of Arts ,Sciences and Commerce, Erandwane, Pune.,411038 entitled "Screening and evaluation of intestinal microbiota in breastfed neonates as potential probiotics"

Please find the following enclosures:

1. Audited utilization certificate
2. Statement of expenditure and annexure
3. Summary of final report
4. Head wise audited statement of accounts
5. Total dues of reimbursed by UGC, Delhi, amounting totally to 8,99,900/-(Rupees Eight lakh ninety nine thousand and eight hundred only)
6. You are cordially requested to approve the accounts and arrange to send (a) Second and final installment of Rs- 2,74,000/-,(b)Excess amount of Rs- 6126/- (c) Revised fellowship difference of Rs- 2,40,000/-(d) HRA @20% of the fellow of Rs- 1,05,600/- as detailed certificate attached, and totally amounting to 6,25,726/-(Rupees Six lakh twenty five thousand seven hundred and twenty six only)

Please approve the same, arrange to send the amount at early date and oblige.
Thanking you

Yours faithfully,

Dr. Mrs. Vaijayanti Rajiv Sapre
Investigator
Major Research Project (UGC)
BVDU, Y. M. College, Pune-38.

Y. M. College No. F-9-151 95-U3 PUNE-38.

Prin. Dr. K. D. Jahav
PRINCIPAL
YASHWANTRAO MOHITE COLLEGE
PUNE-38.

Acknowledgement

Its my great pleasure to express sincere thanks towards “University Grants Commission”, New Delhi for the sanctioning this project and releasing the grant in time.

I express my deep sense of gratitude towards respected honorable Dr. S.S. Kadam, Vice chancellor of Bharti Vidyapeeth Deemed University, Pune (India), Honorable Principal Dr. K.D.Jahav Yashwantrao Mohite College of Arts ,Science and Commerce, Erandwane, Pune., Dr M.G.Bodhankar ,Dean Faculty of Science, Bharti Vidyapeeth Deemed University, Pune for providing infrastructure, all facilities, full cooperation ,encouragement and valuable suggestions throughout the tenure of the project.

I am thankful to National Centre For Cell Science (NCCS), Pune for support given in characterization purpose. I am also thankful to Kamla Nehru Hospital Pune for provision of samples.

I thank all staff members of Microbiology Department for their encouragement and support during the project period.

I also thank Mr. Ashok Koli, Accountant for maintaining the accounts.

Place: Pune Dr. Mrs. Vaijayanti Rajiv Sapre

University Grants Commission,

Bhadur Shah Jafar Marg,

New Delhi-110002.

FINAL REPORT

INDEX

Annexure VIII

University Grants Commission,
Bhadur Shah Jafar Marg - New Delhi-110002
Final Report of the work done during the Major research project in microbiology (April 1,2011-March 31,2014) File No UGC File No. 39-193/2010(SR)dated 27/12/2010

Sr. No.	Description	Details
1.	Project report No.	Final
2.	UGC Reference No	39-193/2010(SR)
3.	Period of report	From April 1,2011-March 31,2014
4.	Title of research project	"Screening and evaluation of intestinal microbiota in breastfed neonates as potential probiotics"
5.	(a) Name of Principal Investigator	Dr. Mrs. Vaijayanti Rajiv Sapre
	(b) Department of university/college where work has progressed	Department of Microbiology, Yashwantrao Mohite College of Arts ,Sciences and Commerce, Erandwane, Pune.,411038
6.	Effective date of starting the project	01/04/2011
7.	Grant approved and expenditure incurred during the period of report	Annexure I
a.	Total Amount Approved	8,99,800 /- (Rupees Eight Lakh ninety nine thousand and eight hundred only)
	Amount received as first installment	6,25,800/-(Six Lakh twenty five thousand and eight hundred only)
b.	Total expenditure	9,05,926/- (Nine Lakh five thousand and nine hundred twenty six rupees only)
	i. Balance Receivable II nd installment	2,74,000/- (Two Lakh seventy four thousand rupees only)
	ii. Difference of revised rate of project fellowship from 2010 onwards	(1,44,000 for first and second year @ 6000 pm and 96,000 for third year@ 8000 pm)= 2,40,000/-

