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### RESEARCH ARTICLE

#### Studies on the identification of Bifidobacteria isolated from Human Breast milk of Indian women.

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#### Abstract

Breast milk is an important nutrient source for neonates and many studies showed that this has beneficial effects on human health. One reason is that this micro flora of human breast milk contains the beneficial *Bifidobacteria*. Based on this information we proposed to isolate and identify various Bifido's from human breast milk. In this present study a total of about 59 Breast milk samples were collected from lactating women during the first week of delivery. The milk samples of about 3ml taken into a sterile tube and subjected for screening of microflora by using standard methods. We have observed mixed flora in these samples therefore we have used a selective media i.e. Bifidobacterium agar supplemented with mupirocin. After incubation the colonies were picked up for Gram's staining, F6PPK tests. Further the DNA was extracted from the identified Bifido's and subjected to PCR using specific primers. Further the genome sequence analysis was carried out to know the variations which were then compared with already existing Bifidobacterium species in databases. The *Bifido's* identified as "Y" & "V" shaped in morphology were Gram positive and the F6PPK test is positive reaction for the Genus Bifidobacterium which was further confirmed by PCR. The DNA sequencing results revealed that isolates from 10 samples out of 59 had *Bifido's*. In these (6) *Bifido's* had 99% similarity & 4 were found to have 98% similarity with the existing *Bifidobacterium animalis* sub sps lactis. Breastmilk is a potential source for Bifidobacteria particularly *Bifidobacterium animalis* sub sps lactis in Indian women.

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#### Introduction:-

Human Breast milk is a heterogeneous biological fluid adapted to fulfill the nutritional requirements of the rapidly growing newborn. It contains right balance of nutrients for growth and development of newborns, and also contains many bioactive substances that benefits neonate immune system. The proactive substances present in colostrum, transitional as well as in mature human milk include immunoglobulin's, immunocompetent cells, (Martin et al 2003) carbohydrates, fatty acids, vitamins, Polyamines and lysozyme, lactoferrin, glycoprotein and antimicrobial peptides (Newburg DS, 2005). In addition to that various studies have been shown that human breast milk contains bacteria approximately  $10^3 - 10^4$  cfu/ml, (Beasley and Saris, 2004; Collado et al., 2009) representing a continuous source of potential commensal bacteria for the infant gut (Martin et al.2003; Perez et al.2007). Breast milk is a continuous source of micro organisms that colonize the infant gut and involves in mucosal immune response. The most

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common encountered micro flora in Human breast milk is *Lactobacilli*, *Bifidobacteria* including commensals like *Streptococci* and *staphylococci* etc (Martin et al.2003, 2004 and2005), (Heikilla and Saris 2003).

There are many studies reported the effect of human milk on health of neonates and their diseases but lack of studies have the microbial benefits of human breast milk micro flora. Most of these micro floras have probiotic properties such as survival in the gastrointestinal conditions particularly low pH, bile tolerance at 0.3%, production of antimicrobial compounds such as bacteriocins and adhesion to intestinal mucosa (Martin and Olivares 2005).Based on the available information we have made an attempt to isolate probiotics particularly *Bifidobacteria* from human breast milk.The aim of the present study was isolation and identification of *Bifidobacteria* from Human breast milk and comparison of these new bacterial species obtained with the already existing ones.

### Materials and Methods:-

This Research work is a cross-sectional study which involves only collection of Breast milk from Lactating mothers during the first week of delivery and there are no experiments involved on these subjects. A total of about fifty nine (59) lactating mothers who were admitted in the Department of Gynecology at Gandhi Medical College and Hospital, Secunderabad, Telangana State, India were enrolled in this study. The inclusion criteria included healthy women with normal and full term pregnancy and who were breastfed exclusively to their newborn, without any maternal or any prenatal problems. The Institute Medical Ethical Clearance (NIN Protocol Number 07/2012/I) approval and a written consent from the subjects were obtained before the commencement of study. This study is a hospital based i.e. women admitted exclusively for delivery. This work was carried out during the period from January 2014 to December2014 at our Institute (NIN, Hyderabad, India). Breast milk samples were collected randomly from fifty nine (59) Lactating women as and when delivery took place either by cesarean or Normal vaginal. The sample size was calculated by assuming 95% of CI and 5% precision with 10% of *Bifidobacterium* isolates and the required samples are 59 using EPI epidemiological tool calculator.

