**Methods:**

***Blood testing:***

*Complete blood count (CBC)* was obtained for each subject at the day of enrollment and weekly for two weeks, resulting in three samples per subject. CBC was assayed using Sysmex KX-21N cell counter and its reagents (Kobe, Japan).

*CD4 and CD8 cytokines in serum* were measured at day of enrollment and weekly for two weeks. Two mls of blood were collected and allowed to clot at room temperature for 10-20 minutes and then centrifuged for 20 minutes at 2000-3000 RPM to collect serum. Sera were stored at -20oC until processing. ELISA was used to assess the concentrations of CD4 and CD8 in samples (Human cluster of differentiation (CD4 and CD8) ELISA Kits, San Diego, California, USA). Samples with unknown amounts of CD4 or CD8 were added to monoclonal antibody enzyme wells, which were pre-coated with a CD4 or CD8 monoclonal antibody and incubated. Then CD4 and CD8 antibodies with biotin were added and combined with Strepavidin-HRP to form immune complexes. The samples were washed to remove uncombined enzymes. Chromogen solutions A and B were added. The resulting chroma or color intensity positively correlated to the concentration of CD4 and CD8. Using the concentration of the standard solution and corresponding optical density (OD) values, a standard curve linear regression equation was calculated. Then, the regression equation was used with the OD values of samples to calculate the corresponding sample’s concentration.

***Stool Samples***

Stool samples were collected at the day of enrollment and weekly for two weeks to isolate and identify bacteria. There were 3 samples collected per subject. Fresh fecal samples were collected directly from the subject’s diaper in two sterile containers. One container was sent to microbiology lab for cultures. The second container was frozen at -80oC to be used for the molecular detection of *Bifidobacterium bifidum* and *Lactobacilus spp.*

**Isolation and identification of bacteria:**

Pour plate technique was used to isolate the organisms1. One gram of the feces was inoculated into 9 ml of thioglycolate (Oxoid, United Kingdom) broth, shaken and homogenized. Then, samples were serially diluted ten folds. One ml aliquot of each sample and its dilutions were plated into Man Rogosa Sharpe (MRS) agar (MRS; Oxoid, UK) for isolation of *lactobacilli.* The MRS-Cys agar plates were supplemented with 0.05% L-cysteine hydrochloride and 50 μg mupirocin (Delchimica, Italy) per liter of MRS for isolation of *bifidobacteria*2*.* Mannitol salt agar (Oxoid, UK) was used for isolation of staphylococci3and MacConkey agar (Oxoid, UK) was used for isolation of Enterobacteriaceae4.

Inoculated MRS and MRS-Cys plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C for 48-72 h in an anaerobic jar using Oxoid anaerogen compact gas packs (Oxoid, UK)1. Inoculated mannitol and MacConkey agar plates were incubated aerobically at 37°C for 24 h. The approximate number of colonies of the different genera was counted and the bacterial burden was expressed as colony forming unit CFU/gm stool5.

Colonies of different morphologies and sizes growing on MRS and MRS-Cys agar were chosen6 and transferred to MRS and MRS-Cys broth for incubation for 24 to 48 h anaerobically. Bacterial isolates were characterized on the basis of their morphology, microscopic appearance after gram staining and catalase reaction.

**Gram Staining:**

Fresh cultures were transferred aseptically into 1.5 ml Eppendorf tubes and centrifuged for 5 min at 6000 rpm. The supernatant was then removed and cells were re-suspended in sterile distilled water that was followed by application of the Gram staining procedure7.

**Slide Catalase Test:**

This test was performed to confirm that lactic acid producing bacteria was catalase negative8. Isolates growing on either mannitol salt agar or MacConkey agar were identified as members of the genus *Staphylococcu*s or *Enterobacteriaceae* respectively by conventional methods9.

