**Probiotics and gut health in infants: A preliminary case-control observational study about early treatment with Lactobacillus reuteri DSM 17938.**

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Abstract: AIMS: we performed this case-control observational study to evaluate the effects of early administration of Lactobacillus reuteri DSM 17938 on microbial composition in infants' gastrointestinal tract.
METHODS: early fecal microbiota composition was analyzed by using selective and differential cultural methods. Genomic DNA from positive Escherichia coli and Cronobacter sakazakii colonies was extracted and DNA was processed by multiplex PCR assay.
RESULTS: fecal samples of 30 hospitalized infants who previously received probiotics and 30 not receiving probiotics were analyzed. We find that the two groups showed differences in gut microbial strains composition and richness. Infant treated with probiotics have a lower total anaerobic gram-negative counts (p=0.03) and a higher total anaerobic gram-positive counts (p=0.02). Enterobacteriaceae and enterococci were significantly higher (p=0.04) in the control group. No significant differences were observed for total aerobic counts, lactobacilli and bifidobacteria. C. sakazaki was found only in one infant recruited in the control group. Infants not previously treated with probiotics showed a higher colonization by diarrheagenic E. coli (EPEC) (p=0.04).
CONCLUSIONS: our findings enhanced our understanding of the effects of probiotics on gut health in pediatric subjects. Early administration of L.reuteri in infancy could improve gut health by reducing pathogens colonization.

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Dipartimento di Scienze della Sanità Pubblica e Pediatriche

September 10th 2014

To Guest Editors,

Vassilios Fanos, Michele Mussap and Maurizio Ferrari

Dear Professors,

We are hereby submitting an original paper entitled "**Probiotics and gut health in infants: a case-control observational study about early treatment with Lactobacillus reuteri DSM 17938**" by Savino F. et al. for publication in special issue of Clinica Chimica Acta Journal: "**Neonatal Laboratory Medicine: the past and future decade**".

Our trial underlines the efficacy of an early probiotic administration in improving gut health in infancy.

Informed consent was obtained from each child's parent.

The study has been approved by a research ethics committee.

There are no prior publications or submissions with any overlapping information, including studies and patients.

These data have not been published previously and are not under consideration for publication by any other journal. The manuscript has not been and will not be submitted to any other journal while it is under consideration by *Clinica Chimica Acta Journal.*

All authors have read and approved the version of the article being submitted.

No an honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

All authors declare no conflict of interest.

No affiliations with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials of the research discussed in the manuscript were present.

Looking forward to hearing from you,

Your sincerely,

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***Highlights (for review)**

- We evaluated the effect of early administration of probiotic on infants'gut microflora
- Infants previously treated show differences in gut microbial composition and richness
- Early administration of L.reuteri improves gut health reducing pathogens colonization
- Improve our knowledge of gut microbiome could be useful for promoting infants' health

# Probiotics and gut health in infants: a case-control observational study about early treatment with *Lactobacillus reuteri* DSM 17938.

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**Keywords:** Infants, formula fed, gut microbiota; microbial pathogens; *Lactobacillus reuteri* DSM 17938; probiotic

**Abbreviations:**

AAD - antibiotic-associated diarrhea

CFU- colony forming units

NEC - necrotizing enterocolitis

PCR - polymerase chain reaction

DEC - diarrheagenic *E. coli*

EPEC - enteropathogenic E.coli

LAB - lactic acid bacteria

## 1 .INTRODUCTION

The human gastrointestinal tract represents one of the most densely populated microbial ecosystems detected to date. Although this microbial group has been recognized to have a crucial impact on human health, its precise composition in the first period of life is still subject to intense investigation. Before birth, the gastrointestinal tract of the fetus is sterile, and only during the initiation of the delivery process and thereafter, acquisition of microbes is ongoing and time-dependent [1, 2].

