Original article

Preliminary study of neuro-immune cross-talk by gut microbiota for perioperative management in infants with congenital heart disease

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Abstract

Background：Congenital heart disease involves abnormalities in the heart structure that occur before birth. Most infants require one or more heart surgeries to correct the congenital heart defects. Down syndrome and congenital heart disease is the most widely studied congenital anomaly. We evaluated the potential for neuro-immune cross-talk mediated by changes in gut microbiota to identify methods for effective perioperative management.

Methods：Four infants with Down syndrome and congenital heart disease that underwent cardiopulmonary bypass (CPB) were included. We evaluated the dynamics of microbiota pre-CPB, post-CPB, and upon discharge. We further measured factors potentially associated with the function of the intestinal mucosa and immune cross-talk, including fecal secretory immunoglobulin A (IgA) and short chain fatty acids, and the serum neutrophil to lymphocyte ratio (NLR) and C-reactive protein (CRP).

Results：CPB altered the abundance of fecal microbiota, especially those in the order Neisseriales. Furthermore, serum NLR showed faster normalization than CRP following CPB. There was no significant change in fecal sIgA levels observed during hospitalization overall.

Conclusion：Stress from an operation appears to affect the intestinal microbiota and cause dysbiosis in young patients with Down syndrome and congenital heart disease. In addition, NLR may be a better biomarker for perioperative management.

Key words

congenital heart disease, neuro-immune cross-talk, short chain fatty acids (SCFAs), the neutrophil to lymphocyte ratio (NLR), C-reactive protein (CRP)

Rinsho Hyoka (*Clinical Evaluation*). 2016；44：151-64.

1. Introduction

Recent studies have shown that after heart surgery, approximately 40% of infant patients will have brain abnormalities that show up on MRI (magnetic resonance imaging) scans [1]. The damage is most often caused by a stroke, which can be triggered and exacerbated from multiple events during surgery and recovery, when the brain is most susceptible to injury. These brain injuries can lead to deficiencies in the child's hyperactivity and speech delays, as well as impact motor skills and mental development [1].

Early exposure to environmental microbial components, even potentially pathogenic microorganisms, is suggested to play an important role in the maturation process and to dramatically reduce the incidence of inflammatory, autoimmune, and atopic diseases. This is because the gastrointestinal tract is the major site of interaction between the host immune system and external factors. The most dramatic changes in intestinal microbiota occur early in life, and can even influence the normal functional development of the adult immune system following heart surgeries performed to correct congenital heart defects [2]. Therefore, we here consider the role of neuro-immune cross-talk mediated by gut microbiota for perioperative management in infants with Down syndrome (DS) and congenital heart disease, and discuss the results in the context of inter-organ communication.

Recent studies have focused on the role of specific metabolites for inter-organ communication, such as short chain fatty acids (SCFAs), and their influence in perioperative management in infants with congenital heart disease [3, 4]; however, the role of global metabolites has not been widely explored. It is well known that the gut microbiota is altered in young patients requiring urgent surgery, which can be driven by many factors such as surgical stress with cardiopulmonary bypass (CPB), inflammation, intravenous nutrition, and antibiotics [5]. In this study, we focus on the influence of surgical stress due to CPB for neuro-immune cross-talk mediated by gut microbiota.

Therefore, we hypothesized that a subject receiving CPB would exhibit increased or decreased permeability to proinflammatory bacterial products in the intestine [3, 6]. Down syndrome (DS) is the most common cause of congenital heart defects [6]. Accordingly, this study was designed to examine the growth dynamics of the gut microbiota and their influence on inter-organ communication in perioperative management for infants with DS and congenital heart disease that received CPB.

Previous reports showed that the main mechanism of intestinal barrier dysfunction following CPB involves a decrease in the expression of tight junction proteins in the intestinal epithelium, which subsequently leads to changes in the structure and function of the intestinal mucosa, increased intestinal permeability, bacterial translocation, and endotoxemia. Given this background, we evaluated the changes in the colonic environment, including fecal microbiota, SCFA concentrations, pH level, and secretory immunoglobulin A (sIgA) concentrations, and immune reaction markers, including the neutrophil to lymphocyte ratio (NLR) and C-reactive protein (CRP), which might be associated with the function of the intestinal mucosa [4] following CPB in four subjects.

