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# **Comparing the Gut Flora of Irish Breast and Formula Fed Neonates aged between Birth and 6 Weeks Old**

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## **Abstract**

Ireland has one of the lowest breastfeeding rates in the developed world. A gut flora consisting of high levels of the bacteria *Bifidobacteria* and *Lactobacilli* is regarded as a healthy gut. These bacteria are reported to impart beneficial health properties to humans. This project looked at the establishment of eight selected representative bacterial groups of the gut flora at three time points in the 0-6 week range, differentiating between breast fed and formula fed babies. Microbiological methods were used to specifically enumerate *Lactobacilli* sp., *Bifidobacteria* sp., *Enterococci* sp., *Staphylococci* sp., *Bacteroides* sp., *Clostridia* sp. and coliforms present in the gut. The data indicated major trends present, including the fact that *Bifidobacteria* sp. and *Lactobacilli* sp. were more prevalent in the gut flora of breast fed neonates whereas *E.coli* and *Enterococci* sp. were more prevalent in the gut flora of formula fed neonates. The higher prevalence of *Bifidobacteria* and *Lactobacilli*, which are often incorporated into probiotic foods, in Irish breast fed neonates thus re-enforces the advantages of breastfeeding over formula feeding.

## **Key Words**

Gut flora, neonate, breastfeeding, formula feeding, probiotics, *Bifidobacteria*, *Lactobacilli*.

## Introduction

‘You are what you eat’, as the saying goes, and at no point in life is this more relevant than at birth. Diet is a major dictator of the health and wellbeing of every human. Modification of our diet using probiotics is now becoming more common, with companies selling products that contain bacteria with reported beneficial properties<sup>1,2,3</sup> including *Bifidobacteria* sp. and *Lactobacilli* sp.<sup>4,5</sup>. These bacteria are well documented as preventing diarrhoea and colon cancer, stimulating the immune system, and inhibiting the growth of toxin producing organisms such as *Clostridia difficile*, *Escherichia coli*, *Salmonella* sp. and *Shigella* sp.<sup>6,5,7,8,9</sup>. Several previously reported studies have examined the effect of the feeding regime of newborn infants on the establishment and composition of the gut flora and these have shown that there are differences between the gut flora of a breast fed neonate and a formula fed neonate<sup>10,11,12,13,14,15</sup>. Despite the fact that these studies have indicated that breastfeeding provides a more beneficial bacterial composition in the gut for the infant than formula feeding, Ireland has one of the lowest breast feeding initiation rates in the developed world, at 35% compared with our nearest neighbour, the UK, at 70% and Scandinavian countries where the rate can be as high as 95%<sup>16</sup>. The study presented in this paper is the first study to examine the Irish neonatal gut in the 0-6 week age group, to establish if a statistically significant link could be found in the percentage presence and dominance of several bacterial groups which occur in the gut flora over the selected time period, with the aim to provide further evidence of the beneficial effect of breast milk.

## **Materials and Methods**

*Subject selection.* The study was approved by the ethics committee of the National Maternity Hospital, Holles Street, Ireland. Signed consent was obtained from all informed parents prior to the inclusion of their infants in this study. All babies were of normal vaginal delivery and were from four different wards within the one hospital. A random selection of 105 infants varying in age from zero to six weeks were each sampled once during the study, with 49 being exclusively fed breast milk and 56 being exclusively given formula milk in the period from birth to sampling.

*Collection of specimens.* Samples were collected at several time points between the years 2001 & 2002. Fresh faecal samples were collected in the nursery from the neonates' nappies using sterile swabs, during the first 5 days of life. Faecal samples from 6 week old infants were obtained by swabbing nappies which had been soiled either immediately preceding the visit and were then provided in sealed plastic bags by the parent, or alternatively were freshly soiled during the hospital check-up visit. All swabs were placed in sterile Aimes medium for transport back to the laboratory where they were frozen at  $-70^{\circ}\text{C}$  within three hours of sampling. Recovery rates were determined to ensure the viability of cells throughout the storage period.

