Intestinal microbiota in neonates requiring urgent surgery: assessing the role of probiotics using fecal DNA sequencing

TITLE PAGE

Title:

Intestinal microbiota in neonates requiring urgent surgery: assessing the role of probiotics using fecal DNA sequencing.

Running Title:

Analysis of intestinal microbiota in infants

Authors:

Hiroshi Murakami[1*], Yumi Shimomura[2*], Mitsuharu Matsumoto[2], Geoffrey J Lane[1], Atsuyuki Yamataka[1], Manabu Okawada[1]

Institutions:
[1]Department of Pediatric General & Urogenital Surgery
Juntendo University School of Medicine, Tokyo, Japan
[2]Research Laboratories, Kyodo Milk Industry Co., Ltd

* These authors contributed equally to this work.

Correspondence and address reprint request to:
Manabu Okawada
Department of Pediatric General & Urogenital Surgery
Juntendo University School of Medicine
2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel: +81-3-3813-3111
Fax: +81-3-5802-2033
E-mail: manabu-o@juntendo.ac.jp

ABSTRACT

Purpose: To assess the impact of urgent surgery on neonates and the value of an orally administered probiotic preparation of *Bifidobacterium animalis* subsp. lactis LKM512 (LKM) using fecal DNA sequencing to analyze intestinal microbiota.

Methods: Subjects for this study were 13 neonates born at our institution. Surgical cases required surgery within 3 days of birth. Groups studied were surgical cases administered LKM (n=4; LKM+), surgical cases not administered have surgery and were not administered LKM (n=2; CS), and normal healthy neonates (n=3; CN). Stool specimens (20mg) were collected 5 times (after birth, and on days 3, 7, 10, and 14 after surgery in surgical cases, and after birth, and on days 4, 8, 11, and 15 of life in controls).

Results: Clinical data were similar for LKM+ and LKM-. *Enterobacteriaceae*, *Streptococcaceae*, *Staphylococcaceae and Bifidobacteriaceae* were identified in descending order of abundance in CS stool. *Streptococcaceae*, *Staphylococcaceae*, *Enterococcaceae and Bifidobacteriaceae* were identified in

descending order of abundance in LKM+ stool. *Bifidobacteriaceae*, *Enterobacteriaceae*, *Staphylococcaceae* and *Streptococcaceae* were identified in descending order of abundance in LKM- stool. Unexpectedly, *Bifidobacteriaceae* was significantly more abundant in LKM- than LKM+ ($p < 0.05$).

Conclusion: Surgical stress appears to affect intestinal microbiota considerably. Probiotic administration in neonates requires clarification.

Key words: intestinal microbiota in infants, next-generation DNA sequencing

INTRODUCTION

It is well known that following birth, the intestinal microbiota undergoes rapid development as a result of a combination of internal and external factors affecting nutrition and immune functions of the neonatal gastrointestinal system. Early exposure to environmental microbial components, even potentially pathogenic microorganisms, is suggested to play an important role in the maturation process and dramatically reduce the incidence of inflammatory, autoimmune, and atopic diseases, with the gastrointestinal tract being the major site of interaction between the host immune system and external factors [1]. Thus, microbial colonization plays an important role in regulating and fine-tuning the immune system throughout life.

The most dramatic changes in intestinal microbiota occur early in life [2] and can even influence the normal functional development of the adult immune system. Therefore, failure or delay in development of the intestinal microbiota may be associated with health problems. In term neonates during the first days of life, the most abundant colonizers are *staphylococci*, *γ-proteobacteria,* and

bifidobacteria [3]. Previous studies on gut microbiota in preterm infants report that *Enterobacteriaceae* dominate with decreased numbers of *lactobacilli* and *bifidobacteria* [4, 5]. Research on the etiology of necrotizing enterocolitis (NEC) has shown that prematurity is associated with a delay in the establishment of *bifidobacteria*, which may predispose the premature intestine to NEC [6].

Probiotic therapy has been proposed as an effective way to enhance the intestinal environment during times of stress but there are few reports about probiotic use in children [1, 7]. Therefore, the use of a beneficial blend of pre, pro and synbiotics may balance intestinal microbiota and help prevent morbidity or assist the body overcome physical stresses [1].

Recently, there have been a number of studies on microbiota using 16S rRNA gene specific sequencing [8] and the broad understanding of the role of commensal microbiota in health and disease is improving as a result of recent advances in genome sequencing technologies and metagenomic analysis [3,9]. These reports, however, were limited to either premature infants or healthy

babies, with no studies concerning the development of gut microbiota in neonates requiring urgent surgery (i.e., within 3 days of birth), so the value and effectiveness of probiotic administration in neonates is unknown. Gut microbiota in neonates requiring urgent surgery is affected by many factors, such as surgical stress, inflammation, intravenous nutrition, and antibiotics and it is generally believed that postoperative stool production in these neonates is limited because of limited or no oral feeding making it difficult to study gut microbiota in neonates undergoing surgery and by inference the effectiveness of probiotic administration.

The purpose of this study was to conduct a comprehensive investigation of the intestinal microbiota of neonates undergoing surgery by using a next-generation DNA sequencing system and assess the value of probiotic administration.