### Sample Collection:-

The human milk samples were collected preferably within the first week of delivery. The breast skin and nipples were cleaned with soap and sterile water containing chlorhexidine. Before collection, first few drops were discarded and about 3-5ml of milk was taken into a sterile container by manual expression using sterile gloves. The milk samples were transported immediately in an ice box to the laboratory for further processing and investigation.

### Isolation of *Bifidobacteria* from Human breast milk:-

About 500µl of Breast milk sample was serially diluted to  $10^3$  using sterile phosphate buffer saline (PBS) and 100µl of the diluted sample was plated on to a *Bifidobacterium* agar (Himedia-India) and incubated at 37°C for 24-48 hrs in an anaerobic chamber. After incubation the culture plates were observed for colony growth and various colonies were picked up for Gram's staining to identify morphological characteristics of these bacteria. Since we could observe multiple microfloras viz. *Lactobacilli*, *Streptococci*, and *Staphylococci* etc therefore we have supplemented Bifido agar with mupirocin (50µg/ml) which is an antibiotic that prevents the growth of *Streptococcus* and *Staphylococcus* sps. Which are commonly as commensals and repeated sub-culturing to obtain pure colonies of *Bifido*.

### Identification of the Bifido isolates by F6PPK Assay:

After morphological confirmation of the above Bifido was further subjected to Fructose 6-Phosphate Phospho Ketolase (F6PPK) test. The F6PPK test was carried out according to the protocol of Gerald W and Tannock (1999) which was modified by the addition of CTAB (SIGMA) to the phosphate buffer for a better cell disruption .Use of CTAB to disrupt cell membranes is an effective alternative traditional cell disruption procedures. (J.I.Orban &J A Patterson 2000).

### Molecular Identification:

#### DNA extraction from samples:

2ml of bacterial culture was centrifuged and dissolved the pellet in lysozyme (500 µl) and incubated at 37°C for 45 mins and 100µl of SDS (10 %), 200µl of NaCl were then added and the mixture was incubated for 1hr. The DNA was extracted from this bacterial cell lysate by adding phenol- chloroform. Further DNA was precipitated with 70 % ethanol and dissolved in 50µl TE buffer. The integrity, purity, and concentrations of nucleic acids were confirmed by gel electrophoresis and nanodrop.

**PCR Amplification for genus Confirmation:**

The PCR was carried out using the thermal cycling conditions. A total volume of 25µl reaction mixture containing 10 mM of Tris-HCl (pH8.3), 50 mM of KCl, 4mM of MgCl<sub>2</sub>, 0.4 µM of dNTP's, 0.05 U/µl Taq DNA polymerase, 1 µl of template DNA 1µl of forward and reverse primers (1µmol) and 9.5µl of nuclease free water (Table-1).The PCR amplification program consisted of initial denaturation of 5 min at 96°C (one cycle) and 34cycles of 30sec at 96°C for denaturation, 35 sec for annealing at 58°C and 30sec for 72°C for extension .final cycle was 72°C for 8min and further samples were cool down at 4°C.Further species was identified by using 16s rRNA gene sequencing. (Details are depicted in Table 2 &3).

**Gel electrophoresis:**

About 2 µl of PCR products and 2 µl of loading dye were mixed and loaded into wells along with 1kb DNA ladder to see the amplification of PCR product. The PCR products were electrophoreses at 60 mA for 45 min and the amplified products were visualized under U.V light.