*Culture and identification of bacteria.* The specimens were thawed out at room temperature before serial tenfold dilutions were performed from  $10^{-1}$  to  $10^{-7}$  in sterile Ringers solution. 0.2mls of each dilution was applied to duplicate plates

of each selective culture media and distributed over the surface using a sterile spreader. The bacteria selected for the study, the culture media and incubation conditions are summarised in Table 1.

After incubation, as indicated in Table 1, plates which contained between 30 and 300 colonies were enumerated. To ensure that the correct colonies were being enumerated and identified, picks of each different colony morphology type were taken and analysed by Gram stain and then stored at  $-70^{\circ}\text{C}$  in Tryptic Soy Agar and 10% glycerol for later analysis by API system (bioMérieux). Bacterial counts were initially expressed as colony forming units (cfu)/ml. In order to compare bacterial composition between swabs that varied in faecal matter content, a percentage presence was calculated for each bacterial group relative to the total count for each bacterial swab. An independent “t” test was used to statistically analyse the data gathered. A  $p$  value of  $<0.05$  was considered to be statistically significant.

## Results

The number of samples that tested positive for the selected bacteria in both feeding regimes over the three different time periods was determined (Table 2). The average percentage presence calculated for each bacterial group studied in both feeding regimes at the three different time points is presented in Table 3. The rate of dominance of each bacterial group, in samples that tested positive for the particular bacterial species, is presented in Table 4.

In 0-1 day old breast and formula fed neonates no statistically significant differences were determined between the numbers of samples testing positive for each bacterial species. It did appear that the gut flora of the breast fed neonate developed quicker than that of the formula fed neonate, in that more breast fed neonates tested positive for the selected groups of bacteria than formula fed neonates. *Staphylococci* sp. were found in 58% of all breast fed neonates tested as opposed to only 25% of all formula fed neonates tested (Table 2). The  $p$  value for this result was 0.08 indicating a trend which is almost statistically significant.

In 2-5 day old neonates (Table 2) *Bifidobacteria* sp. were more likely to be present in breast fed neonates (71%) than formula fed neonates (48%). This result, while not statistically significant, ( $p=0.16$ ) shows a strong trend. *Bifidobacteria* sp. were also present on average, in higher percentages (Table 3) in breast fed neonates (36%) compared to formula fed neonates (19%). Although this latter result was not statistically significant, the removal of two “outliers”

(Figure 1) from the total of 27 formula fed neonates tested resulted in a statistically significant  $p$  value of 0.03.

Other trends observed in the analysis of 2-5 day old neonates (Table 3) were that *Bacteroides* sp. had a higher average percentage presence in breast fed neonates (7%) compared with formula fed neonates (0.08%) with a  $p$  value of 0.16. In addition, *Lactobacilli* sp. were more likely to be dominant (Table 4) when present in formula fed neonates (43%) compared to breast fed neonates (0%) with a  $p$  value of 0.15.

*Enterococci* sp. tested positive in more 6 week old formula fed neonates (94%) compared to breast fed neonates (75%) of the same age (Table 2) with a  $p$  value of 0.13. *Enterococci* sp. were also more likely to be dominant ( $p=0.12$ ) in the 6 week old formula fed neonates giving a value of 17% as opposed to 0% in breast-fed infants (Table 4). *E.coli* were also more likely to be present in higher average percentages in 6 week old formula fed neonates (20%) compared to breast fed neonates (7%) of the same age (Table 3) with a low  $p$  value of 0.13, indicating a possible underlying trend. The average percentage presence of *Lactobacilli* sp. was higher in 6 week old breast fed neonates at 25% as compared to 7% in formula fed neonates which gave a  $p$  value of 0.1. However, with the removal of one particular outlying value (Figure 2) from the total of 17 formula fed data points, the  $p$  value became statistically significant ( $p=0.03$ ). In addition, this study indicated that *Lactobacilli* sp., when present in 6 week old neonates (Table 4), were more likely to be dominant ( $p=0.15$ ) in breast fed neonates (45%) compared with formula fed neonates (17%).