METHODS

Subjects

The subjects for this study were 13 neonates born at our institution. Eight

required surgery within 3 days of birth, and were termed surgical babies (SB; n=8; 5 males, 3 females), 2 were suspected of having indications for surgery on prenatal diagnosis but did not actually have surgery (CS; n=2; 2 males), and 3 were healthy normal neonates (CN; n=3; 3 females). CS were recruited from the Neonatal Intensive Care Unit at Juntendo Hospital, Tokyo, Japan (Table 1) and CN were recruited from the maternity ward at Juntendo Hospital. Fecal samples were collected from the 13 subjects between April 2014 and March 2015 (Table 1). The relative abundance of bacteria according to taxonomic families was compared between groups.

Table 1

Probiotic preparation LKM

The probiotic used was a 1g preparation of *Bifidobacterium animalis* subsp. *lactis* LKM512 (LKM) for oral administration. The LKM preparation used in this study to improve the intestinal environment which produce polyamine that is the key factor [8,10,11].

Study design for surgical babies

Indications for surgery in SB are shown in Table 1. All SB were fed a blend of

Table 1

infant formula and breast milk when allowed to have oral intake. For this study, SB were allocated randomly to 2 groups according to LKM use; LKM+ (n=4) or LKM- (n=4). LKM+ received LKM 1.0g per day orally (containing approximately 6×10^9 colony-forming units of *B. animalis* subsp. *lactis*) when oral feeding was allowed.

All SB were prescribed ampicillin (ABPC) as a postoperative antibiotic according to our standard protocol for postoperative management [12]. For SB, a blood specimen for measuring white blood cell count (WBC), C-reactive protein (CRP), total protein (TP), and albumin (Alb) and a fecal specimen (20mg) were collected in the neonatal intensive care unit (NICU) between 24 and 48 hours of life and on postoperative days (POD) 3, 7, 10, and 14 (Figure 1).

Fig 1

Study design for controls

All controls were fed a blend of infant formula and breast milk throughout the study. For CS, a blood specimen for measuring WBC, CRP, TP, and Alb and a fecal specimen (20mg) were collected in NICU between 24 and 48 hours of life and days 4, 8, 11, and 15 of life. For CN, it was difficult to collect specimens

from healthy neonates in the maternity ward reliably and the only specimens obtained were stool specimens between 24 and 48 hours of life and on days 4 and 8 days of life. Because of limited data for CN, our analysis focused mainly on comparing microbiota sequencing results for LKM+, LKM-, and CS.

Quantitative analysis of intestinal microbiota in feces

Extraction of fecal DNA

Fresh fecal samples (20mg) were harvested in transport tubes and stored at -70 degrees Celsius and sent to Research Laboratories, Kyodo Milk Industry Co., Ltd. DNA was extracted from each 20mg specimen of stool by adding the specimen to 550μL of 60mM Tris-HCL-30mM EDTA (pH8.0) buffer and suspending it by vortexing at maximum speed for 1 minute. The suspension was transferred to a 2mL screw tube containing 300mg of glass beads (ϕ = 0.1mm) to which 50μL of 10% SDS solution and 500μL phenol saturated with TE buffer (pH8.0) had been added. The tube was vortexed at maximum speed for 1 minute and incubated at 70°C for 10 minutes. After incubation, the tube was

shaken vigorously at 4000rpm for 1 minute using a micro smash MS-100 bead beater (Tomy Seiko Co., Ltd., Tokyo, Japan). The tube was then placed on ice for 5 minutes and centrifuged at 15000rpm for 5 minutes at 4°C. 500µL of the upper liquid phase was transferred to a new 1.5mL centrifuge tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added. The tube was vortexed at maximum speed for 1 minute then cooled on ice for 5 minutes. The mixture was then centrifuged at 15000rpm for 5 minutes at 4°C. 350µL of the upper liquid phase was transferred to a new 1.5mL centrifuge tube and mixed with 11.6µL 3M sodium acetate and 3µL of Ethachinmate (Nippon Gene, Tokyo, Japan). The solution was mixed with 0.7 volume of isopropanol and centrifuged at 15000rpm for 5 minutes. The precipitated DNA was rinsed twice with 1mL of 70% ethanol, dried, and dissolved in 100µL of nuclease free water. The DNA sample was stored at -20°C until use.

16S rRNA amplicon library preparation and next generation sequencing

The V3 region of bacterial 16S rRNA gene was amplified by PCR with fusion

primers. The forward primer contained Ion A adapter followed by key, barcode, adapter (GT), and 342f primer sequence (5'- CCTACGGGAGGCAGCAG-3') [13]. The reverse primer has Ion truncated P1 adapter followed by barcode, adapter (CC), and 518r primer sequence (5'- ATTACCGCGGCTGCTGG-3') [13]. PCR was performed in 25µL reaction mixtures containing 23.5µL of Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA), 0.5µL of primer mixture (5µM each), and 1µL of DNA solution. Thermal cycling conditions were 3 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 1 min at 68°C. The amplicon was purified using PureLink™ PCR Purification Kit (Invitrogen). The purified sample was loaded onto 2.0% agarose gel and electrophoresed at 50V for 1.5 hours at 25°C. The target DNA fragments were excised from the gel and purified with a FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The purity of DNA samples was assessed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a High Sensitivity DNA Kit (Agilent). DNA concentration was measured with a Qubit® 2.0 Flourometer (Invitrogen) by using a Quant-iT™ dsDNA HS

Assay Kit (Invitrogen). Molecular concentrations were adjusted according to the Ion Torrent™ protocol (Life Technologies, Gaithersburg, MD, USA). Emulsion PCR was performed using Ion PGM™ Template OT2 400 Kit (Life technologies) according to the manufacturer's protocol. Library sequencing was prepared using an Ion PGM™ Sequencing 400 Kit (Life Technologies) and Ion 318™ Chip V2 (Life Technologies) according to the manufacturer's instructions. Sequencing was performed on an Ion PGM ™ System (Life Technologies).