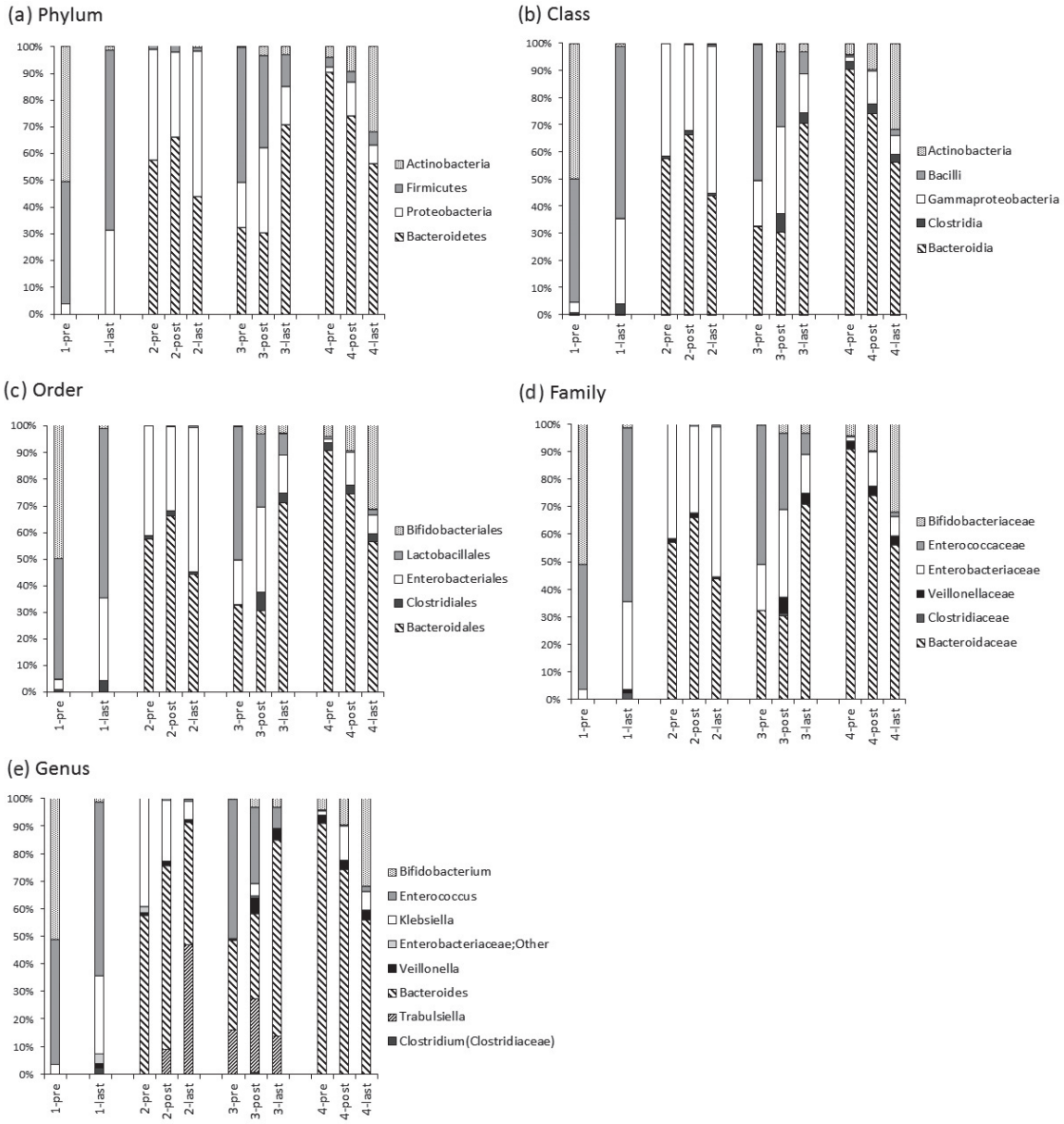
2. Results

We analyzed the fecal specimens of four subjects, although the fecal sample of subject No. 1 post-CPB was not available.

2.1 Alterations of the Fecal Microbiota of Subjects during Hospitalization

A total of 440,248 reads were generated in two runs on Ion 318 Chip V2, and a total of 279,099 reads were used in the final analysis. There was an average of 25,373 (range 18,505–32,213) sequence reads per fecal sample. The predominant bacterial phylum, class, order, family, and genus (with an abundance of more than 1%) detected are shown in Fig. 1, and all of the detected bacterial genera are listed in Table 1. There was high individual variation among the subjects, and CPB showed a clear

Fig. 1 Aggregate microbiota composition



Aggregate microbiota composition at the phylum (a), class (b), order (c), family (d), and genus (e) levels. Only major (more than 1% abundance) taxonomic groups are shown; these covered 97% of all reads assigned to each level.