**Results:-**

Based on the morphological and physical characteristics, about ten (10) colonies were identified as bifidobacterium. These colonies of Bifidobacteria were appeared as round, white and cream in color and “Y”&“V” shape which were Gram's positive bacilli upon microscopic examination (Figure-1). Further these were found to be catalase negative and non spore forming bacteria. These isolates analyzed by F6PPK test were found to be positive for enzyme reaction which showed the change in color from yellow to dark brown indicating the Genus Bifido (Figure-2). Further the DNA extracted (Figure-3) and amplified PCR product of DNA was found to be 529 bp. (Figure-4). Thus confirming these isolates belongs to *Bifidobacteria* genus The species was identified using 16S rRNA gene sequencing and the product obtained was found to be 1500 bp (Figure-5) which was then compared with existing strains of Bifidobacterium that are available in Genome Banks viz. NCBI-GENBANK. Finally these comparisons revealed that about Six (6) Bifidos were found to have 99% and remaining four (4) had 98% similarity with the existing *Bifidobacterium animalis sub sps lactis* strain.

The clinical history of mothers in whom Bifido's were present in their breast milk revealed that all eight (8) out of ten (10) had undergone cesarean deliveries except two of them were with normal (vaginal) delivery. Their age ranged from 19-30 yrs and mean age was  $23.5 \pm 3.47$  as given in the Table-1. We have noted that most of them were house wives and one woman happened to be a staff nurse and the other a daily laborer. Further all the newborns (6 male and 4 females) had normal weights i.e. 2.5kgs and above except one baby with 2.0 kgs.

**Table1:-** showing the Genus specific primers for Bifidobacterium

Target	Primers	Sequences	Product length	Annealing Temperature
<i>Bifidobacteria</i>				
Bifidobacterium	g-Bifid-F g-Bifid-R	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	529-560	58°C

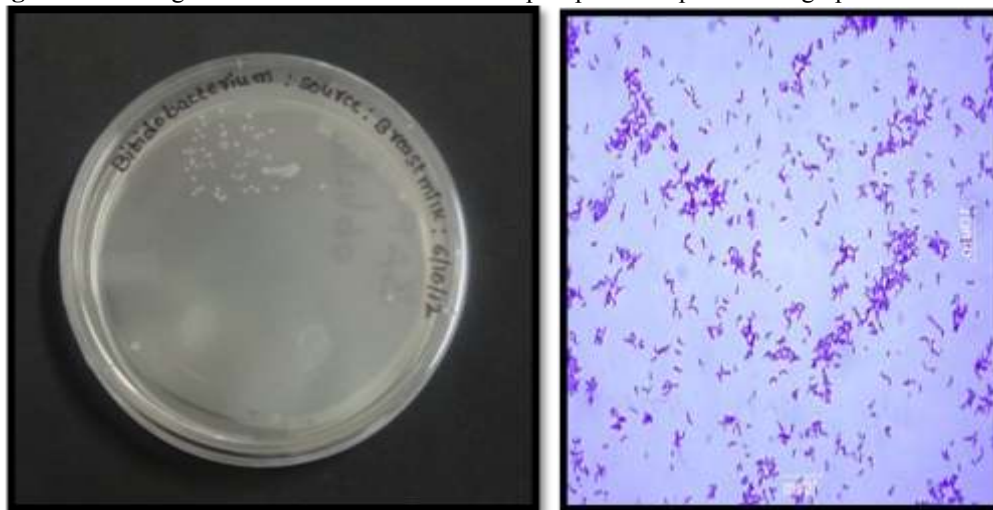
**Table 2:-** Primers used for amplification of 16S rRNA region.

Target	Primers	Sequences	Product length	Annealing Temperature
<i>Bifidobacteria</i>				
Bifidobacterium	16SF	AGAGTTTGATCTHYGGYTYAG	1500	60 °C
16S rRNA region	16SR	ACGGCTACCTTGTTACGACTT		