Sequence analysis

Sequence data was obtained in FASTQ format and analyzed using QIIME software [14]. Raw sequences were sorted by using barcodes, screened using average quality score ≥20 and priming sites to obtain sequences of ≥ 130bp. The trimmed sequences were clustered into operational taxonomic units (OTUs) at the level of 97% similarity by using the UCLUST algorithm [15] and the farthest neighbor algorithm. The most abundant sequences in each OTU were selected to represent other OTUs. The representative sequences were aligned

with the PyNAST algorithm [16]. The aligned sequences were checked for potentially chimeric sequences using the algorithm. Non-chimeric sequences were assigned taxonomy using RDP classifier at 50% confidence cutoff value [17].

Statistical Analysis

Data were reported as mean ± standard deviation (SD). Results were analyzed using the t-test for comparison of two means, and a one-way analysis of variance (ANOVA) for comparison of multiple groups. A post-hoc Bonferroni test was used to assess statistical difference between groups. The chi-square test was used for categorical data (Prism soft- ware; GraphPad Software, Inc., San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

Ethics

The study was approved by the Juntendo Hospital Institutional Review Board (IRB: ID2013038), and informed written consent was obtained from the parents.

RESULTS

One SB was a preterm neonate weighing 2930g at birth and other SB were full-term weighing from 2426-3300 g. Two SB were delivered by cesarean section and the others were delivered vaginally. The 2 mothers who had cesarean sections were given a dose of 2.0g ampicillin intravenously at the starting operation. Mean duration of follow up for SB was 344.3 days (range: 223-426). Biochemical data were similar for LKM+, LKM-, and CS (Figure 2). Fig 2 All fecal DNA sequencing successful using 20mg of stool. The V3 region of bacterial 16S rRNA gene was amplified and the average of amplification was 41290.5 (range: 0 - 111993). Relative abundance ratios of taxonomic families of microbiota identified in feces in CN were similar to CS. In CS stool, *Enterobacteriaceae*, *Streptococcaceae*, *Staphylococcaceae and Bifidobacteriaceae* were identified in descending order of abundance. When CS was compared with LKM+, there were significantly more *Enterobacteriaceae and Bacteroidaceae* in CS than LKM+ ($p < 0.05$) and significantly more *Streptococcaceae* in LKM+ than CS ($p < 0.05$). Between CS and LKM-, there

were significantly more *Bacteroidaceae* in CS than LKM- ($p < 0.05$). Between LKM+ and LKM-, there were significantly more *Streptcoccaceae* in LKM+ than LKM- ($p < 0.05$) and unexpectedly there were significantly more *Bifidobacteriaceae* in LKM- than LKM+ ($p < 0.05$) (Figure 3 a,b). Fig 3

In LKM+, 2 cases developed egg allergy and 1 case developed generalized colitis around 12 months old. In LKM-, 2 cases developed colitis at the same time. In CS, no cases developed allergies (Table1) or colitis. No significant differences in weight gain were observed. Table 1

DISCUSSION

The immune system is not fully functional at birth, rendering neonates highly susceptible to infections. After birth, there is an age-dependent maturation of the immune system in which different key functions are fine-tuned after exposure to direct stimulation from environmental signals not previously encountered during fetal life [1].

The most widely recognized application of probiotics in the pediatric population

is for preventing NEC in premature infants. A meta-analysis conducted as part of this study found that probiotics are associated with a 49.1% reduction in the incidence of NEC and a 26.9% reduction in overall mortality in VLBW infants, which are consistent with the results of previous meta-analyses, including a recent published review in the Cochrane Database of Systematic Reviews [18]. The mechanism for the efficacy of probiotics in NEC remains unclear but is believed to be related to a protective effect on the mucosa of the gastrointestinal tract. Commensal bacteria are known to inhibit enteric pathogens and may help suppress the growth and invasion of pathogenic bacteria, thereby improving intestinal barrier function. Probiotics also modulate proinflammatory cytokines, which help regulate immune responses and maintain homeostasis. Thus, probiotic supplementation may facilitate the acquisition and subsequent population of the gastrointestinal tract by normal commensal bacteria and modulate the balance of the gastrointestinal tract [18].

Our study follows other recent 16S rRNA gene sequencing based studies analyzing the composition of gut microbiota in surgical babies, but although our

study is essentially a pilot study, our findings are valuable because we are the first to describe the initial colonization of the neonatal gut in SB and the effects of surgery at this time using next-generation DNA sequencing and assess the value of probiotic administration. We observed major changes in gut microbiota in SB irrespective of probiotic use during the first weeks of life in response to major stresses (NICU stay, surgery, delayed feeding, and drug use, including broad-spectrum antibiotics). By the second week of life, however, gut microbiota had a more stable composition with a major shift in variety occurring between one and two weeks of life, but still not as extensive as the shift that occurs in the first week of life.