Table 1　Fecal microbiota of subjects (genus level)

	Pre-CPB			
	1	2	3	4
k__Bacteria;Other;Other;Other;Other;Other	0.004661	0	0	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Actinomyces	0.013983	0	0.003104	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	0.032628	0	0	0.003856
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia	0.107206	0	0	0.003856
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	49.48728	0	0.201782	4.099182
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Atopobium	0.004661	0	0	0.007712
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Eggerthella	0.703831	0	0.031043	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.004661	57.39557	32.38755	90.33241
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	0	0.003228	0	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus	0.004661	0	0	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;Other	0.004661	0	0	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Granulicatella	0.004661	0	0.003104	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;Other	0.550014	0	0.149008	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	43.82866	0	50.23748	0.262224
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Lactococcus	0.144495	0.006456	0.006209	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.531369	0.154949	0.009313	0.632423
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	0.279668	0.003228	0	0.003856
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53	0.004661	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	0	0	0	0.015425
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	0.004661	0	0	0.0617
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Oribacterium	0.046611	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	0.004661	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	0.419502	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella	0.004661	1.126606	0.170739	2.761067
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Finegoldia	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Parvimonas	0	0	0	0
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	0.004661	0	0	0.03085
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium	0.018645	0	0.003104	0.003856
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0.027967	2.056298	0.32906	0.011569
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Klebsiella	3.752214	38.99542	0.121069	1.592627
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Morganella	0.004661	0	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Serratia	0	0.025825	0	0.007712
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus	0	0	0	0.115687
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__	0	0	0	0.003856
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Odoribacter	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__[Prevotella]	0	0	0.006209	0
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other;Other	0	0	0.003104	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__	0	0	0	0.003856
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__Gemella	0	0	0	0.003856
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0	0	0.003104	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;Other	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Butyrivibrio	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0	0	0.003104	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Peptostreptococcus	0	0	0.009313	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__[Clostridium]	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Acidaminococcus	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megasphaera	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Anaerococcus	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Peptoniphilus	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Sutterella	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Acidovorax	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria	0	0	0.003104	0.042419
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Trabulsiella	0	0.232423	16.3195	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	0	0	0	0

Table 1 Fecal microbiota of subjects (genus level)