	iii HRA @20% for first and second year on Rs. 14,000/- (2800/- pm and yr on 16,000/-(3200/- pm)	67,200 +38,400= 1,05,600/- (One lakh five thousand and six hundred only)
	iv . Excess amount	6126/-(Six thousand one hundred twenty six/-)
	Total dues to be receivable from UGC(i+ii+iii+iv)	2,74,000/- + 2,40,000/-+ 1,05,600 /-+ 6126/-= 6,25,726/-

C (i)

Brief objective of the project:

1. To isolate the intestinal bacteria of the breastfed neonates
2. To check the morphological, biochemical characters of the isolated organisms.
3. To screen the bacteria on the basis of their tolerance to acid, bile, different enzymes, artificial gastric juice, phenol, NaCl and evaluate optimum growth temperature.
4. To study the bacteria for their antagonistic properties against pathogens and check the antibiotic resistance pattern.
5. To evaluate the potential probiotic characteristics of the faecal isolates in comparison with commercial probiotics.
6. To identify the selected isolates on the basis of 16 S rRNA.

C (ii)

Work done so far and results achieved and publications, if any, resulting from the work

Isolation and identification

Prior to the commencement of this study, a total of 92 bacterial strains were isolated and identified from the intestine of breastfed neonate. While total ten bacterial strains were isolated and identified from commercial products. Strains included 12 isolates of *Lactobacillus acidophilus*, 16 isolates of *Lactobacillus plantarum*,16 isolates of *Lactobacillus casei* ,2 isolates *of Lactobacillus gasseri*,3 isolates of *Lactobacillus pentosus*, 29 isolates of *Enterococcus faecium*, 8 isolates of *Enterococcus faecalis* and 2 isolates of *Bacillus coagulance.* All the isolates were identified on the basis of classical biochemical method and further selected 30 isolates were identified further on the basis of 16SrRNA. (Appendix 1 - Table 1, 2,3,4 and Fig 1 and 2 were showing the details of isolation and identification of isolates)

Acid tolerance

The typical transit time of a small food bolus in the stomach is approximately 20 minutes. This period of time was therefore chosen to test the resistance of bacterial strains to simulate the gastric contents. Stomach acidity varies according to individuals and whether individuals has fasted prior to ingestion. To account for these inter-individual differences, the ability of isolated strains and commercial probiotic strains to survive at various acidic pH was investigated

Stresses to organisms begin in the saliva, with pH between 6.5 and to 7.5 and time up to 1 min and continues in the upper stomach between 4.0-6.5 pH for up to 30 to 60 min, lower stomach 1.5- 4.0 pH for 1 to 3 hrs and duodenum 7.0 to 8.5 pH. The probiotic microorganism may have to survive in small intestine at pH 4.0- 7.0 for 1 to 5 hrs and in large intestine at pH 4.0 -7.0 for 10 to several days.Survival at pH 3.0 for 2 h and in bile concentration of 1000 mg/L is considered optimum for acid and bile tolerance for probiotic strains (Usman and Hosono, 1999). It was observed that normal pH of small intestine is 2to 3, duodenum, 6 to 6.5 and stomach 2-3. All the isolated LAB were able to tolerate the intestinal acidic stress. Strains of LAB isolated in this study showed varying levels of viability at pH 2.0 after 2 h incubation. It is not surprising that all the isolated LAB were able to tolerate and grow in pH 2.5 and above as it is natural habitats of the isolates of gastrointestinal tracts of human. *L. acidophilus* , *L. casei* , *E.faecalis*