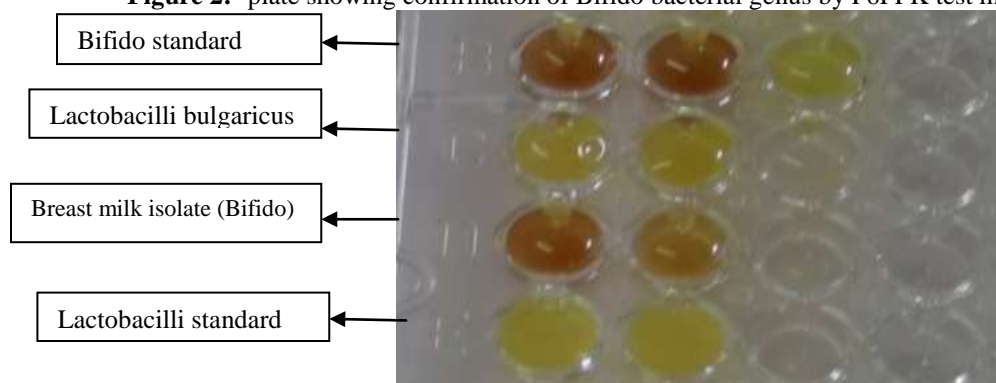
**Table 3:-**showing the PCR Reaction setup.

TEMPERATURE	TIME
95°C	3mins
1 cycle	
98°C	20sec
60°C	15sec
72°C	1.30mins
35cycles	
72°C	3 mins

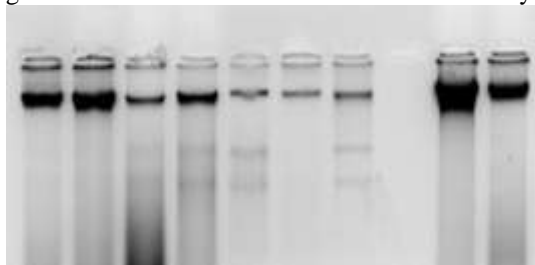
**Figure1:-**showing isolated colonies of Bifido's in petriplate and photomicrograph of stained flora