Initial neonatal gut colonization is a crucial stage for developing a healthy physiology, and is beneficially influenced by breast-feeding [3]. The colonization, development and maturation of the newborn gastrointestinal tract that begin immediately at birth and continue for two years, are modulated by numerous factors including mode of delivery, feeding regime, maternal diet/weight, probiotic and prebiotic use and antibiotic exposure [4, 5].

Many other factors affect the composition of the microbiota in the intestinal tract before getting a stable population as in the human adult [6]. Early colonization with beneficial bacteria could help establish an effective ecosystem and bring about maturity in the physical structure of the gut [7].

Probiotic administration during the neonatal period can improve gut function by improving the intestinal immune status and maintaining microbial balance during gastrointestinal disturbances [7, 8]. Although progress has been made in disclosing the mechanisms through which probiotics act on the intestinal immunity and microbial composition of clinical models, knowledge about the interaction between probiotics and intestinal microbial metabolism, as well as microbial composition in healthy infants, is lacking [4, 8]. Conventional studies using culture techniques revealed that, compared with adults, the early gut microbiota of infants has a lower proportion of anaerobic bacteria (especially *Bacteroides* and *Clostridium*) [2]. The sterile neonatal gut is colonized first by facultative anaerobic bacteria (*Enterobacteriaceae*, *Enterococcus* and *Streptococcus*) due to the abundance of oxygen. After about 1 week, the increase of these facultative anaerobic bacteria creates a reduced environment favorable for the establishment of anaerobic genera (bifidobacteria, *Bacteroides* and *Clostridium*). With time, a large number of anaerobic species will colonize the infant gut and multiply [1- 4]. Increasing evidence suggests that this process is a critical step for future child's health and it may be influenced by environmental, maternal and dietary factors [5, 6].

Probiotics are live microorganisms that, when ingested in adequate amounts, could supply beneficial intestinal bacteria: many recent clinical trials suggest that this temporary colonization may provide health benefit [7 – 9]. Recent researches have demonstrated that probiotics can bring a direct action on gut microbial composition, influencing clinical outcomes in pediatric

gastroenterology. These diseases include necrotizing enterocolitis (NEC), antibiotic-associated diarrhea (AAD) and colitis, infantile colic, acute gastroenteritis and irritable bowel syndrome [10 – 13].

As far as skin and airways are concerned, the possible actions of probiotics need to be further investigated and clarified to better understand if some strains may improve conditions such as asthma and atopic dermatitis [14 – 16].

Among the gastrointestinal microbiota, bifidobacteria represent an important commensal group, being among the first microbial colonizers of the gut [17]. However, the prevalence and diversity of members of the others genus in the infant intestinal microbiota has not yet been fully characterized, while some inconsistencies exist in literature regarding the gut health after probiotic administration [9 – 13].

The present study was conducted to evaluate the effects of early administration of *Lactobacillus reuteri* DSM 17938 on microbial composition in the gastrointestinal tract of infants using a case-control observational study of subjects admitted at hospital during early infancy.

## *2.* MATERIALS AND METHODS*:*

### 2.1 Selection of Subjects and study design

From January 2013 to March 2014, 1241 infants were assessed for eligibility. 1181 infants were excluded because they did not fit the inclusion criteria (n=1146), parents refused to participate (n=25) or the amount of fecal sample was not sufficient (n=10) **(Figure 1)**

The 60 eligible patients were divided into two groups (30 cases and 30 controls). 30 infants, who received *L.reuteri* DSM 17938 in the previous month ($10^8$ cfu/day administered in 5 drops, once a day, 30 minutes before the feed in the morning), were consecutively recruited in the treatment group, while 30 infants who never received *L.reuteri* were inserted in the control group. A pediatrician interviewed parents to detect the assumption of probiotics.

The control group subjects were matched to cases (1:1) by age, month of entry by a pediatrician and type of feeding. To prevent variability in the intestinal microbiota caused by diet, all infants enrolled were exclusively standard formula fed and none of them has even started weaning.