E.faecium,L.pentosus, L.plantarum survived and grew best under the acidic conditions. (Fig 2.1, in Appendix II is showing the effect on survival and growth of different acid and bile conc. on 92 isolates and commercial probiotics)

Bile tolerance

To show probiotic properties, these organisms should reach to the lower intestinal tract and maintain themselves over there. Therefore, the first criteria in looking for probiotic strains is being resistant to acid and bile. The average bile concentration is around 0.3%, and may range up to the extreme of 2.0% during the first hour of digestion (Gotcheva *et al*., 2002). However, it was also considered that the concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Lankaputhra and Shah, 1995). Survival at pH 3.0 for 2 h and in bile concentration of 1000 mg/L is considered optimum for acid and bile tolerance of probiotic strains (Usman and Hosono, 1999). All the isolated LAB were able to tolerate and can grow in 0.3% bile. Majority of isolates were able to tolerate 0.1 to 2% bile up to 120 min. All of them were growing at bile conc. of 0.1 to 1.5% However, very few isolates were able to grow at bile conc. of 2%. While comparing with commercial probiotic isolates all the isolates showed same pattern of acid and bile tolerance. (Table 2.2 in Appendix II is showing the effect on survival and growth of different acid and bile conc. on 92 isolates and commercial probiotics)

Enzyme tolerance

The probiotic bacteria were able to survive in presence of different gastric enzymes. The organisms may entrap these enzymes at the time of the their entry in to the mouth up to the colonization in the intestine. The mucosal layer on the inner lining of small intestine has projections ie. villi. The nutrients are picked up and transported in to the blood stream there. The villi are tiny pores which lead to gland called crypts. These crypts secrete a highly alkaline intestinal juice that contains water, mucus, lysozyme ,phospholipase and several enzymes which may protect the body from infection by bacteria. While pepsine is released by chief cells in the stomach and degrade food proteins in to peptides .It is activated at pH 2 and inactivated at pH 6.5. All the faecal isolates and commercial probiotic isolates were able to tolerate pepsin (100ppm),Lysozyme (1 mg/ml),Trypsin(1mg/ml),Lipase(1mg/ml),diastase (1mg/ml) at 0,15,30,60,90 and 120 min at 37^0C in5% CO_2. (Growth of the isolates and commercial probiotics in presence of different enzymes were showing in Table 2.3 Appendix II)

Tolerance to artificial gastric juice

Gastric juice is watery strongly acidic(pH ranging 1 to 3), almost colourless liquid secreted by glands in the lining of stomach. Its essential constituents are digestive enzymes like pepsin, rennin, hydrochloric acid and mucus. Up to 2 litres gastric juice is secreted by every human being within 24 hrs. However ,quantity and composition vary according to type of food. It was difficult to collect the natural gastric juice. So, to mimic the gastric conditions we prepared artificial gastric juice as per formulation described by Corcoran *et.al*. (Corcorant *et.al*. 2007).We prepared artificial gastric juice with pH 1 and 2.All the faecal isolates and commercial probiotic isolates were able to tolerate artificial gastric juice on exposure for 30,60,90 and 120 min at pH 1 and 2.(Table 2.3 ,Appendix II is showing the survival in artificial gastric juice with pH 1 and 2 of 92 isolates and commercial probiotics)

Antibiotic sensitivity

As early as 1969, the Swann report drew attention to the potential transfer of antibiotic resistant bacteria from animals to the food chain and to humans. Teuber *et al.* (1999) suggested that the potential of commensal bacteria to transfer their antibiotic resistance genes from food to the indigenous human microflora should be investigated .Moreover, resistant food contaminants that originate from animals and are consumed by humans, can also act as a gene pool (donors) of antibiotic resistant genes (Aarestrup F.,2000).