We were puzzled by our probiotic findings. Our probiotic preparation, LKM512 would appear to be have sufficient acid tolerance [11] because it was detected in feces and did not appear to cause any side effects during administration, however, we could not observe any remarkable advantages associated with its administration, such as preventing infections, enhancing weight gain, or

supporting growth. We are planning to repeat our study with administration at a different period to determine when LKM512 administration is beneficial to SB. Our technique for analyzing fecal specimens would appear to be accurate because of reliable reproducibility. Thus, we plan to perform larger studies with more subjects to assess the effect of physical stresses on gut microbiota in surgical cases so we can understand what factors are important for maintaining gut microbiota in the pediatric population and deal effectively with derangements, especially in surgical cases. The interaction between commensal microorganisms and the immune system is likely to be complex but supporting natural defense mechanisms will enhance recovery and directly influence the quality of life of our patients.

In conclusion, the impact of surgical stress on intestinal microbiota in neonates would appear to be considerable and warrants further investigation and the role of probiotic administration in infants requires clarification. New-generation DNA sequencing was particularly effective for identifying stool microbiota and will facilitate larger scale studies by reducing costs and labor.

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FIGURE LEGENDS

Table 1:

Clinical characteristics of the subjects for this study. VD: vaginal delivery, CS: cesarean section, M: male, F: female, ARM: anorectal malformation, DA: duodenal atresia.

Figure 1:

Our postoperative management for (a) ARM, (b) DA, and (c) Lymphangioma. A blood specimen for biochemistry and fecal specimen for DNA sequencing were collected from SB in the NICU between 24 and 48 hours of life (After birth) and on postoperative day (POD): POD3, POD7, POD10, and POD14. ABPC: ampicillin.

Figure 2:

All data were similar for LKM+, LKM-, and CS.

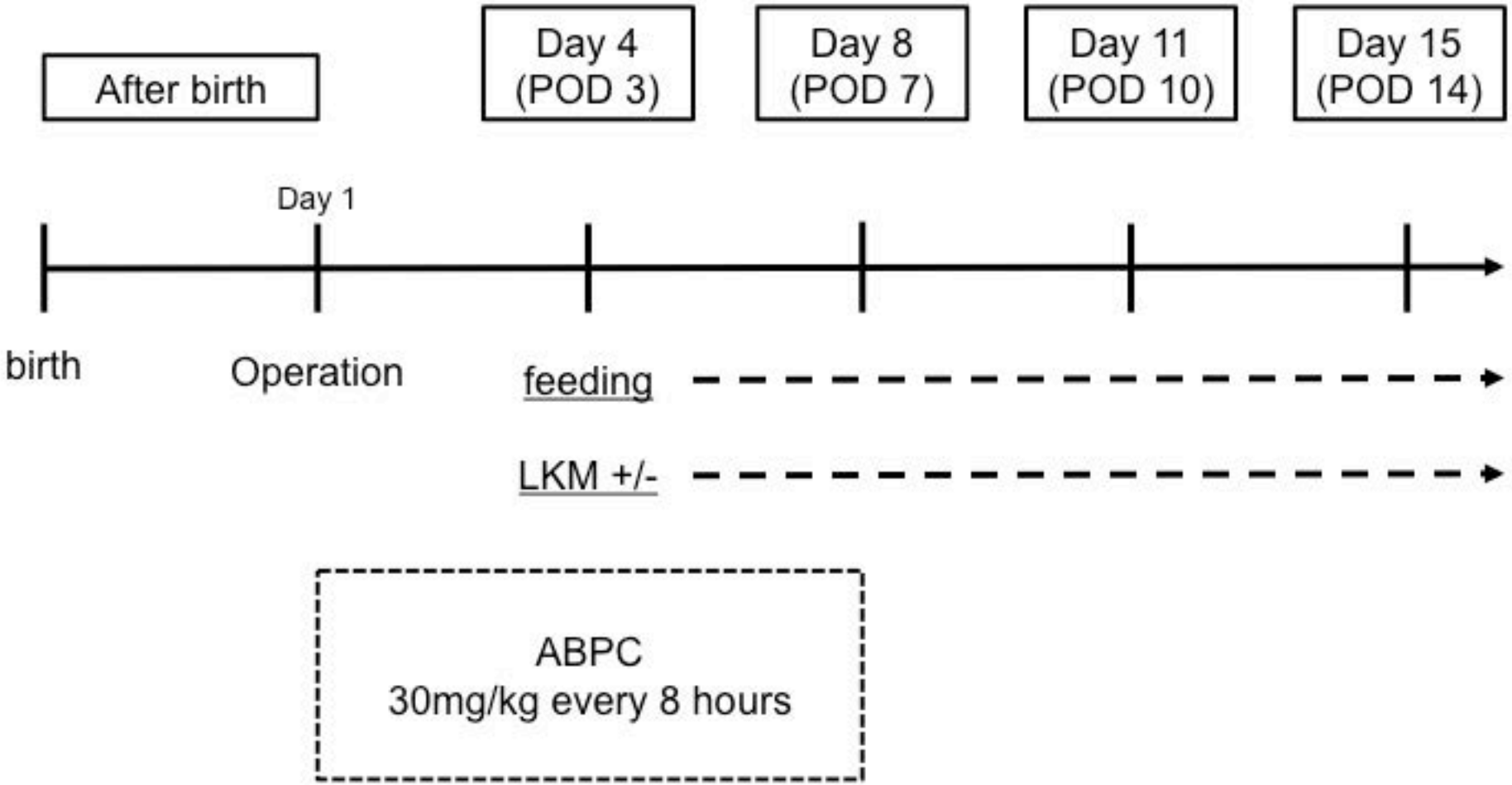
(a) WBC: white blood cells count, (b) CRP: C-reactive protein, (c) TP: total