The infants recruited had an average age of 91 ± 58 days in case group and 94 ± 53 days in control one. The population was divided according to sex, kind of delivery and nationality as shown in **Table 1**.

Involved infants were hospitalized at our Department of Pediatrics (Regina Margherita Children Hospital, Turin, Italy). They were admitted to the hospital for various diseases: congenital hypothyroidism, bronchiolitis, epilepsy, urinary tract infections, gastroesophageal reflux disease,

mononucleosis, herpes stomatitis, pneumonia, ALTE (Apparent Life-Threatening Events), poor growth. The following inclusion criteria were used for the selection of subjects:

- absence of gastroenteritis;
- no antibiotic therapy in the week before the hospitalization and until the time of sample collection;
- APGAR score at 5 minutes after birth greater than 8;
- absence of diseases due to perinatal hypoxia;
- age < 6 months of life.
- exclusively standard formula fed.

The parents of the infants involved in the study were informed about the purpose of the research work and their written informed consent was obtained. The local ethics committee (Comitato Interaziendale AA.SS.OO approved the study. O.I.R.M. /S. Anna-Ordine Mauriziano di Torino) before the start.

The necessary data on patients enrolled in the study were retrieved from medical records of patients and placed in a special enrollment cards. If data were missing, an interview was conducted with the parents of the patient. The following data were recorded:

• general personal data: name and last name, date and place of birth, date of enlistment;

• information on parents': ages and nationalities, the number and outcome of any other pregnancies;

• reason for admission;

• therapy in action and possible therapy in the week before hospitalization (with particular attention to the administration of antibiotics and / or probiotics);

• history physiological: sex, current age, gestational age, mode of delivery, course of pregnancy , conditions at birth, birth weight and recruitment, type of nursing or any kind of complementary feeding , APGAR on the first and the fifth minute;

• medical history: presence / absence of fever and general condition;

• microbiological examinations carried out.

**2.2 Sample Collection**

Infants' health status was recorded each afternoon and the occurrence of diarrhea was visually assessed [18].

Fecal samples were collected into a sterile Eppendorf tube under aerobic and anaerobic conditions and immediately transported to the microbiological laboratory of the Department of Public Health and Pediatric of University of Turin at +2÷6° C in a cooler for analysis. One gram of each sample was weighted and diluted 1:10 with normal saline solution (0.9% sodium chloride). After serial

dilutions in normal saline, 100 µl of each dilution were streaked on selective and differential media for bacteria isolation and identification.

### 2.3 Microbiological analysis

Total aerobic count on Brain Heart Infusion Agar (BHA; Biolife, Milan, Italy) was performed; Azide Maltose Agar (KF; Biolife, Milan, Italy) and Mac Conkey Agar (MC; Biolife, Milan, Italy) to detect enterococci and *Enterobacteriaceae*, respectively, were used. All plates were incubated at 37°C for 24 h under aerobic conditions. The lactobacilli and bifidobacteria strains were obtained from Rogosa Bios Agar (RG; Biolife, Milan, Italy) after incubation at 37° C under microaerophilic and anaerobic conditions for 3 days, respectively. Schaedler CNA Agar with 5% Sheep Blood (SCNA, Becton Dickinson, NJ, USA) and Schaedler Kanamycin-Vancomycin Agar with 5% Sheep Blood (SKV, Becton Dickinson, NJ, USA) were used to evaluate total anaerobic gram-positive and gram-negative counts, respectively. All plates were incubated at 37°C for 3-7 days under anaerobic conditions [18]. *E. coli* strains were isolated using Tryptone Bile X-Gluc Agar (TBX, Biolife S.r.l., Milan Italy). *Cronobacter sakazakii* strains were detect using selective enrichment medium Enterobacter Sakazakii Selective Broth (ESSB, Biolife S.r.l., Milan Italy) and Enterobacter Sakazakii Isolation Agar plates (ESIA, Biolife S.r.l., Milan Italy). All plates were incubated at 37°C for 24 h under aerobic conditions.