The acquired antibiotic resistance is found in *Enterococcus, Lactococcus* and *Lactobacillus* species (J. Ukovi,1997, S. Mathur,2005). Such resistant bacteria may interact with the resident human microflora and possibly transfer or acquire antibiotic resistant determinants by horizontal gene transfer. Large number of probiotic bacteria are consumed to maintain and restore the microbial balance in the intestines. It must be kept in mind that they have a potential to transfer multiple antibiotic resistance genes to pathogenic bacteria. For these and other applications the safety aspects of these bacteria are of concern, including the presence of potentially transferable antibiotic resistances (Ukovi J,1997, Mathur S.,2005,).Different studies show that bacteria that normally reside in the human colon are able to transfer resistance genes among themselves (Davison ,1996 Kidwell M.,2000, Ochman H.,2000., Finlay B.1997). This type of transfer becomes a huge problem when these harmless commensal bacteria may transform into pathogens (Manges A.,2001).Once acquired, resistance genes are not easily lost. Instead, they become a relatively stable part of a genome. Additional resistant determinants may join those already

prevailing, thus broadening the multidrug resistant phenotype and further diminishing treatment options (Dzidic S.,2003). The absence of the acquired antimicrobial resistance has become an important criterion for evaluating the safety of lactic acid bacteria used as starter culture or probiotics. To avoid risk of transfer of antibiotic resistance from LAB species, to pathogenic bacteria, The European Commission has, as advised by the European Food Safety Authority (EFSA), requested that bacterial strains harbouring transferable antibiotic resistance genes should not be used in animal feeds (European Parliament and Council Regulation EC 429/2008; EC, 2001). No legislation exists so far regarding microorganisms intentionally added to fermented food and probiotics for human use. However, based on the precautionary principle, it is recommend that these products follow similar requirements to feed additives (EFSA, 2007).

Using the disc diffusion method, antibiotic resistance among faecal isolates was detected against 12 antibiotics. 98% isolates were sensitive to bacitracin and 96% were sensitive to tetracycline. 91% of the isolates were resistant to norfloxacin and all the isolates sensitive to Penicillin G. The isolates were resistant to vancomycin (10%),gentamycin (66%),streptomycin (95%),nalidixic acid (100%),colistin (83%) and ampicillin (85%).All the isolates were sensitive to penicillin. Similar results were found with commercial probiotics. . (Table 3.1 and Fig 3.1 Appendix III were showing the details of antibiotic sensitivity of isolated organisms.)

BSH activity

Bile salt hydrolase (BSH) is an enzyme produced by several bacterial species in the human or animal gastrointestinal tract that catalyzes the glycine- or taurine-linked bile salt deconjugation reaction. BSH activity has been observed mainly in *Lactobacilii* spp, *Bifidobacterium longum*, *Clostridium perfrigens, Bacteroides vulgates, B.fragills, Listeria monocytogens, and Xanthomonas maltophilia.* Since bile acts as a biological detergent by which it shows antimicrobial property, microbes produce BSH against it to cope up with its toxicity. As deconjugation has recently been included by WHO experts as one of the main activities of intestinal microbiota for them to be considered as probiotic organisms.(FAO/WHO,2002),microbial BSH activity contributes for its probiotic properties in the gastrointestinal tract. Tanka *et al* carried out a semi-quantitative screening of more than 300 lactic strains of genera *Bifidobactrium ,Lactobacillus* and the spp of *Lactococcus lactis,Leuconostoc mesenteroid* and *S.thermophilus*. It was observed that BSH activity was found in the strains isolated from the intestine or feces of mammals. The LAB in presence of environment rich in conjugated and unconjugated bile acids show maximum BSH