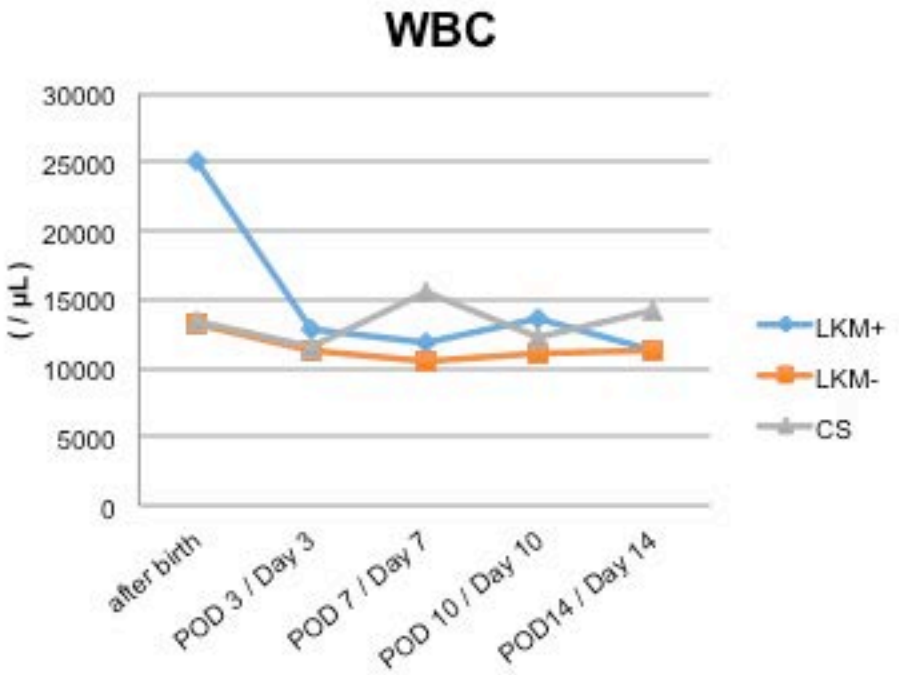
protein, (d) Alb: albumin.

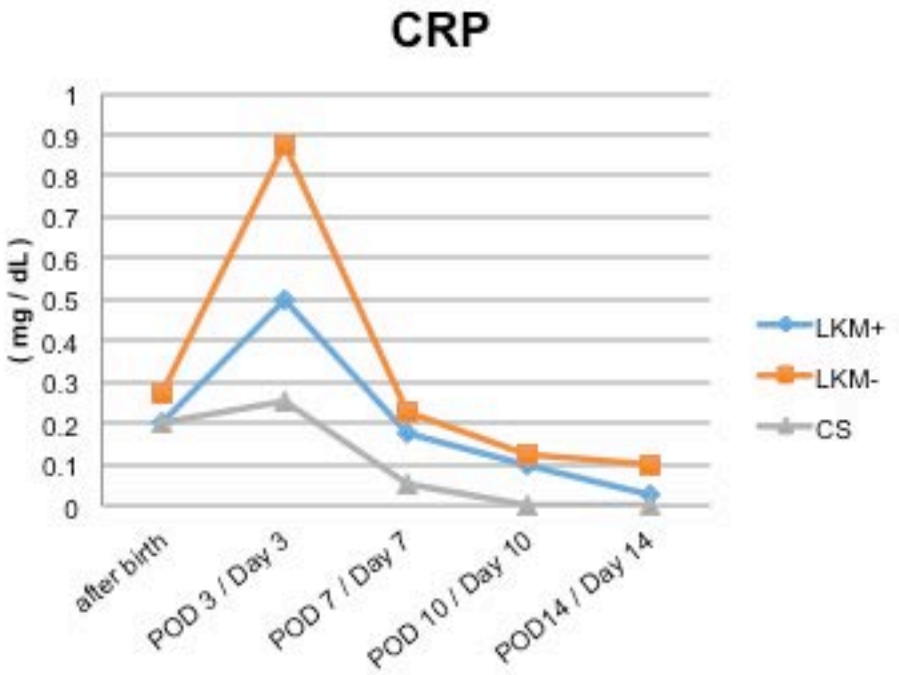
Figure 3:

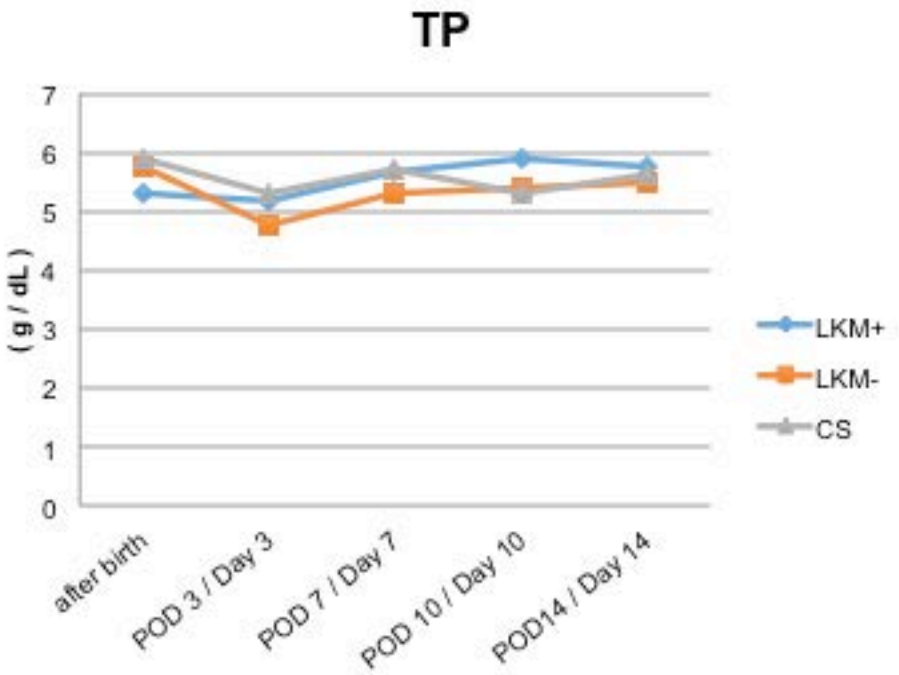
(a) Relative abundance according to taxonomic families in microbiota in LKM+, LKM- and CS stool.

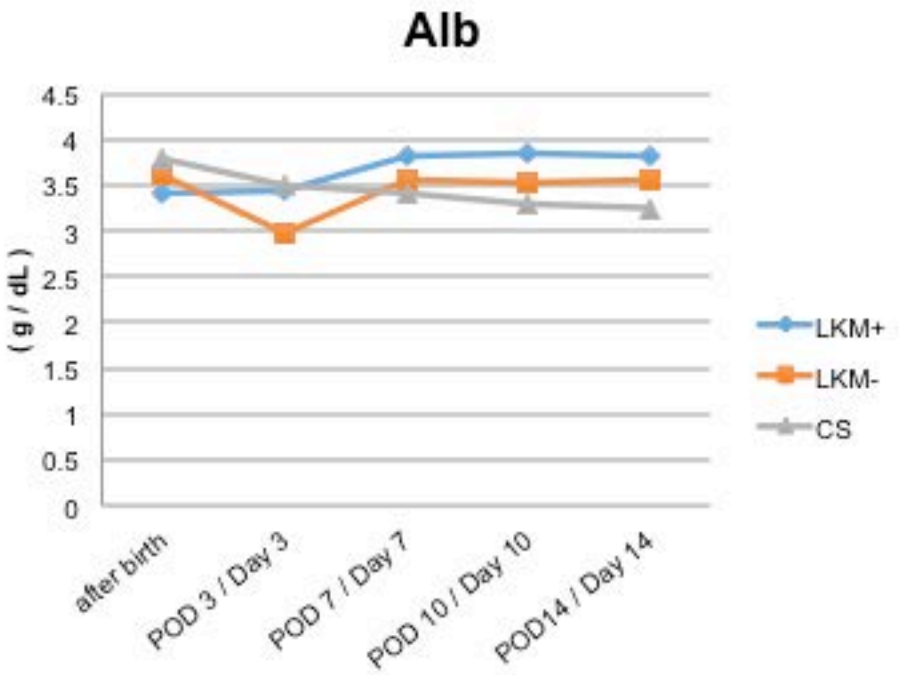
(b) Comparison of overall relative abundance according to taxonomic families in microbiota in LKM+, LKM- and CS stool.