The microbial counts were recorded as colony forming units per gram (cfu/g) of sample. The qualitative analysis was performed by biochemical methods (API System, Biomérieux, Rome, Italy).

### 2.4 DNA Isolation and PCR

Genomic DNA from positive *E. coli* and *C. sakazakii* colonies was extracted with NucleoSpin Tissue method genomic DNA kit (MACHEREY-NAGEL GmbH & Co. KG., Düren, Germany).

One µl of DNA was processed by multiplex PCR assay. For *E.coli lt* and *st* (ETEC), *eaeA* and *bfpA* (EPEC), $stx_1$ and $stx_2$ (EHEC), *ial* (EIEC) genes was identified (**Table 2**) [19]. The reaction mixture was obtained with 12, 5µl PCR Master Mix (2X) (Fermentas, Waltham, MA USA), 1µM (each) primer, 1µl template DNA and water nuclease-free to 25µl. The thermocycle program was set up as follows: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min of annealing (ranging from 55°C to 58°C), 1 min at 72°C; 7 min at 72°C after the final cycle before cooling at 4°C.

Primers ESSF and ESSR were designed to amplify a 469-bp fragment of the *ompA* gene specific to *C. sakazakii* (The sequences of PCR primer for target genes are reported in Table 2) [19]. PCR was performed in a 50µl reaction mixture containing 25µl PCR Master Mix (2X) (Fermentas, Waltham,

MA USA), 1μM (each) primer, 50 ng of template DNA and water nuclease-free to 50μl. The samples were subjected to PCR with a program that consisted of denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min [20].

The amplification products were electrophoresed through a 2% agarose gel and visualized with UV transilluminator after ethidium bromide staining.

Positive controls for PCR were *E. coli* ATCC 35401 (ETEC $st^+/lt^+$), *E. coli* ATCC 43887 (EPEC $bfpA^+/eaeA^+$), *E. coli* ATCC 35150 (EHEC $stx1^+/stx2^+/eaeA^+$), *E. coli* ATCC 43893 (EIEC $ial^+$) and *C. sakazakii* ATCC 51329. *E. coli* ATCC 25922 was used as negative control.

### 3. Statistical Analysis

To describe the characteristics of the studied samples, the main information was shown using classical descriptive indicators. Data are shown as mean ± SD for continuous variables and as number and percentage for categorical variables. Differences between groups were evaluated by Student's t test for independent samples and associations between categorical variables were evaluated by Yates' chi-squared test. All reported p values are 2-sided, and differences were considered to be significant when $p \leq 0.05$. All statistical calculations were performed with commercially available software (SPSS for Windows version 21.0; SPSS Inc., Chicago, IL, USA).

### 4. RESULTS

From January 2013 to March 2014, 60 infants were enrolled (30 previously treated with probiotics and 30 which received no probiotic supplementation) and their stools were collected and analyzed.

Participants' characteristics are reported in **Table 1**: the two groups do not differ for age, gender and type of delivery.

We find that the two groups showed differences in gut microbial strains and richness. No differences were observed for total aerobic counts (p=0.49). Infant treated with probiotic showed a lower total anaerobic gram-negative counts (p=0.03) and a higher total anaerobic gram-positive counts (p=0.02). The faecal counts of *Enterobacteriaceae* and enterococci were found to be significantly higher in the control group compared with the treated group (p=0.04). No differences were observed for lactobacilli and bifidobacteria. Medians of microbial species amount were calculated and reported in **Table 3**.

Among *Enterobacteriaceae*, *E.coli* was isolated in 23 of 60 stool samples analyzed (38.3%), 16 of these belonging to the control group (26.7%) and 7 in treated infants' samples (11.7%) with a statistically significant difference (p=0.04). All *E.coli* strains were processed by multiplex PCR for

the detection of virulence genes (data not shown). Four diarrheagenic *E. coli* (DEC) were observed in samples belonging to the control group (13.3%) and none in the other group. Four pathogen *E. coli* identified was atypical enteropathogenic *E.coli* (a-EPEC) (only *eaeA* gene). *C. sakazaki* and *Salmonella spp.* were found only in one and two respectively asymptomatic infants recruited in the control group (3.3% and 6.7%). *C. sakazakii* positive colonies were confirmed by PCR. Amplification of the 469-bp *ompA* product in this sample was observed.