activity. Schillinger *et al* detected BSH activity in various strains of *L. acidophilus* and *L. johnsonii* but not in *Lactobacillus casei* strains group.(Schillinger, U.,*et al*., 2005).*Escherichia coli* and *Salmonella enteric, serovar typhimurium* are reported as BSH-negative strains (Begley, M , *et al*.2005) However, *L. lactis* and *S. thermophilus* isolated from bile-salt-originated environments and from outside the gut did not exhibit such activity(Moser, S,*et. a.l.* 2001; Elkins, C.*et al*.2001; Ahn, Y. *et. al*. 2005).It was observed that the probiotic strains and species isolated from other habitats like milk or vegetables where bile salt is absent , normally did not have BSH activity. (Table 4.1 appendix IV is showing Qualitative BSH activity of LAB isolates and commercial probiotics) Lactobacilli were principal genera among the gut microbiota leading to the maximum BSH activity in mice gut, which was proved by eliminating the lactobacilli from the total gut microbiota. High levels of BSH activity have been reported in enterococci genus
(Knarreborg, A.*et al*.,2002; Yoon, M. *et al*.2008).

The cholesterol reduction mechanism involves the ability of probiotics to enzymatically deconjugate the bile acids by bile salt hydrolase (BSH; chloroglycine hydrolase), enzyme that catalyzes the hydrolysis of glycine and/or taurine-cojugated bile salts which mainly occurs in the small and large intestines of human being. Previously reported that the removal of cholesterol by *L. reuteri* CRL 1098 was closely related to the BSH activity of the cells, which hydrolyzed the amide bond of bile salts releasing the corresponding free bile acids. Conjugated bile salts are readily absorbed into the gastrointestinal tract due to higher hydrophilicity, while free bile acids are less soluble and thus less efficiently reabsorbed into the intestines, compared to conjugated bile salts, and thus are more prone to be excreted with the feces. This will increase the need for the synthesis of new bile acids to replace the lost ones. Since cholesterol is the precursor for the *de novo* synthesis of new bile acids, the use of cholesterol to synthesize new bile would lead to a decreased concentration of cholesterol in blood.
In our study nearly all *Lactobacilli* spp and *Enterococcus* sp were able to precipitate unconjugated bile acid and showed precipitation zone around each disc and similar results were found with commercial probiotic isolates.

Antagonistic activity

The inhibitory power of *Lactococcus* was first observed in 1930 when the inhibition of commercial cheese starter cultures by similar dairy bacteria was reported. Antimicrobial compounds were

produced by lactic acid bacteria .Mode of antagonistic activity of lactic acid bacteria depends upon their metabolic products and its action on test organisms such as i) Carbon dioxide inhibits decarboxylation and thus reduces membrane permeability of test bacteria ,ii) Diacetyl interacts with arginine -binding proteins of test organisms and thus inhibit the test bacteria,iii) Hydrogen peroxide oxidize basic proteins iv) Lactic acid produced by LAB act on the cell membrane of test organism and lowers the intracellular pH and v) Bacteriocins, which affect membrane, DNA synthesis and protein synthesis. (Review by Siamansouri, M.,2013).

Lactic acid bacteria (LAB) especially, *Lactobacilli* have gained particular attention nowadays, due to the production of bacteriocins as according to WHO, they were considered as generally safe for consumption (WHO,2002).

The bacteriocins produced by LAB have the tendency to penetrate the outer membrane of Gram-negative bacteria and in combination with other augmenting antimicrobial environmental factors, such as low temperature, organic acid and detergents induce the inactivation of Gram-negative bacteria. It was stated that the bacteriocins produced by LAB offer several desirable properties to make them suitable for food preservation such as : (i) generally recognized as safe substances, (ii) not active and nontoxic on eukaryotic cells, (iii) are readily inactivated by the action of digestive proteases, with slight impact on the microorganism inhabiting the gut , (iv) research has been proved that they can tolerate a wide range of pH and temperature, (v) they have a fairly broad antimicrobial spectrum, against many food-borne pathogen of food origin and food spoiling bacteria, (vi) they exhibit bactericidal action on the target host, typically acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation. Also the collection of studies carried out in recent years undoubtedly point out that the use of bacteriocins in food preservation can offer several advantages (a), an extended shelf life of foods, (b) offer extra protection during abnormal temperature conditions, (c) lessening the risk for spread of foodborne pathogens through the food chain, (d) lessen the economic losses due to food spoilage, (e) decrease the application of chemical additives, (f) the process allows the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (g), permit the marketing of “novel” foods (less acidic, with a lower salt content, and with a higher water content), and (h) they may serve to satisfy industrial and consumers demands

In our study , cell free culture supernatant of LAB exhibited varying degree of inhibitory activity against pathogenic test organisms.