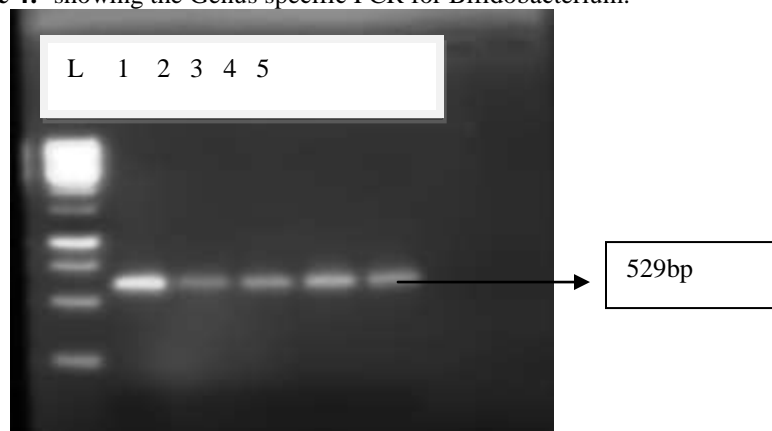


**Figure 2:-** plate showing confirmation of Bifido bacterial genus by F6PPK test indicated by brick red color



**Figure 3:-** showing the confirmation of Probiotic bacterial DNA by gel electrophoresis

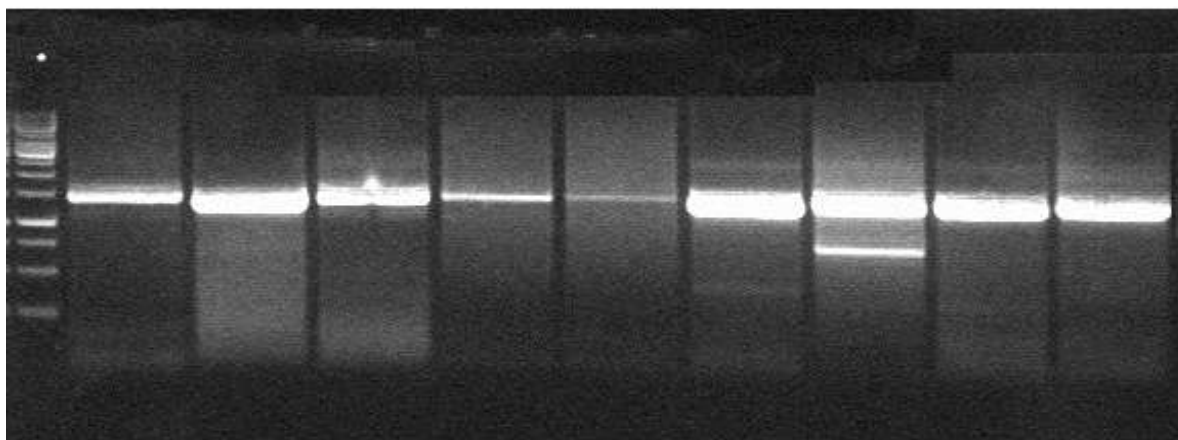


**Figure 4:-** showing the Genus specific PCR for Bifidobacterium.

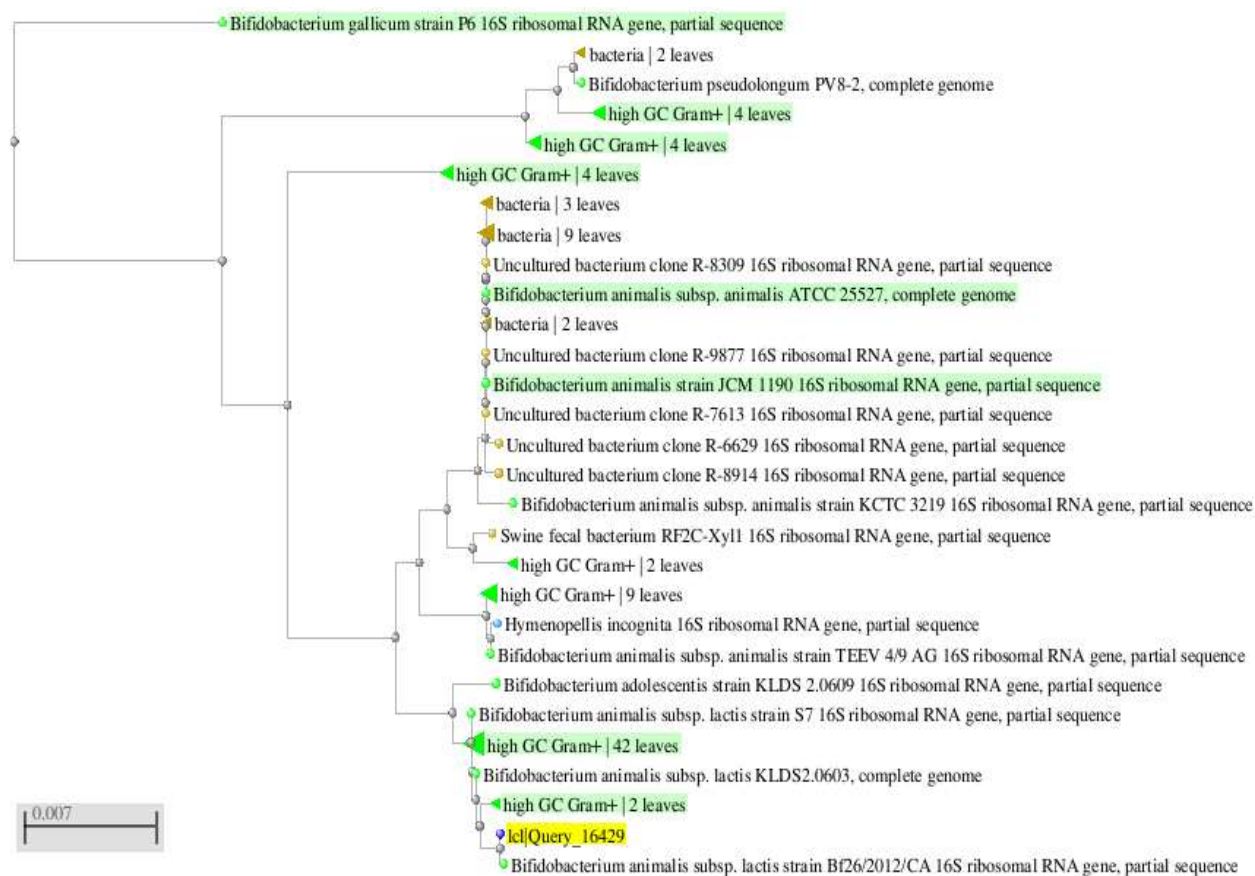
L-Ladder Samples -1, 2,3,4,5

**Figure 5:** showing the PCR amplified product with 1500 bp in all the samples confirming the *Bifidobacterium animalis sub sps lactis*

1KB 73 95 96 97 98 99 101 102 104

**Bacterial Identification:-**

The molecular analysis for sample NIN – 73 shows 99% identity to the sequence *Bifidobacterium animalis subsp. Lactis* the sample is most probably *Bifidobacterium animalis subsp. Lactis*.



**Figure6:-** Phylogenetic tree of isolates prepared using mega 6 software and neighbor-joining algorithm.

## Discussions:-

Probiotics for health benefits should preferably include bacteria of human origin possessing a series of desirable properties and lacking of toxic traits (Silvya Arboyela 2011). Based on this information we concentrated on the isolation and identification of specific probiotics particularly Bifidobacteria from human breast milk. We hereby report that the *Bifidobacterium animalis sub sps lactis* was found to be predominant species in human breast milk samples. Our results are matched with that of earlier reports of Gueimonde M et al (2007) and Fernandez L, (2013) who also reported the same. We could achieve the isolations of Bifidobacteria despite relatively presence of low numbers in some of the breast milk samples. Our findings are in conformity with Gueimonde et al. (2007) who showed the similar results on the presence of Bifidobacteria bifidum in 46% of 20 human milk samples and in another study Martin et al. (2009) obtained B.bifidum from two out of eight breast milk samples.