In the 60 stool samples analyzed, other potentially opportunistic pathogenic *Enterobacteriaceae* were isolated as follows: 21 *Hafnia alvei*, 9 *Klebsiella oxytoca*, 6 *K.pneumoniae*, 10 *Enterobacter aerogenes*, 7 *E. cloacae*, 3 *Serratia odorifera*. These microorganisms were distributed differently in the two groups with a significantly higher rate in no probiotic treated infants. Details about pathogens and opportunistic bacteria are reported in **Table 4**.

## *5.* DISCUSSION

Improve our knowledge of intestinal microbiome could be useful for better understanding the roles of the microbial strains in promoting infants' health [21, 22].

Although data on gut health in infants are scanty [12, 13, 15, 16], many recent clinical trials suggest that probiotic administration in early infancy could be useful for the prevention and treatment of many disorders: modulation of the intestinal microbiota through use of probiotics has been suggested as strategy for preventing NEC, atopic eczema and managing infantile colic symptoms. Early administration of probiotics (mainly *L. reuteri* DSM 17938, *Lactobacillus* GG and *Saccharomyces boulardii*) could modulate the composition of the gut microbiota, improving beneficial flora and reducing potential pathogens [23].

This could have a significant long-time impact; however, studies with adequate follow up are needed.

The timing to start the administration of probiotics may be important to maximize their effectiveness.

Recent studies involving the use of culture-independent techniques have shown that *Bifidobacterium* is only a minor component of the infant gut microbiota [17]. These techniques have also introduced the concept of a core microbiome in which metabolic function is more important than the presence of a particular bacterial strain. A less various gut microbiota with high counts of *Bacteroides*, *Clostridium*, *Enterobacteriaceae* and *Staphylococcus* in early infancy has been associated with an increased risk for atopic disease [24]. Changes in infant's gut colonization are also related to a higher incidence of obesity during childhood [25].

In this study, we have analyzed fecal samples of 30 hospitalized infants, which previously received probiotics, and 30 not receiving probiotics. We find that the two groups showed differences in gut microbial strains and richness.

In the study, no difference was observed for total aerobic counts and for lactobacilli and bifidobacteria; conversely, a significant increase of total anaerobic gram-positive counts in the treated group compared with the control group was detected. These data suggested that the abundance of bifidobacteria and lactobacilli is in the typical range reported for infant fecal samples [26], while the increase of anaerobic gram-positive in the treated group could be due to the use of probiotic therapy. Moreover, the total gram-negative anaerobic bacterial count (which included *Bacteroides spp*.) in control group was moderately higher than that of the probiotic group. Studies demonstrated that growth of gram negative bacilli produces an increased susceptibility to necrotizing enterocolitis and sepsis; hence, the decrease of anaerobic gram-negative bacilli load in the probiotic group suggested a benefit of probiotic therapy in preventing NEC [27].

In the current study, *L. reuteri* probiotic assumption showed significant antibacterial activity against pathogens and opportunistic bacteria such as DEC, *Salmonella spp.*, *C. sakazakii*, *H. alvei*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*. In fact, under anaerobic growth conditions, *L. reuteri* produces a potent antimicrobial compound called reuterin, which is a β-hydroxypropionaldehyde derivative of glycerol [28].