Production of bacteriocin is the important characteristics together with their antagonistic effect against frequent food contaminants like *Shigella* spp ,*Salmonella* spp, *E.coli* spp, and *Pseudomonas* spp. In our study a significant inhibition was observed against all these spp. All the isolated strains of *E.faecium, E.faecalis* were strongly inhibiting *Shigella flexneri* and *E.coli.* (Zone of inhibition± <16 mm).However, pathogenic, multiple antibiotic resistant *Shigella* spp and *Salmonells* spp (which were collected from Bharati Hospital, Pune) were strongly inhibited by some isolates of *E.faecium, E.faecalis* and *B.coagulance.* .

All the fecal isolates of breastfed neonates showed strong inhibition of *S.marcesens* , *E.clocae* and *S.aureus.* However none of the isolate showed inhibition to *K.terrigena, S.epidermitis, L.plantarum* and *S.faecalis. P.vulgaris* is important pathogen in urinogenital infections. The cell free culture supernatant of all lactic acid bacteria exhibited varying degree of inhibitory activity against *P.vulgaris*.

Molds and yeasts are important spoilage organisms in different food and feed systems in dairy products and fermented food .Regarding availability of efficient and safe procedure to prevent fungal growth in raw and prepared foods, LAB producing antimicrobial substances have long been reported. All the isolated LAB showed moderate inhibition of *Aspergillus niger.* However, only *L.pentosus* showed inhibition of *Penicillium crysogenum*. All the commercial probiotics were not able to inhibit *Aspergillus niger.* (Table 5.1 and fig 5.1 showing the details of Antimicrobial activity of LAB isolates and commercial probiotics)

NaCl and Phenol are inhibitory substances which when applied in high amount may inhibit growth of certain types of bacteria. All the isolated lactic acid bacteria showed good growth up to6 % of NaCl .While nine isolates showed growth in 8% NaCl and 4 isolates grew well at 10 % NaCl concentrations.

All the isolated lactic acid bacteria showed different percent cell hydrophobicity. (Fig. 6.1. showing % hydrophobicity of the isolated bacteria and commercial probiotics)

ii . Details of the papers and names of the journals in which papers have been published or accepted for publication

1. Development of gut microbiota in early infancy, Asian Journal of Multidisciplinary Studies, 1, (3), 122-127,(2014).

2. Evaluation of Antimicrobial Activity of Probiotic Microflora from Neonatal Origin, Asian Journal of Multidisciplinary Studies, 2, (8),182-183,(2014).

3. Screening of intestinal Lactic Acid Bacteria of breastfed neonates for antimicrobial activity against *Bacillus subtilis, Staph. aureus* and *E.coli*, Research Journal of Chemistry and Environment, 18 ,(3),37-41,(2014).
4. Cholesterol assimilation of intestinal *Lactobacilus acidophilus* ,Research Journal of Chemistry and Environment 19,(3),10-14 (2014).

5. Antibiotic sensitivity of lactic acid bacteria isolated from the intestine of Indian breastfed neonates,J.Microb.World 16, 30-34(2014).

6. Evaluation of antimicrobial of probiotic microflora from neonatal origin., International conference on Biotechnology for better tomorrow(BTBT-2014) Accepted

7. Review on human probiotics, Bharati Vidyapeeth Deemed University Research Journal, 9,19-28, (2011).

8. A new modified media for isolation and enumeration of Lactic acid bacteria, Published in abstract book of UGC Sponsored National Seminar on New Challenges in Chemistry and Nanosciences -2012(Abstract No.25), organized by Dept of Chemistry ,Y.M.College, Pune.