The successful isolation of Bifidobacteria in our study could be achieved by the modification of medium i.e. Bifido bacterium agar supplemented with mupirocin with a concentration of 50ug/ml which was earlier used by Simpson P.J. et al (2004). Mupirocin is a narrow spectrum antibiotic and active against both Gram-positive and Gram negative bacteria including microorganisms *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Staphylococcus etc.* The mechanism in Bifidobacteria is that this bacterium has isoleucyl-tRNA Synthase gene which provides resistance to mupirocin unlike for other organisms (Faustra et al 2011). F6PPK test is the key taxonomic tool for the identification of Genus Bifidobacteria therefore we followed the modified procedure of Biblioni et al (2000) which saved lot of time and obtained quick results.

The information on the presence of bacteria in human breast milk could be the maternal gut and that the bacteria arrive at the mammary gland through an endogenous route called as entero-mammary pathway (Perez PF et al 2007). It is now understood that during late pregnancy and lactation the maternal dendritic cells and macrophages can enter into the gut epithelium and take up commensal bacteria directly from the gut lumen and translocation of the bacteria from gut to mammary gland (Rescigno M, et al 2001). Human breast milk is an interesting source to



obtain novel and specific probiotic strains for newborns which assists in the development of the gut microbiota and immune system. It can be understood from our study bacterial strains particularly of *Bifidobacterium animalis subsp. lactis* can promote immense health benefits to humans.

### Conclusion:

From this study we could conclude that human breast milk is a good source of *Bifidobacteria*. These Probiotic strains can be treated as original, indigenous of human origin. Therefore it is advisable to study for their probiotic potentiality as these can be vital for human health and have immense clinical applications in future.

### Note:-

Kindly note that this paper was presented and awarded prize in the 3<sup>rd</sup> Biennial Conference and International Symposium on Stress, Microbiome and Probiotics organized by Probiotic Association of India (PAI) and NISER, held during March 11-13<sup>th</sup> 2016 at Bhubaneswar Odisha, India

### Conflict of Interest:

The authors declared there are no conflicts of interest of this particular work.