Our results are in agreement with others who reported the antimicrobial activity of human LAB isolates including *L. casei*, *L. acidophilus*, *L. johnsonii*, *L. reuteri*, *L. rhamnosus*, and *Bifidobacterium spp*. against a wide range of foodborne pathogens [29, 30]

In the present study, atypical EPEC was the only DEC category identified and isolated exclusively in the infants group who did not assume probiotics. Indeed, atypical EPEC is now predominant in industrial countries, while typical EPEC is more frequently isolated in developing areas [31]. This microorganism is associated with infantile diarrhoea, and in comparison with other pathtypes of diarrheagenic *E. coli*, it is isolated more frequently in infants less than one-year-old. The decrease of the infections caused by this pathogenic strain in older children could be due to acquisition of immunity or loss of specific receptors in the gut mucosa with increasing age.

The percentage of EPEC found in asymptomatic infants is comparable to other studies [31, 32].

In this work *C sakazakii* was isolated only in one asymptomatic infant recruited in the control group in agreement with others studies where this microorganism caused asymptomatic infections [33]. It is considered an emerging pathogen, responsible of cases of neonatal infections (sepsis, meningitis, necrotizing enterocolitis) related to use of contaminated infant milk formula, which can represent both the vehicle and the source of contamination. Powered infant formula is a non-sterile product

which can be, once rehydrated, a good medium for microorganisms. *C. sakazakii* may cause infections in all age groups and particularly in subjects from zero to 12 months. The main source of diffusion and transmission to sensitive sites is the intestine; factors contributing to increase the risk of infection include patient's susceptibility, level of contamination of food, tolerance to temperature, speed of growth, infectious dose and the virulence of the microorganism [33].

*Salmonella spp*. was isolated in two asymptomatic infants belonging to the control group. In humans, salmonellosis varies from a self-limiting gastroenteritis to septicemia. Whether the microorganism remains in the intestine or disseminates depends on host factors as well the virulence of the strain. Some people may be asymptomatic carriers of *Salmonella spp.* [34]

This research is affected by some limitations: first, in an observational study the investigator has no control over the composition of the control groups, and cannot randomize the allocation of subjects. This could create bias, and could also mask cause and effect relationships or, alternatively, suggest correlations where there are none (errors in research). Second, new methods such as molecular identification could help us to make an assessment not only quantitative but also qualitative.

## 6 . CONCLUSIONS

In conclusion, one of the important functions of probiotics is their ability to inhibit the growth of different types of foodborne pathogens [30]; our study indicated that early administration of *L. reuteri* DSM 17938 in infancy could influence gut microbiota composition, improving gut health by reducing pathogens colonization.

Further studies are needed to better understand how individual differences could act in the interaction between gut health and microbiota. Evaluating gut microbiota changes in addition to clinical outcomes in future studies concerning *L. reuteri* DSM 17938 effects should be helpful in understand how this strain influences gut health.

## 7. ACKNOWLEDGEMENTS

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**Table 1. Characteristics of participants of the infants enrolled in the study.**

| Variable | Cases, n (%) n= 30 (50.0) | Controls, n (%) n= 30 (50.0) | p value |
|---|---|---|---|
| Mean age ± s.d. (days) | 91 ± 58 | 94 ± 53 | 0.66[¶] |
| Males/females, n (%) | 17/13 (56.7/43.3) | 20/10 (66.7/33.3) | 0.60[#] |
| Delivery (caesarean/spontaneous), n (%) | 16/14 (53.3./46.7) | 20/10 (66.7/33.3) | 0.43[#] |
| Nationality | | | |
| Italian, n (%) | 15 (50.0) | 19 (63.3) | 0.43[#] |
| Foreign, n (%) | 11 (36.7) | 9 (30.0) | 0.78[#] |
| Mixed, n (%) | 4 (13.3) | 2 (6.7) | 0.67[#] |