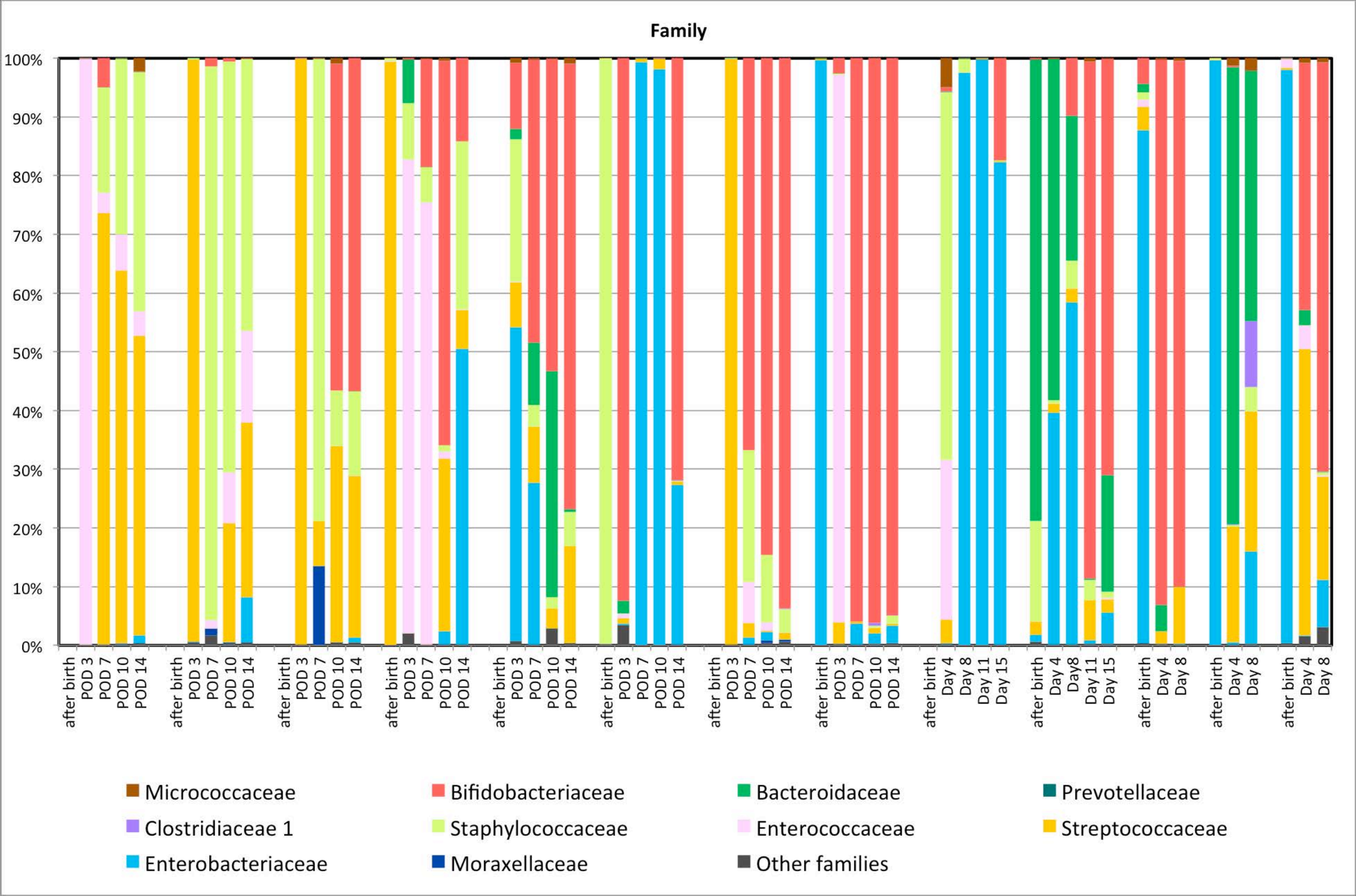

After birth
Day 4 (POD 3)
Day 8 (POD 7)
Day 11 (POD 10)
Day 15 (POD 14)
Day 1
birth
Operation
feeding
LKM +/-
ABPC
30mg/kg every 8 hours

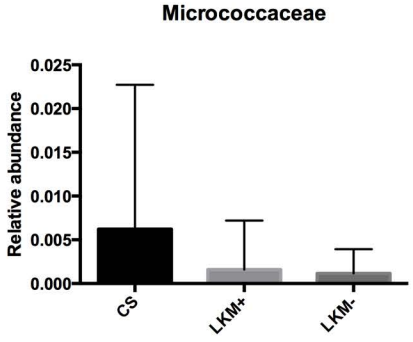

WBC
(/ μL)
30000
25000
20000
15000
10000
5000
0
after birth
POD 3 / Day 3
POD 7 / Day 7
POD 10 / Day 10
POD14 / Day 14
LKM+
LKM-
CS

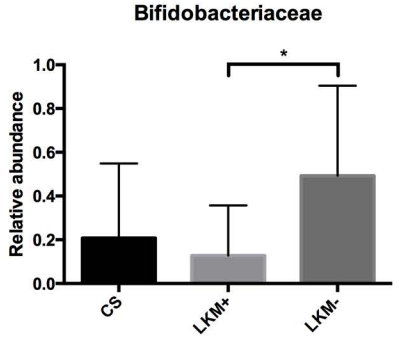

CRP
(mg / dL)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
after birth
POD 3 / Day 3
POD 7 / Day 7
POD 10 / Day 10
POD14 / Day 14
LKM+
LKM-
CS

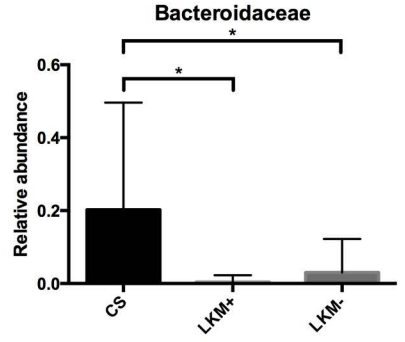

TP
(g / dL)
7
6
5
4
3
2
1
0
after birth
POD 3 / Day 3
POD 7 / Day 7
POD 10 / Day 10
POD14 / Day 14
LKM+
LKM-
CS

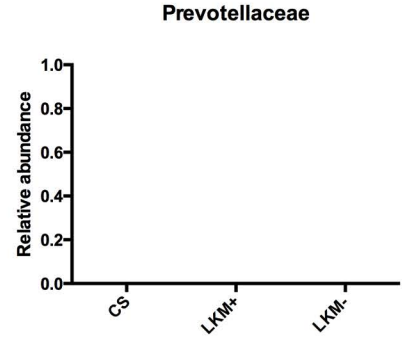

Alb
(g / dL)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
after birth
POD 3 / Day 3
POD 7 / Day 7
POD 10 / Day 10
POD14 / Day 14
LKM+
LKM-
CS