## Discussion

Random sampling of infants of various age profiles set out to analyse the selected bacterial groups known to comprise a major proportion of the neonatal intestinal gut flora (Fuller, 1992). While several other species are known to colonise the gut at lower levels, the study was designed to only assess the predominant neonatal gut flora in the Irish infants. The provision of updated additional evidence supporting the beneficial effects of breast feeding will aid in promoting the uptake rates for exclusive breast milk feeding regimes. This study showed that by day 5 the faecal floras of both feeding regimes are becoming well established. The results presented, indicate the differences between the gut flora of breast and formula fed neonates immediately from the introduction of feeds and that these differences continue to be seen at least until the 6 week point examined in this study.

*Staphylococci* sp. were found to be testing positive in more 0-1 day old breast fed neonates than formula fed neonates ( $p=0.08$ ) in this study. This result was similar to the findings of another study<sup>14</sup> where all five of the 1 day old breast fed neonates studied had been colonised with *Staphylococci* sp., compared to just 3 out of 6 formula fed neonates. The close contact between mother and child during feeding presumably results in transfer of this bacterium from the mothers' skin to the child.

The breast fed neonates in this study were dominated by the presence of *Bifidobacteria* sp. at both the 2-5 day old and the 6 week old time periods.

Previous studies<sup>13,14,15</sup> have also reported *Bifidobacteria* sp. to be present in higher amounts in breast fed neonates compared to formula fed neonates at the fifth day of life.

The considerably higher Bifidobacterial level in infants as young as 2 days of age is earlier than reported in previous studies. However, in the studies by Lundequist<sup>12</sup> and Yoshioka<sup>14</sup> it was reported that by the third week and fifth week of life the bacterial group was present in higher amounts in formula fed neonates. Oligosaccharides naturally present in breast milk have been shown to stimulate the growth of *Bifidobacteria* sp<sup>18,19</sup>.

Another study in 2002<sup>19</sup> also found that human milk provides peptides that are highly stimulating to *Bifidobacteria* sp., are present in much lower levels in bovine milk, which could provide a partial explanation for the higher levels of *Bifidobacteria* sp. being found in breast fed neonates compared to formula fed neonates.

In agreement with the work of Rubaltelli *et al.*<sup>13</sup>, Lundequist *et al.*<sup>12</sup>, and Yoshioka *et al.*<sup>14</sup>, *Bacteroides* sp. were found to be present in higher average percentages in 2-5 day old breast fed neonates, than for formula-fed infants with a lower *p* value of 0.16 (Table 3). *Lactobacilli* sp., as with *Bifidobacteria* sp., were found more likely to be the dominant bacterial group when present in 2-5 day old breast fed neonates compared to formula fed neonates of the same age, in agreement with previously reported studies<sup>12,13,14</sup>. By the sixth week *Lactobacilli* sp. when detected, were more likely to be dominant and on average were present in higher percentages in breast fed neonates compared to formula fed neonates.

This result was similar to the findings of Lundequist<sup>12</sup>, and Yoshioka<sup>14</sup> but is not in agreement of the findings of Benno<sup>10</sup>. Breast milk has recently been shown to be a possible source of *Lactobacilli* sp. in a study by Martin<sup>22</sup>. The finding that *Enterococci* sp. and *E.coli* are more likely to be present in higher levels in 6 week old formula fed neonates compared to breast fed neonates is similar to the findings of previous studies<sup>12,13,14</sup>.

The composition of the complex ecosystem that forms the human gut flora is influenced by several factors. This study, which examined the effect of breast milk versus formula milk in the evolving gut of a newborn infant, strongly suggests that breast milk plays an important role in encouraging the growth and dominance of healthy bacteria such as the *Lactobacilli* and *Bifidobacteria* in the neonatal gut. Breast milk is composed of many components that inhibit disease-causing bacteria such as *Clostridia* sp. and which also play a role in encouraging the growth of healthy bacteria like the *Lactobacilli* and *Bifidobacteria*<sup>20</sup>. These latter bacteria have been shown to be less likely to cause disease and are reported to aid the immune system by either inhibiting the growth of disease-causing organisms by lowering the pH of the gut or by producing anti-microbials<sup>10</sup>. These organisms have been shown to inhibit the growth of *Escherichia coli*, *Salmonella enteritidis* and *Clostridium jejuni*<sup>21</sup> all of which are common enteropathogens. In contrast, the gut flora of the formula fed neonate seems to be dominated more by *E.coli* and *Enterococci*, bacteria which would generally not be considered as contributing to a healthy gut.