Post-CPB				Leaving hospital (Last)				Mean		
1	2	3	4	1	2	3	4	Pre	Post	Last
	0	0.004025	0	0.008001	0	0	0	0.001165	0.001342	0.002
	0	0.004025	0.046434	0.020003	0	0	0	0.004272	0.01682	0.005001
	0	0	0.004643	0	0	0.017583	0	0.009121	0.001548	0.004396
	0	0	0.055721	0.004001	0.193979	0	0.226966	0.027766	0.018574	0.106236
	0	3.051284	9.337853	1.072172	0.028738	2.910018	31.44556	13.44706	4.129712	8.864121
	0	0	0	0	0	0	0	0.003093	0	0
	0	0.253603	0	0.176028	0	0.008792	0	0.183719	0.084534	0.046205
	66.1142	30.35987	74.05275	0.004001	43.86809	70.62288	55.99568	45.03005	56.84227	42.62266
	0	0	0	0.004001	0	0	0	0	0	0.001
	0	0	0.004643	0.004001	0.003592	0.008792	0.021616	0.000807	0.001548	0.0095
	0	0	0	0	0.003592	0.272539	0.005404	0.001165	0	0.070384
	0	0	0	0.004001	0	0	0	0.001165	0	0.001
	0.003564	0	0	0.036006	0	0	0.005404	0.001941	0.001188	0.010352
	0	0.161018	0	0.832133	0	0.057145	0	0.174756	0.053673	0.22232
	0.295837	27.26834	0.529346	61.52984	0.610676	7.648688	1.74007	23.58209	9.364506	17.88232
	0.046336	0.012076	0	0.780125	0.079029	0.197811	0	0.03929	0.019471	0.264241
	0.014257	0.008051	0.046434	0.260042	0.061068	0.039562	0.297217	0.332014	0.022914	0.164472
	0	0	0.009287	0	0	0	0.005404	0.071688	0.003096	0.001351
	0	1.058691	0	2.620419	0	0	0	0	0.352897	0.655105
	0	0	0	0	0	0	0	0.001165	0	0
	0	0.004025	0.032504	0.004001	0	0	0	0.003856	0.012176	0.001
	0	0	0.130015	0	0	0	0	0.01659	0.043338	0
	0	0	0	0	0	0	0	0.011653	0	0
	0	0	0	0	0	0	0	0.001165	0	0
	0	0	0	0.004001	0	0	0	0.104876	0	0.001
	0.010693	0	0	0.004001	0.014369	0	0	0	0.003564	0.004592
	1.361563	5.716126	3.292162	1.368219	0.592715	3.683678	3.139692	1.015768	3.456617	2.196076
	0.003564	0.084534	0.004643	0.016003	0.086213	0.004396	0	0	0.030914	0.026653
	0	0	0	0.004001	0	0	0	0	0	0.001
	0	0.008051	0.004643	0	0	0	0	0.008878	0.004231	0
	0	0	0	0	0	0	0	0.006401	0	0
	0.174651	0.897673	0.116085	3.616579	0.344852	0.246165	0.086463	0.606223	0.396136	1.073515
	22.12717	4.210611	12.26319	27.58041	6.911416	0.180228	6.971089	11.11533	12.86699	10.41079
	0	0	0	0	0	0	0	0.001165	0	0
	0.02495	0.740681	0.01393	0.004001	0.003592	0	0.005404	0.008384	0.259854	0.003249
	0	0	0.004643	0.044007	0	0.08352	0	0.028922	0.001548	0.031882
	0	0	0	0	0.010777	0	0	0	0	0.002694
	0	0.004025	0	0	0	0.008792	0	0.000964	0.001342	0.002198
	0	0	0	0	0	0	0.005404	0	0	0.001351
	0	0	0	0	0	0	0	0.001552	0	0
	0	0	0	0	0	0.008792	0	0.000776	0	0.002198
	0	0	0	0	0	0	0	0.000964	0	0
	0	0	0.009287	0	0	0	0	0.000964	0.003096	0
	0	0	0	0	0	0	0	0.000776	0	0
	0	0	0	0	0	0	0.005404	0	0	0.001351
	0	0	0.004643	0	0	0	0.005404	0	0.001548	0.001351
	0	0	0	0	0	0.008792	0	0	0	0.002198
	0	0	0.004643	0	0	0.004396	0.005404	0.000776	0.001548	0.00245
	0	0.004025	0	0	0	0	0.005404	0	0.001342	0.001351
	0	0	0	0	0	0	0	0.002328	0	0
	0.409894	0	0	0	0.215533	0	0	0	0.136631	0.053883
	0	0	0	0	0	0	0.005404	0	0	0.001351
	0	0	0	0	0.003592	0	0	0	0	0.000898
	0	0	0.004643	0	0	0	0	0	0.001548	0
	0	0	0	0	0.057475	0	0	0	0	0.014369
	0	0.048305	0	0	0	0.08352	0	0	0.016102	0.02088
	0	0.04428	0.009287	0	0	0	0	0	0.017856	0
	0	0	0	0	0	0	0.005404	0	0	0.001351
	0	0	0	0	0.010777	0	0.005404	0	0	0.004045
	0	0	0	0	0	0	0.005404	0	0	0.001351
	0	0	0	0	0.003592	0.004396	0.005404	0.011381	0	0.003348
	9.413316	26.05668	0.009287	0	46.89633	13.89951	0	4.13798	11.82643	15.19896
	0	0	0.004643	0	0	0	0	0	0.001548	0
	0	0	0.004643	0	0	0	0	0	0.001548	0

impact on the fecal microbiota. For example, 3, 7, and 6 bacterial genera disappeared or were reduced to an abundance of less than 10% following CPB in subject No. 2, No. 3, and No. 4, respectively. Furthermore, 6, 13, and 11 new bacterial genera appeared or showed a more than 10-fold increase following CPB in subject No. 2, No. 3, and No. 4, respectively. On the other hand, some specific bacteria increased in abundance during the period from immediately post-CPB to discharge from the hospital. Specifically, the abundance of members of the order Neisseriales, family Neisseriaceae, and genus *Neisseria* was significantly higher at discharge compared to the post-CPB levels ($p < 0.05$). Moreover, the class β-Proteobacteria ($p = 0.0864$) and the family Streptococcaceae ($p = 0.0752$) also tended to be more abundant at discharge compared to the post-CPB levels (Table 2).

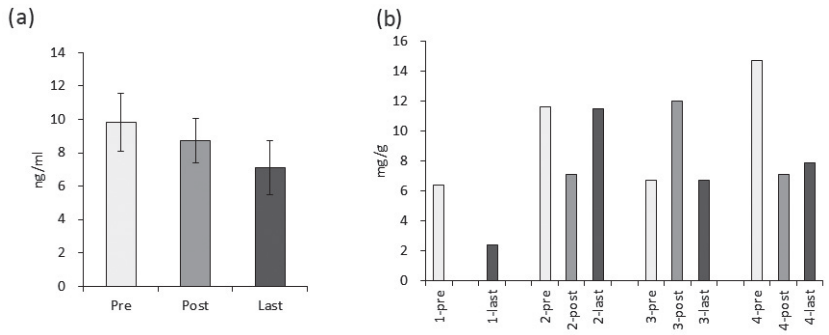
2.2 Fecal SCFA Concentrations and pH Level