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### References:

1. Beasley, S.S. and Saris, P.E. (2004). Nisin-producing *Lactococcus lactis* strains isolated from human milk. *Applied and Environmental Microbiology*. 70, 5051-5053.
2. Bibiloni, R., Perez PF, Antoni DE G Lan. (2000) Enzymatic-colorimetric assay for the quantification of bifidobacteria. *Journal of food prot.* 63, 322-326
3. Collado, M.C., Delgado, S., Maldonado, A. and Rodriguez, J.M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Letters in Applied Microbiology*. 48: 523-528.
4. Fausta, Serafini, Francesca Bottacini, Alice Viappiani, Enrico Baruffini, Francesca Turrone, Elena Foroni, Tiziana Lodi, Douwe van Sinderen, Marco Ventura. (2011) Insights into Physiological and Genetic Mupirocin Susceptibility in *Bifidobacteria*. *Appl Environ Microbiol*. 77(9): 3141–3146.
5. Fernandez L, Langa S, Martín V, Maldonado A, Jiménez E, Martín R, Rodríguez JM. (2013). The human milk microbiota: origin and potential roles in health and disease. *Pharmacol Res*; 69:1–10.
6. (a) Gueimonde M., Laitinen K., Salminen S. & Isolauri E. (2007) Breast milk: a source of bifidobacteria for infant gut development and maturation? *Neonatology*, 92(1), 64-66.  
(b) Gueimonde M., Laitinen K., Salminen S. & Isolauri E. (2007) Breast milk: a source of bifidobacteria for infant gut development and maturation? *Neonatology*. 92(1), 64-66.
7. Heikkilä, M.P. and Saris, P.E. (2003). Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *Journal of Applied Microbiology*. 95: 471-478.
8. Martín, R., Langa, S., Reviriego, C., Jiménez, E., Marin, M.L., Xaus, J., Fernandez, L. and Rodriguez, J.M. (2003) Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics* 143: 754-758.
9. Newburg DS. (2005) Innate immunity and human milk. *Journal of Nutrition*; 135:1308–12.
10. Martín, R., Langa, S., Reviriego, C., Jiménez, E., Marin, M.L., Xaus, J., Fernandez, L. and Rodriguez, J.M. (2003) Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics*. 143: 754-758.
11. Martín R, Langa S, Reviriego C, Jiménez E, Marín ML, Olivares M, Boza J, Jiménez J, Fernández L, Xaus J and Rodríguez JM. (2004) The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics. *Trends Food Sci. Technol.*; 15: 121-127.
12. Martín R, Olivares M, Marin ML, Fernandez L, Xaus J, Rodríguez JM. (2005). Probiotic potential of 3 *Lactobacilli* strains isolated from breast milk. *Journal of Human Lactation*. 21(1):8-17.
13. Martín R, Olivares M, Marin ML, Fernandez L, Xaus J, Rodríguez JM. (2005). Probiotic potential of 3 *Lactobacilli* strains isolated from breast milk. *J Hum Lact. Feb*; 21(1): 8-17.
14. Martín, R., E. Jiménez, H. Heiling, L. Fernandez, M.L. Marin, E.G. Zoetendal and J.M. Rodríguez. (2009). Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing

- gradient gel electrophoresis and quantitative real-time PCR. Applied and Environmental Microbiology. 75: 965-969.
15. Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, Serrant P, Segura-Roggero I, Schiffrin EJ and Donnet-Hughes A. (2007) Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? Pediatrics; 119(3): 724-732.
  16. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP and Ricciardi-Castagnoli P (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial minelayers to sample bacteria. Nat. Immunol.; 2: 361–367.
  17. Simpson P.J, Fitzgerald GF, Stanton C, Ross RP.J (2004). The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria. Microbial Methods. 57(1): 9.
  18. Silvia Arbolea, Patricia Ruas-Madiedo, Abelardo Margolles, Gonzalo Solís, Seppo Salminen, Clara G. de los Reyes-Gavilán and Miguel Gueimonde (2011). Characterization and *in vitro* properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. Int J Food Microbiol.. Volume 149, Issue 1, 28–36.