## Conclusion

Despite the fact that the study could not follow the same infants throughout the 0-6 week period the results obtained are similar to those of studies that did. Overall, this study's results supports the findings of studies done in other countries and indicates that exclusive breastfeeding of Irish neonates offers the type of healthy gut bacteria considered desirable to humans throughout life. **It is also hoped that the publication of this study's findings will help improve breast feeding rates in Ireland.**

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**Table I. Culture media and incubation conditions.**

| <b>Culture Medium</b>                         | <b>Bacterial group isolated</b>   | <b>Temperature</b> | <b>Atmosphere</b> |
|---|---|--------------------|-------------------|
| Eosin Methylene Blue                          | <i>Escherichia coli</i> and other members of the <i>Enterobacteriaceae</i> family | 37°C               | Aerobic           |
| Rogosa Agar                                   | <i>Lactobacilli</i> sp.   | 37°C               | Anaerobic         |
| Bacteroides Bile Aesculin Agar                | <i>Bacteroides</i> sp.  | 37°C               | Anaerobic         |
| Baird Parker Media                            | <i>Staphylococci</i> sp.  | 37°C               | Aerobic           |
| Reinforced Clostridial Agar                   | <i>Clostridia</i> sp.   | 37°C               | Anaerobic         |
| Reinforced Clostridial Agar with Analine Blue | <i>Bifidobacteria</i> sp.   | 37°C               | Anaerobic         |
| Slanetz and Bartley                           | <i>Enterococci</i> sp.  | 44°C               | Aerobic           |

**Table II. Number of samples testing positive in the gut flora of 0-1, 2-5 days and 6 Week old breast and formula fed neonates.\***

| Positive Neonates (%)         |                            |                             |         |                            |                             |         |                            |                             |         |
|-------------------------------|----------------------------|-----------------------------|---------|----------------------------|-----------------------------|---------|----------------------------|-----------------------------|---------|
| Faecal Flora                  | 0-1 Day old Neonates       |                             |         | 2-5 Day old Neonates       |                             |         | 6 Week old Neonates        |                             |         |
|                               | Breast Fed Neonates (n=19) | Formula Fed Neonates (n=12) | P Value | Breast Fed Neonates (n=14) | Formula Fed Neonates (n=27) | P Value | Breast Fed Neonates (n=16) | Formula Fed Neonates (n=17) | P Value |
| <i>Escherichia coli</i>       | 3 (16%)                    | 0 (0%)                      | 0.16    | 6 (43%)                    | 9 (33%)                     | 0.56    | 6 (38%)                    | 10 (59%)                    | 0.23    |
| <i>Enterobacteriaceae</i> sp. | 3 (16%)                    | 2 (17%)                     | 0.95    | 6 (43%)                    | 8 (30%)                     | 0.41    | 12 (75%)                   | 11 (65%)                    | 0.54    |
| <i>Staphylococci</i> sp.      | 11 (58%)                   | 3 (25%)                     | 0.08    | 13 (93%)                   | 23 (85%)                    | 0.49    | 16(100%)                   | 15 (88%)                    | 0.17    |
| <i>Bacteroides</i> sp.        | 1 (5%)                     | 0 (0%)                      | 0.44    | 3 (21%)                    | 2 (7%)                      | 0.21    | 12 (75%)                   | 10 (59%)                    | 0.34    |
| <i>Bifidobacteria</i> sp.     | 5 (26%)                    | 2 (17%)                     | 0.55    | 10 (71%)                   | 13 (48%)                    | 0.16    | 5 (31%)                    | 6 (35%)                     | 0.81    |
| <i>Enterococci</i> sp.        | 6 (32%)                    | 2 (17%)                     | 0.37    | 8 (57%)                    | 12 (44%)                    | 0.45    | 12 (75%)                   | 16 (94%)                    | 0.13    |
| <i>Lactobacilli</i> sp.       | 1 (5%)                     | 1 (8%)                      | 0.75    | 4 (29%)                    | 7 (26%)                     | 0.86    | 11 (69%)                   | 12 (71%)                    | 0.91    |
| <i>Clostridia</i> sp.         | 0 (0%)                     | 0 (0%)                      | -       | 1 (7%)                     | 2 (7%)                      | 0.98    | 1 (6%)                     | 1 (6%)                      | 0.97    |