The SCFA concentrations of subjects No. 4 and No. 1 were not determined post-CPB, because an insufficient quantity of fecal sample was obtained. Therefore, there were only 2 post-CPB samples available, making statistical analysis impossible (Fig. 2a). Nevertheless, large individual differences in SCFAs between the subjects were noted. Acetate was detected from all samples and the concentrations of both subjects (No. 2 and No. 3) increased during the period from post-CPB to discharge. Butyrate was not detected in the pre-CPB samples,

Table 2　List of bacteria that had difference between test periods

Classfication level	Bacteria	Mean of relative abundance (%)			Paired t-test (p-value)		
		Pre-CPB	Post-CPB	Last	Pre-CPB	Post-CPB	Last
Phylum	Not detected						
Class	Proteobacteria;c__Betaproteobacteria	0.0114	0.0000	0.0087	0.3820	0.7767	0.0864
Order	Proteobacteria;c__Betaproteobacteria;o__Neisseriales	0.0114	0.0000	0.0033	0.3820	0.4679	0.0135
Family	Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	0.3713	0.0424	0.4301	0.3376	0.7249	0.0752
Genus	Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae	0.0114	0.0000	0.0033	0.3820	0.4679	0.0135
	Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria	0.0114	0.0000	0.0033	0.3820	0.4679	0.0135

Fig. 2　The change in fecal SCFA content during hospitalization



The change in fecal SCFA content during hospitalization. (a) All subjects, (b) individual subjects. Vertical bars represent SEM.

except in subject No. 1. However, the butyrate concentration increased in all samples post-CPB except for subject No. 4 (Fig. 3b).

The fecal pH in subjects No. 1 and No. 2 increased during hospitalization, although that in No. 3 was stable (Table 3).

2.3 Fecal sIgA Concentrations

The fecal sIgA contents are shown in Fig. 3. There was no significant change in fecal sIgA levels observed during hospitalization overall (Fig. 3a); however, a change was observed in each subject (Fig. 3b). The fecal sIgA contents of subject No. 2 and No. 4 decreased by CPB and increased by discharge. In contrast, the sIgA level of subject No. 3 increased following CPB and had decreased by discharge.

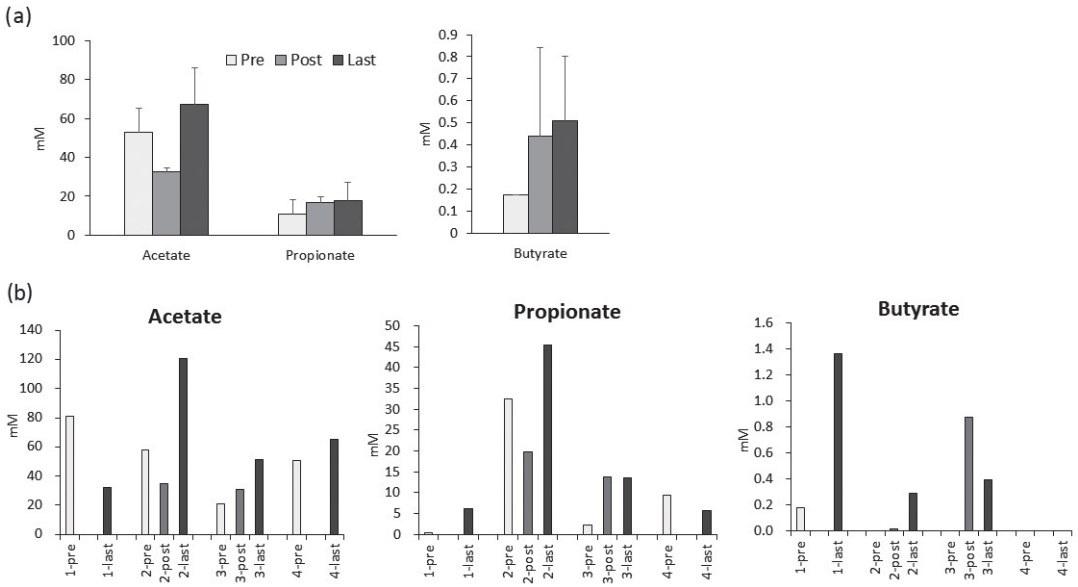
2.4 Serum NLR and CRP

The NLR and CRP levels in serum obtained from all subjects pre-CPB, post-CPB Day 1–2 (average of the first and second days after CPB), post-CPB Day 3–4 (average of the third and fourth days after CPB), and the day of hospital discharge (Last) were determined. Both NLR ($p = 0.051$) and CRP ($p < 0.05$) rapidly increased immediately following

Table 3 Fecal pH of subjects during hospitalization

subject No.	Pre-CBP	Post CBP	Last
1	5.5	ND	7.3
2	6.4	7.5	6.2
3	6.7	6.6	6.8
4	5.8	ND	6.2