**Molecular Detection of *Bifidobacterium bifidum and Lactobacillus spp:***

Quantitative real-time PCR for *Bifidobacterium bifidum* and *Lactobacillus spp* DNA was performed at the National Research Center as follows:

*DNA Extraction:* QIAamp® DNA Stool Mini Kit for DNA purification from stool samples (QIAGEN Group, Germany) was used for DNA extraction from the stool samples. Stool samples were lysed in buffer ASL. Then, DNA-damaging substances and PCR inhibitors in the stool sample were adsorbed to InhibitEX matrix. After inhibitors and DNA-degrading substances had been adsorbed to InhibitEX matrix, it was pelleted by centrifugation and the DNA in the supernatant was purified on QIAamp mini spin columns. Proteins were digested and degraded during 70°C incubation with proteinase K. Buffering conditions were then adjusted to allow optimal binding of DNA to the QIAamp membrane, and the samples were loaded onto the QIAamp spin column. DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation step. DNA bound to the QIAamp membrane was washed in two centrifugation steps to remove any residual impurities without affecting DNA binding. Then, purified DNA was kept at –20°C for further testing.
 On the day of the test, specimens were allowed to thaw at room temperature. Stool samples (200 mg each) were placed in a micro- centrifuge tube and 2 ml Buffer ASL was added to each tube. This was vortexed continuously until the stool sample was thoroughly homogenized. Then, 1.6 ml of the stool lysate was pipetted into a 2 ml micro-centrifuge tube. The suspension was heated for 5 min at 95°C, then vortexed for 15s. The sample was centrifuged at full speed for 1 min to pellet stool particles. The lysis temperature were increased to 95°C for cells that were difficult to lyse such as Gram-positive bacteria. Then, 1.2 ml of the supernatant were pipetted into a new 2 ml micro-centrifuge tube. One InhibitEX tablet was added to each sample and vortexed immediately and continuously until the tablet was completely suspended. The suspension was incubated for a minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. Samples were centrifuged at full speed for 3 min to pellet stool particles and inhibitors bound to InhibitEX matrix. All of the supernatant was pipetted into a new 1.5 ml micro- centrifuge tube and the pellet was discarded. Samples were then centrifuged at full speed for 3 min; 15 μl proteinase K was pipetted into a new 1.5 ml micro-centrifuge tube and 200 μl of the supernatant was added into the 1.5 ml micro-centrifuge tube containing proteinase K. 200 μl buffer AL were added and vortexed for 15 seconds. Then it was incubated at 70°C for 10 min. 200 μl of ethanol (96–100%) was added to the lysate, and mixed by vortexing. The complete lysate was applied to the QIAamp spin column and centrifuged at full speed for 1 min. The QIAamp spin column was carefully opened and 500μl buffer AW1 was added without wetting the rim. The cap was closed and the tube containing the spin column was centrifuged at full speed for 1 minute. The QIAamp spin column was placed in a new 2ml collection tube and the collection tube with filtrate was discarded. The QIAamp spin column was carefully opened again and 500 μl buffer AW2 was added. The tube containing the spin column was centrifuged at full speed for 3 minutes. This step was performed to eliminate any chance of possible buffer AW2 carryover, as residual buffer AW2 in the eluate may cause problems in downstream applications. The QIAamp spin column was placed in a clean 1.5 ml micro-centrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp spin column was opened carefully and 200μl buffer AE was added directly onto the QIAamp membrane and incubated at room temperature for 1 minute. The micro-centrifuge tube containing the QIAamp spin column was then centrifuged at 8000 rpm for 1 minute. DNA concentration was measured in each DNA elute tube using spectrophotometer (Nano drop 2000). The eluted DNA was placed at -20°C until PCR testing was performed.
**Quantitative Real Time PCR**

PCR was done via the Applied Biosystem StepOne instrument (Applied Biosystems, Foster City, CA, USA). First, the PrimerDesignTM (Genesig, Chandler’s Ford, UK) kit for bifidobacterium was used. The kit protocol was followed and detected *B. bifidum* via the FAM channel. A positive control template was used to generate a standard curve of *B. bifidum* copy number and CT value. If a negative result was obtained, the test results were deemed invalid and the rest was repeated. To confirm the absence of contamination, a negative control reaction was used with each kit. The same protocol was repeated for *Lactobacillus spp.* using the PrimerDesignTM (Genesig, Chandler’s Ford, UK) kit.

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