\* The total number of samples tested is indicated by 'n'. The number of samples testing positive is given in each column with the associated percentage value given in brackets. 'p' values were calculated using the independent 't' test.

**Table III. Average percentage presence of each bacterial group in the gut flora of 0-1, 2-5 days and 6 Week old breast and formula fed neonates.\***

| Faecal Flora                     | Average Percentage Presence (%) |         |         |                      |         |         |                     |         |         |
|----------------------------------|---------------------------------|---------|---------|----------------------|---------|---------|---------------------|---------|---------|
|                                  | 0-1 Day old Neonates            |         |         | 2-5 Day old Neonates |         |         | 6 Week old Neonates |         |         |
|                                  | Breast                          | Formula | P Value | Breast               | Formula | P Value | Breast              | Formula | P Value |
| <i>E.coli</i>                    | 7%                              | 0%      | 0.32    | 9%                   | 19%     | 0.36    | 7%                  | 20%     | 0.13    |
| <i>Enterobacteriaceae</i><br>sp. | 4%                              | 1%      | 0.53    | 7%                   | 16%     | 0.36    | 16%                 | 22%     | 0.48    |
| <i>Staphylococci</i> sp.         | 30%                             | 14%     | 0.2     | 18%                  | 20%     | 0.75    | 10%                 | 5%      | 0.36    |
| <i>Bacteroides</i> sp.           | 0.01%                           | 0%      | 0.44    | 7%                   | 0.08%   | 0.16    | 20%                 | 10%     | 0.25    |
| <i>Bifidobacteria</i> sp.        | 8%                              | 4%      | 0.45    | 36%                  | 19%     | 0.16    | 14%                 | 20%     | 0.5     |
| <i>Enterococci</i> sp.           | 9%                              | 7%      | 0.43    | 20%                  | 12%     | 0.39    | 7%                  | 15%     | 0.12    |
| <i>Lactobacilli</i> sp.          | 5%                              | 0.10%   | 0.66    | 2%                   | 11%     | 0.28    | 25%                 | 7%      | 0.1     |
| <i>Clostridia</i> sp.            | 0%                              | 0%      | -       | 0.60%                | 1.20%   | 0.67    | 1.20%               | 0.40%   | 0.53    |