Fig. 3 The change in fecal sIgA content during hospitalization



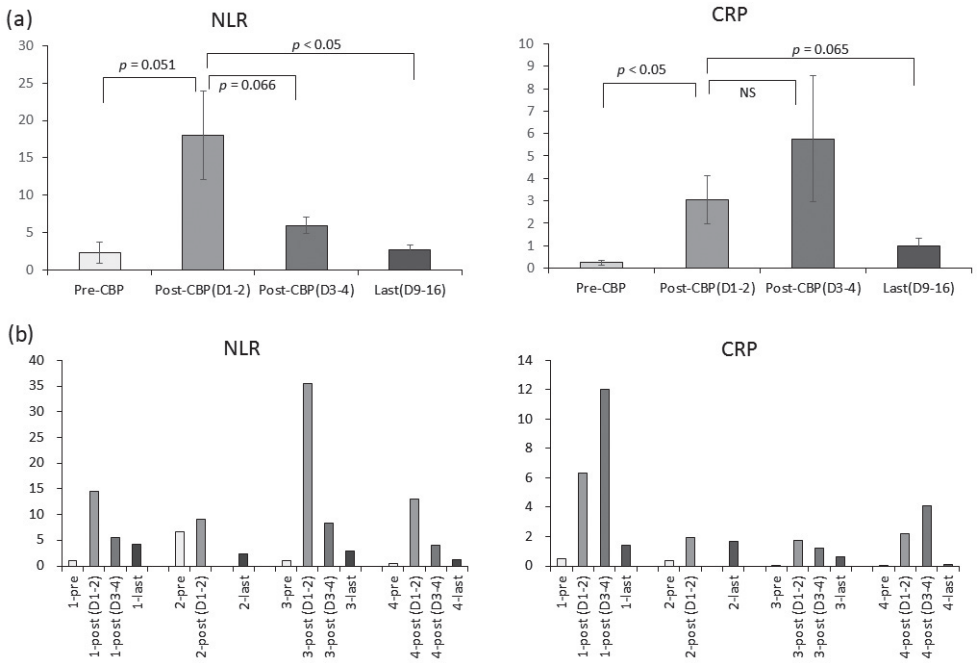
The change in fecal sIgA content during hospitalization. (a) All subjects, (b) individual subjects. Vertical bars represent SEM.

CPB (Fig. 4a). At post-CPB Day 3–4, the NLR tended to decrease rapidly (p = 0.066), although CRP was not decreased compared with the post-CBP Day 1–2 levels (Fig. 4a). Upon discharge, the NLR was decreased significantly ($p < 0.05$) and CRP tended to be decreased (p = 0.065) compared with the post-CBP Day 1–2 level (Fig. 4a). Both the NLR and CRP level increased following CPB in all subjects (Fig. 4b). At post-CPB Day 3–4, the NLR in all subjects decreased, although CRP in 2 subjects (No. 1 and No. 4) increased compared with the post-CBP Day 1–2 levels (Fig. 4b). At the last day of hospitalization, both the NLR and CRP of all subjects had decreased compared with the post-CBP Day 1–2 level (Fig. 4b).

3. Discussion

To the best of our knowledge, there is only report on the microbiota of DS, showing that DS subjects were dominated by Firmicutes (relative abundance of 83 ± 2%)[7]. However, in the present study, 3 out of 4 subjects with DS and congenital heart disease did not show a predominance of the phylum Firmicutes. There were no common patterns of the microbiota among the subjects observed at the class, order, and family levels. This is probably due to the use of antibiotics in these patients. Thus, further studies are required to clarify the characteristics of the DS intestinal microbiota. However, the alteration of intestinal microbiota due to CPB showed an interesting pattern.

Fig. 4　The change in NLR and CRP levels in serum during hospitalization



The change in NLR and CRP levels in serum during hospitalization. (a) All subjects, (b) individual subjects. Vertical bars represent SEM.
Serum was obtained from all subjects pre-CPB, post-CPB Day 1–2 (average of the first and second days after CPB), post-CPB Day 3–4 (average of the third and fourth days after CPB), and the day of hospital discharge (Last).

CPB is commonly used to support circulation during heart surgery. However, CPB also causes damage to the intestinal mucosa, resulting in bacterial translocation and endotoxemia [8]. A previous study showed that these effects could synergistically contribute to the development of systemic inflammatory response syndrome and multiple organ dysfunction syndrome, which are the leading causes of severe complications after cardiac surgery [8]. There have been some attempts to mitigate this problem with measures to protect intestinal barrier function during CPB, including inhibition of inflammation and elevation of intestinal perfusion or oxygen supply, but the outcome was not positive [8].