Statistical analysis: [#]Yates' chi-squared test, [¶]Student's t-test

**Table 2. The sequences of PCR primer for target genes: details of nucleotide sequence for each primer pair**

| TARGET GENE | OLIGONUCLEOTIDE SEQUENCE (5' to 3') | AMPLICON SIZE (bp) | REFERENCE |
|---|---|---|---|
| *elt*[a] | F,TCTCTATGTGCATACGGAGC<br>R,CCATACTGATTGCCGCAAT | 322 | Rappelli et al. (2001) |
| *sta*[a] | F,TCTTTCCCCTCTTTTAGTCAGTC<br>R,CCAGCACAGGCAGGATTAC | 170 | Rappelli et al. (2001) |
| *eaeA*[a] | F,TGATAAGCTGCAGTCGAATCC<br>R,CTGAACAGATCGTAACGGC | 229 | Rappelli et al. (2001) |
| *bfpA*[a] | F,CACCGTTACCGCAGGTGTGA<br>R,GTTGCCGCTTCAGCAGGAGT | 450 | Rappelli et al. (2001) |
| *stx*$_1$[a] | F,GAAGAGTCCGTGGGATTACG<br>R,AGCGATGCAGCTATTAATAA | 130 | Rappelli et al. (2001) |
| *stx*$_2$[a] | F,GGGTACTGTGCCTGTTACTGG<br>R,GCTCTGGATGCATCTCTGGT | 510 | Rappelli et al. (2001) |
| *ompA*[b] | GGATTTAACCGTGAACTTTTCC<br>CGCCAGCGATGTTAGAAGA | 469 | Mohan Nair et al. (2006) |

[a] gene for *E.coli*; [b] gene sequence for *C. sakazakii*

**Table 3. Comparison of gut microbial counts detected in subjects who received probiotics versus those did not receive**

| Microrganisms | Probiotic therapy (cases group) | No probiotic therapy (controls group) | p-value |
|---|---|---|---|
| Total aerobic counts | 1.9E+09 | 1.7E+09 | *p= 0.49* |
| Total anaerobic gram-negative counts | 2.5E+06 | 5.3E+06 | *p = 0.03* |
| Total anaerobic gram-positive counts | 2.8E+07 | 6.5E+06 | *p = 0.02* |
| *Enterobacteriaceae* | 6.9E+08 | 1.8E+09 | *p = 0.04* |
| Enterococci | 6.4E+08 | 1.8E+09 | *p = 0.04* |
| Lactobacilli | 3.8E+09 | 2.9E+09 | *p= 0.67* |
| Bifidobacteria | 5.9E+09 | 1.3E+09 | *p = 0.33* |

**Table. 4 Pathogens and opportunistic pathogens isolated**

| **Bacterial species** | **Probiotics treatment n (%) n=30(50.0)** | **No probiotics treatment n (%) n=30 (50.0)** | **OR (95%*CI)** | **p value** |
|---|---|---|---|---|
| Diarrheagenic *E. coli* (a-EPEC*)* | 0 (0.0) | 4 (13.3) | NA | NA |
| *Salmonella spp.* | 0 (0.0) | 2 (6.7) | NA | NA |
| *Cronobacter sakazakii* | 0 (0.0) | 1 (3.3) | NA | NA |
| *Hafnia alvei* | 4 (13.3) | 17 (70.0) | 8.2 (2.10 - 40.39[1]) | 0.001 |
| *Klebsiella oxytoca* | 1 (3.3) | 8 (26.7) | 10.2 (1.22 - 483.7[1]) | 0.03 |
| *Klebsiella pneumoniae* | 1 (3.3) | 5 (16.7) | 6.7 (0.58 - 283.6[1]) | 0.20 |
| *Enterobacter aerogenes* | 2 (6.7) | 8 (26.7) | 5.0 (0.87 - 52.6[1]) | 0.08 |
| *Enterobacter cloacae* | 1 (3.3) | 6 (20.0) | 7.0 (0.77 - 344.7[1]) | 0.11 |
| *Serratia odorifera* | 0 (0.0) | 3 (10.0) | NA | NA |

*CI, Confidence interval; NA, not applicable; [1] 95% confidence interval

**Figure(s)**

**Figure 1 Diagram showing the flow of participants through each stage of the study.**