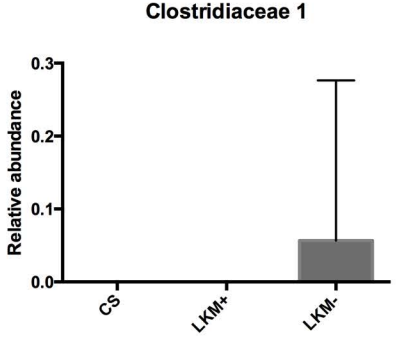

Family
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
after birth
POD 3
POD 7
POD 10
POD 14
Day 4
Day 8
Day 11
Day 15
Day8
Micrococcaceae
Bifidobacteriaceae
Bacteroidaceae
Prevotellaceae
Clostridiaceae 1
Staphylococcaceae
Enterococcaceae
Streptococcaceae
Enterobacteriaceae
Moraxellaceae
Other families

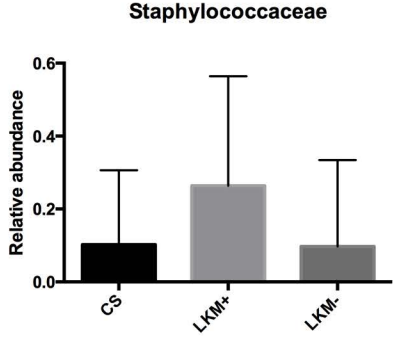

Micrococcaceae
Relative abundance
0.025
0.020
0.015
0.010
0.005
0.000
CS
LKM+
LKM-

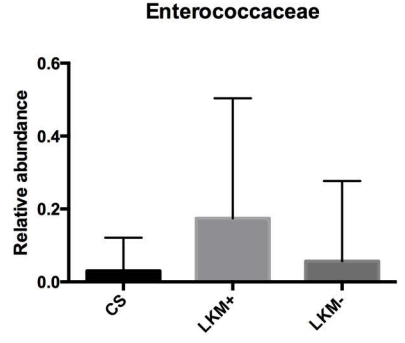

Bifidobacteriaceae
Relative abundance
1.0
0.8
0.6
0.4
0.2
0.0
*
CS
LKM+
LKM-

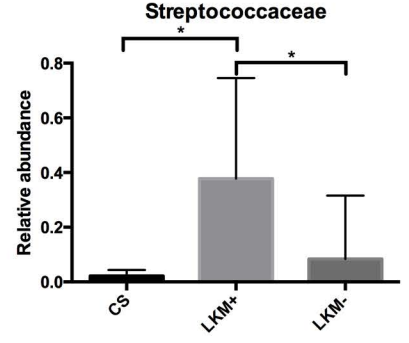

Bacteroidaceae
Relative abundance
0.6
0.4
0.2
0.0
*
*
CS
LKM+
LKM-

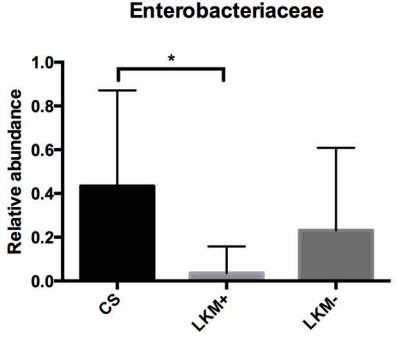

Prevotellaceae
Relative abundance
1.0
0.8
0.6
0.4
0.2
0.0
CS
LKM+
LKM-

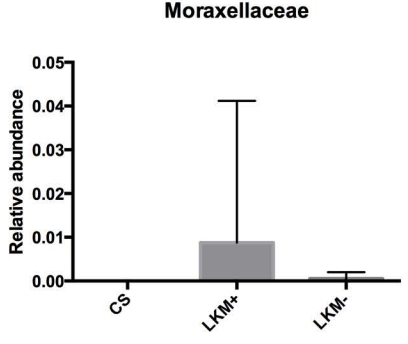

Clostridiaceae 1
Relative abundance
0.3
0.2
0.1
0.0
CS
LKM+
LKM-


Staphylococcaceae
Relative abundance
0.6
0.4
0.2
0.0
CS
LKM+
LKM-


Enterococcaceae
Relative abundance
0.6
0.4
0.2
0.0
CS
LKM+
LKM-


Streptococcaceae
Relative abundance
0.8
0.6
0.4
0.2
0.0
*
*
CS
LKM+
LKM-


Enterobacteriaceae
Relative abundance
1.0
0.8
0.6
0.4
0.2
0.0
*
CS
LKM+
LKM-


Moraxellaceae
Relative abundance
0.05
0.04
0.03
0.02
0.01
0.00
CS
LKM+
LKM-

Case	LKM+ No.1	LKM- No.1	CS No.1
Sex	M	F	M
Pathology/ Treatment	ARM/ colostomy	DA/ duodenoduodenostomy	Bilateral hydronephrosis
Gestation	Full term	Full term	Full term
Delivery	VD	VD	VD
Birth Weight (g)	2986	2755	2774
Duration of follow- up (Days)	426	370	424
Allergies	egg white	-	-
	LKM+ No.2	LKM- No.2	CS No.2
	M	M	M
	ARM/ colostomy	DA/ duodenoduodenostomy	Bowel distention
	Full term	Full term	Full term
	CS	VD	VD
	2814	2426	2916
	383	265	421
	egg / dairy products	-	-
	LKM+ No.3	LKM- No.3	
	M	M	
	ARM/ colostomy	ARM/ cut back	
	Pre term	Full term	
	VD	VD	
	2930	3300	
	367	244	
	-	-	
	LKM+ No.4	LKM- No.4	
	F	F	
	ARM/ colostomy	Lymphangioma/ injection of OK432	
	Full term	Full term	
	CS	CS	
	3160	3161	
	320	223	
	-	-	