Recent studies have investigated the effect of gut microbiota on the brain and behavior. The results of these studies suggest that intestinal microbiota have a great impact on gut-brain communication, which led to the coining of the term "microbiota-gut-brain axis" [9, 10]. We also previously found that cerebral low-molecular metabolites are influenced by intestinal microbiota [11]. In the present study, we showed that the alteration of microbiota may influence the severity of cerebral pathology initiated during CPB. The intestinal microbiota also play an important role in intestinal barrier function. Therefore, the alteration of intestinal microbiota during CPB and hospitalization also likely influence intestinal permeability. Indeed, the increase in NLR and CRP of all subjects by CPB and their subsequent decrease during hospitalization after CPB (Fig. 4) was accompanied by alterations of the microbiota (Fig. 1), suggesting a causal relationship. Interestingly, *Neisseria* species, whose relative abundance was significantly changed during hospitalization (Table 2), have been reported to modulate the immune response via activation of NOD receptors [12]. To our knowledge, this represents the first demonstration that stress from an operation affects the intestinal microbiota in young patients with DS and congenital heart disease, based on studying alterations pre- and post-CPB.

SCFAs are important anions in the colonic lumen, which influence both the morphology and function of the colonocytes. Increased SCFAs lowers the pH, which indirectly modifies the composition of the colonic microbiota and increases the absorption of minerals [13]. For example, a low pH was suggested to be an important factor to decrease the incidence of infections caused by the O157 subtype of *Escherichia coli* [14].

SCFAs are saturated aliphatic organic acids containing one to four carbons (acetate C2, propionate C3, and butyrate C4). Importantly, absorption of SCFAs through colonic epithelial cells alters the pH of the colon, which in turn has an important influence on the composition and population of the gut microbiota. This is because most of the SCFAs absorbed in the colon interact with bicarbonate; therefore, the resultant luminal pH is determined by the rate of SCFA production by the microbiota and the neutralizing capability of the bicarbonate [2]. Due to its continuous absorption, a decline in SCFA concentration from the proximal to the distal colon leads to a corresponding increase in pH from the cecum to the rectum. Animal and human fecal studies have demonstrated that gut pH has an important effect on the growth and composition of the gut microbiota [2]. The luminal pH from the ileum to the cecum is relatively low due to the higher SCFA concentration, which prevents the overgrowth of pH-sensitive pathogenic bacteria (e.g., Enterobacteriaceae and Clostridia), and at pH 5.5, butyrate-producing bacteria (phylum Firmicutes) dominate [2]. However, as the luminal pH increases to 6.5 in the more distal colonic sites due to reduced production of SCFAs (since fermentable dietary fibers are less available here) and because of their absorption in exchange with bicarbonate, the butyrate-producing bacteria are drastically reduced, which

corresponds with an increase in acetate- and propionate-producing bacteria (Bacteroidetes phylum)[2].

Considering the homeostatic impact of indigenous microbiota and secretory immunity, in the process of evolution, the mucosal immune system has developed two levels of anti-inflammatory defense: first, immune exclusion performed by sIgA (and secretory IgM) antibodies to modulate or inhibit surface colonization of microorganisms and dampen the penetration of potentially dangerous antigens; and second, suppressive mechanisms to avoid local and peripheral hypersensitivity to innocuous antigens, particularly food proteins and components of commensal bacteria. Therefore, our data suggest that it is possible for the immune system of the gut to produce an environment under the gut, given the observation of the high NLR along with the increase in the SCFA butyrate immediately after surgery.

The link between brain abnormalities and heart surgery remains unclear. However, we can speculate that the role of acetate is to help avoid brain abnormalities after heart surgery[15]. A previous study explored the role of the SCFA acetate, a product of the fermentation of carbohydrate in the colon, and the results suggested that acetate derived from the colon induces an anorectic signal in the hypothalamic arcuate nucleus by supporting the glutamate–glutamine transcellular cycle, leading to an increase in lactate and GABA production[15, 16]. Therefore, it appears that both GABA and the SCFA acetate are very important for a child's mental development and other functions[15, 16].

Overall, our preliminary findings and the results of previous work suggest that changes in the intestinal environment might contribute to earlier postoperative recovery with respect to enteral nutritional intake and defecation. Therefore, further studies should assess the value of probiotic administration with monitoring of GABA production for perioperative management[16].

In conclusion, our study showed that the NLR reaches normalization faster than CRP following CPB in infants with DS. Therefore, NLR is potentially a better biomarker for monitoring post-operative inflammation or early infection during perioperative management. These preliminary observations suggest that gut microbiota, sIgA, and three SCFAs (butyrate, acetate, and propionate) may be involved in a three-way interaction for neuro-immune crosstalk. This hypothesis and its relevance to perioperative management will be explored in future research.