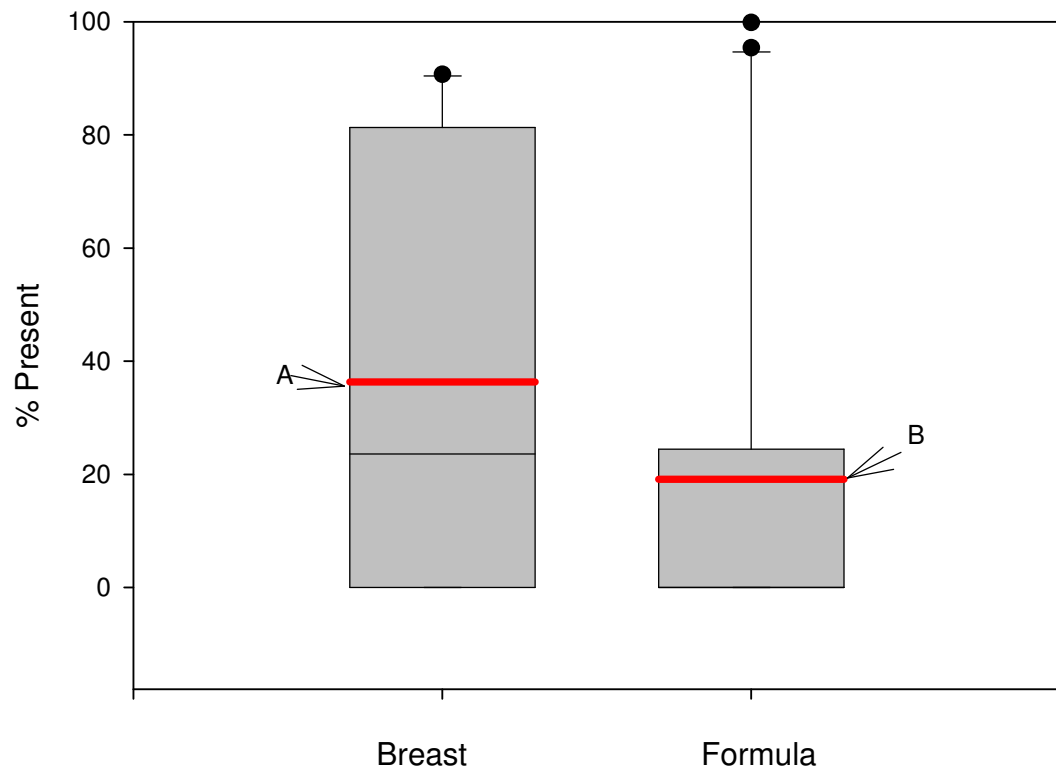
\* Percentage presence was calculated for each bacterial group relative to the total count for each bacterial swab. 'p' values were calculated using the independent 't' test.

**Table IV. Comparison of dominance, within the number of samples testing positive for each bacterial group, in breast and formula fed neonates aged 0-1, 2-5 days and 6 Weeks old.\***

| Dominance (%)                 |                            |                             |         |                            |                             |           |                            |                             |           |
|-------------------------------|----------------------------|-----------------------------|---------|----------------------------|-----------------------------|-----------|----------------------------|-----------------------------|-----------|
| Faecal Flora                  | 0-1 Day Old Neonates       |                             |         | 2-5 Day Old Neonates       |                             |           | 6 Week Old Neonates        |                             |           |
|                               | Breast Fed Neonates (n=19) | Formula Fed Neonates (n=12) | P Value | Breast Fed Neonates (n=14) | Formula Fed Neonates (n=27) | P Value   | Breast Fed Neonates (n=16) | Formula Fed Neonates (n=17) | P Value   |
|                               | <i>Escherichia coli</i>    | 1/3 (33%)                   | 0 (0%)  | -                          | 2/6 (33%)                   | 5/9 (56%) | 0.43                       | 0/6 (0%)                    | 4/10(40%) |
| <i>Enterobacteriaceae sp.</i> | 1/3 (33%)                  | 0/2 (0%)                    | 0.5     | 1/6 (17%)                  | 4/8 (50%)                   | 0.22      | 2/12(13%)                  | 4/11(36%)                   | 0.3       |
| <i>Staphylococci sp.</i>      | 6/11(55%)                  | 2/3 (67%)                   | 0.73    | 2/13(15%)                  | 6/23(26%)                   | 0.47      | 1/16(16%)                  | 0/15 (0%)                   | 0.34      |
| <i>Bacteroides sp.</i>        | 0/1 (0%)                   | 0 (0%)                      | -       | 1/3 (33%)                  | 0/2 (0%)                    | 0.5       | 4/12(33%)                  | 1/10(10%)                   | 0.21      |
| <i>Bifidobacteria sp.</i>     | 1/5 (20%)                  | 0/2 (0%)                    | 0.58    | 6/10(60%)                  | 6/13(46%)                   | 0.53      | 4/5 (80%)                  | 4/6 (67%)                   | 0.66      |
| <i>Enterococci sp.</i>        | 2/6 (33%)                  | 1/2 (50%)                   | 0.72    | 3/8 (38%)                  | 3/12(25%)                   | 0.57      | 0/12 (0%)                  | 3/16(17%)                   | 0.12      |
| <i>Lactobacilli sp.</i>       | 1/1(100%)                  | 0/1 (0%)                    | -       | 0/4 (0%)                   | 3/7 (43%)                   | 0.15      | 5/11(45%)                  | 2/12(17%)                   | 0.15      |
| <i>Clostridia sp.</i>         | 0 (0%)                     | 0 (0%)                      | -       | 0/1 (0%)                   | 0/2 (0%)                    | -         | 0/1 (0%)                   | 0/1 (0%)                    | -         |