C(iii)

Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons

Yes, the progress has been according to the original plan of work

1. The project has been implimented to evaluate intestinal microbiota of breastfed neonate with commercial probiotics on the basis of potential probiotic criterion.
2. The main objective of the study was to isolate the intestinal flora of breastfed neonate. We successfully isolated probiotic *Lactobacillus* spp and *Enterococcus* spp intestinal flora. We followed all the guidelines for evaluation of candidate probiotic strains by FAO/WHO,2002 in vitro including strain identification by phenotypic and genotypic methods(Genus,Species and Strain identification).We also isolated the organisms in commercial probiotics and successfully compared them with fecal isolates. Thus the isolated strains of *Lactobacillus* spp and *Enterococcus* spp have the potential to be used as probiotic strains.

C (iv)

Please indicate the difficulties, if any, experienced in implementing the project

- Bifidobacteria were isolated from the infant faecal sample. As these organisms are strict anaerobes and sensitive to air during transfers and hence could not be further studied for the want of anaerobic glove box. Cost of the anaerobic glove box is around 15 lacs and hence could not be met in the budgetary provision of the project.

C(vi)

The project has been completed in given time according to plan of work

C (vii)

(a) **Manpower trained**: Manpower trained in installation and use of CO_2 incubator and cultivation of anaerobic bacteria

(b) **Ph. D. awarded**: Project fellow (Mrs. Smita Hemant Nilakhe) registered for Ph.D. at Bharati Vidyapeeth University Pune In 2011. She has submitted her Ph.D thesis entitled "Cholesterol Reducing Potential of Intestinal Microbes of Neonate Origin" in June 2015.

(c) Publication of results:

1. Development of gut microbiota in early infancy, Asian Journal of Multidisciplinary Studies, 1, (3), 122-127,(2014).

2. Evaluation of Antimicrobial Activity of Probiotic Microflora from Neonatal Origin, Asian Journal of Multidisciplinary Studies, 2, (8),182-183,(2014).

3. Screening of intestinal Lactic Acid Bacteria of breastfed neonates for antimicrobial activity against *Bacillus subtilis, Staph. aureus* and *E.coli*, Research Journal of Chemistry and Environment, 18 ,(3),37-41,(2014).
4. Cholesterol assimilation of intestinal *Lactobacilus acidophilus* ,Research Journal of Chemistry and Environment 19,(3),10-14 (2014).

5. Antibiotic sensitivity of lactic acid bacteria isolated from the intestine of Indian breastfed neonates,J.Microb.World 16, 30-34(2014).

6. Evaluation of antimicrobial of probiotic microflora from neonatal origin., International conference on Biotechnology for better tomorrow(BTBT-2014) Accepted

7. Review on human probiotics, Bharati Vidyapeeth Deemed University Research Journal, 9,19-28, (2011).

8. A new modified media for isolation and enumeration of Lactic acid bacteria, Published in abstract book of UGC Sponsored National Seminar on New Challenges in Chemistry and Nanosciences -2012(Abstract No.25), organized by Dept of Chemistry ,Y.M.College, Pune.

(d) **Other impact:**

3. The project has been implimented to evaluate intestinal microbiota of breastfed neonate with commercial probiotics on the basis of potential probiotic criterion.
4. While isolating the intestinal flora of breastfed neonates it was found that characters of all the isolates matched with that of classical probiotic strains and nearly all the strains are promising potential human and animal probiotics. *Lactobacillus acidophilus. Lactobacillus plantarum, Lactobacillus casei, Lactobacillus pentosus, Lactobacillus gasseri, Enteroococcus faecium ,Enterococcus faecalis* are isolated in the present study. These genera are generally regarded as safe for consumption. All the *Enterococcus* sp has been identified on the basis of 16 S rRNA
5. During evaluation of commercial probiotics with identified strains, our strains were proved more superior than commercial probiotic isolates on the basis of antagonistic activity where all the faecal isolates showed inhibition of fungal isolates.
6. Overall the study showed that *Lactobacillus spp.*and *Enteroococcus spp.* were resistant to the gastric environment and able to produce bacteriocin which inhibited a wide range of pathogens.

7. As early as 1969, the Swann report (Anonym ,1969) drew attention to the potential transfer of antibiotic resistant bacteria from animals to the food chain and to humans. Moreover, resistant food contaminants that originate from animals and are consumed by humans, can also act as a gene pool (donors) of antibiotic resistance genes (Aarestrup F.,2000). Large numbers of probiotic bacteria are consumed to maintain and restore the microbial balance in the intestines. It must be kept in mind that they have a potential to transfer antibiotic resistances to pathogenic bacteria. For these and other applications the safety aspects of these bacteria are of concern, including the presence of potentially transferable antibiotic resistances (Ukovi J,1997, Mathur S.,2005, Salyers A.,2005).).Different studies shows that bacteria which normally reside in the human colon are able to transfer resistance genes among themselves (Davison J., Kidwell M.,2000, Ochman H.,2000., Finlay B.1997). This type of transfer becomes a huge problem when these harmless commensal bacteria transform into pathogens (Manges A.,2001).Once acquired, resistance genes are not easily lost. The absence of the acquired antimicrobial resistance has become an important criterion for evaluating the safety of lactic acid bacteria used as starter culture or probiotics. To avoid risk of transfer of antibiotic resistance from LAB species, to pathogenic bacteria, The European Commission has, as advised by the European Food Safety Authority (EFSA), requested that bacterial strains harbouring transferable antibiotic resistance genes should not be used in animal feeds (European Parliament and Council Regulation EC 429/2008; EC, 2001). No legislation exists so far regarding microorganisms intentionally added to fermented food and probiotics for human use. However, based on the precautionary principle, it is recommend that these products follow similar requirements to feed additives (EFSA, 2007). The intestinal lactic acid bacteria such as *Lactobacilli*, *pediococci* and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin. Some lactobacilli have high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, , and vancomycin (Danielsen and Wind, 2003). For a number of lactobacilli a very high frequency of spontaneous mutation to nitrofurazone , kanamycin and streptomycin resistance was found (Curragh and Collins, 1992).The *Lactobacilli* species have been found susceptible to many cell wall synthesis inhibitors, like penicillins and ampicillin (Danielsen and Wind 2003, Coppola *et al.* 2005),

Lactobacilli species are usually susceptible to chloramphenicol, erythromycin and clindamycin, antibiotics that inhibit protein synthesis, (Coppola *et al*. 2005).Here, the promising results were obtained where all the isolates were showing safe antimicrobial resistance. All the isolates in the present study were checked for antibiotic sensitivity pattern. The results agreed with the criterion laid down by WHO for the antibiotic susceptibility pattern of probiotics.

8. As deconjugation has recently been included by WHO experts as one of the main activities of intestinal microbiota for them to be considered as probiotic organisms(FAO/WHO,2002).Microbial BSH activity contributes for its probiotic properties in the gastrointestinal tract. It was observed that BSH activity was found in the strains isolated from the intestine or faeces from mammalians from which environment rich in conjugated and unconjugated bile acids. Schillinger *et al* detected BSH activity in various strains of *L. acidophilus* and *L. johnsonii* but not in *Lactobacillus casei* strains group.(Schillinger, U.,*et al*., 2005).Lactic acid bacteria isolated from intestine have shown BSH activity. It was observed that the probiotic strains and species isolated from other habitats like milk or vegetables where bile salt is absent , normally did not have BSH activity. As all the isolates were BSH positive each and every criterion suggested by FAO/WHO has been fulfilled.