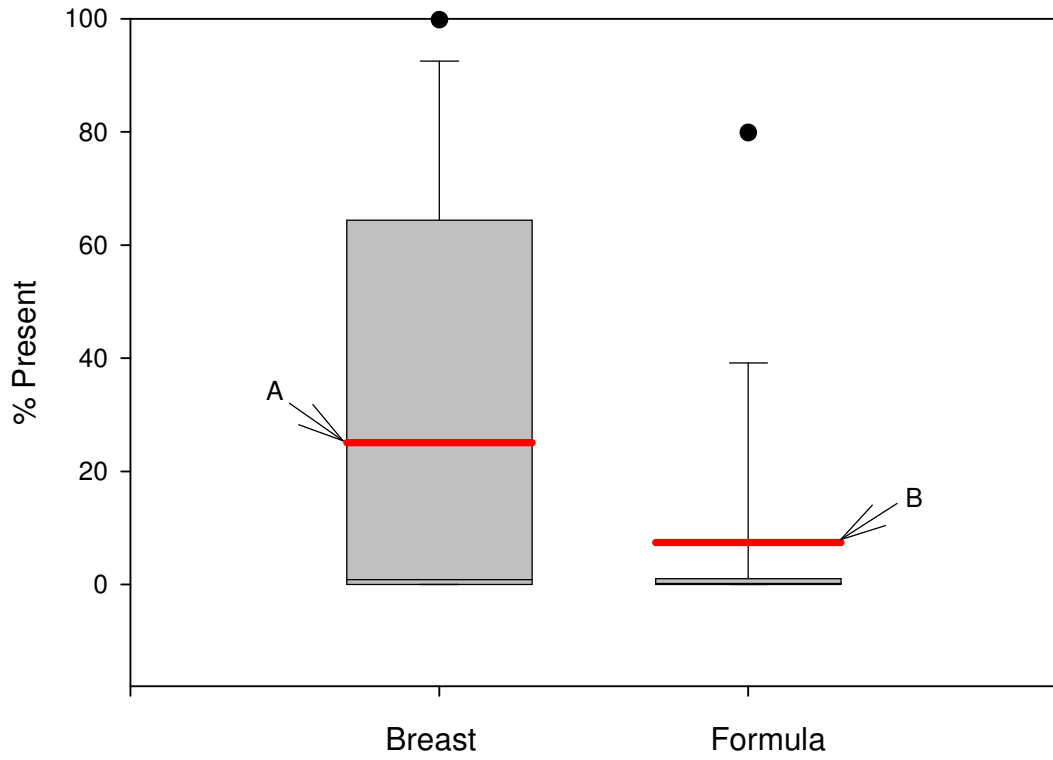
\* The number of samples which showed dominance for each bacterial group tested is represented as a fraction of the total number of samples which tested positive for that particular species. The percentage value is given in brackets. ‘p’ values were calculated using an independent ‘t’ test.

**Figure I. Percentage presence of *Bifidobacteria* sp. in 2-5 day old breast and formula fed neonates.\***



\* Figure I shows the percentage presence range of *Bifidobacteria* sp. in 2-5 day old breast and formula fed neonates. The average percentage presence is indicated for breast-fed (A) and formula-fed (B) infants.

**Figure II. Percentage presence of *Lactobacilli* sp. in 6 week old breast and formula fed neonates.\***





\* Figure II shows the percentage presence range of *Lactobacilli* sp. in 6 week old breast and formula fed neonates. The average percentage presence for breast-fed infants (A) and formula-fed infants (B) is indicated.