4. Methods

4.1 Subjects

This study was approved by the ethics committee for clinical investigation of Sakakibara Heart Institute (approval number 15-021. 23; approved on July 2015, Tokyo, Japan). Infants with DS and congenital heart disease who were admitted to our pediatric intensive care unit and scheduled to undergo cardiac surgery between 1 to 52 weeks after birth were enrolled in this study. Signed informed consent was obtained from the parents.

4.2 Fecal sample collection

Fresh fecal samples were collected in a tube from each subject immediately before (pre-CPB) and after (post-CPB) CPB as well as upon hospital discharge (Last). These samples were stored at –80℃ until use.

4.3 Extraction of fecal DNA

DNA was extracted from each 20-mg stool specimen by adding the specimen to 550 μL of 60 mM Tris–HCl + 30 mM EDTA (pH 8.0) buffer (TE buffer), followed by suspension by vortexing at maximum speed for 1 min. The suspension was transferred to a 2-mL screw-cap microtube containing 300 mg

of glass beads (/ = 0.1 mm) with 50 μL of 10% SDS solution and 500 μL phenol saturated with TE buffer (pH 8.0). The tube was vortexed at maximum speed for 1 min and incubated at 70℃ for 10 min. After incubation, the tube was shaken vigorously at 4,000 rpm for 1 min using a micro-smash MS-100 bead beater (Tomy Seiko Co., Ltd., Tokyo, Japan). The tube was then placed on ice for 5 min and centrifuged at 15,000 rpm for 5 min at 4℃. Five hundred microliters of the upper liquid phase was transferred to a new 1.5-mL 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 min and then cooled on ice for 5 min. The mixture was then centrifuged at 15,000 rpm for 5 min at 4℃, and 350 μL of the upper liquid phase was transferred to a new 1.5-mL centrifuge tube and mixed with 11.6 μL of 3 M sodium acetate and 3 μL of Ethachinmate (Nippon Gene, Tokyo, Japan). The solution was mixed with a 0.7-volume of isopropanol and centrifuged at 15,000 rpm for 5 min. The precipitated DNA was rinsed twice with 1 mL of 70% ethanol, dried, and dissolved in 100 μL of nuclease-free water. The DNA sample was stored at −20℃ until use.

4.4 16S rRNA amplicon library preparation and next-generation sequencing

The V1-V2 region of the bacterial 16S rRNA gene was amplified by PCR with fusion primers. The forward primer contained an Ion A adapter followed by a key, barcode, adapter (GT), and 27Fmod primer sequence (AGRGTTTGATYMTGGCTCAG)[17]. The reverse primer has an Ion truncated P1 adapter followed by an adapter (CC) and 338R primer sequence (TGCTGCCTCCCGTAGGAGT)[17]. PCR was performed in 25-μL reaction mixtures containing 23.5 μL of Platinum® PCR SuperMix High Fidelity (Invitrogen™) (ThermoFisher Scientific, Waltham, MA, USA), 0.5 μL of primer mixture (5 μM each), and 1 μL of DNA solution. Thermal cycling conditions were 3 min at 94℃, followed by 25 cycles of 30 s at 94℃, 45 s at 55℃, and 1 min at 68℃. The amplicon was purified using PureLink PCR Purification Kit (Invitrogen™, ThermoFisher Scientific). The purified sample was loaded onto a 2.0% agarose gel and electrophoresed at 50 V for 1.5 h at 25℃. 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 the 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 Fluorometer (Invitrogen™, ThermoFisher Scientific) using a Quant dsDNA HS Assay Kit (Invitrogen™, ThermoFisher Scientific). Molecular concentrations were adjusted according to the ion torrent protocol (ThermoFisher Scientific). Emulsion PCR was performed using Ion PGM Template OT2 400 Kit (ThermoFisher Scientific) according to the manufacturer's protocol. The library sequencing was done using Ion PGM Sequencing 400 Kit (ThermoFisher Scientific) and Ion 318 Chip V2 (ThermoFisher Scientific) according to the manufacturer instructions. Sequencing was performed on an Ion PGM System (ThermoFisher Scientific).

4.5 Sequence analysis

Sequence data were obtained in FASTAQ format and analyzed using QIIME software[18]. Raw sequences were sorted according to the barcode, and screened using an average quality score of ≥20 and the priming sites to obtain sequences of around 300 bp. The trimmed sequences were clustered into operational taxonomic units (OTUs) at the level of 97% similarity by using the UCLUST method[19] and the furthest-neighbor algorithm. The most abundant sequence in the OTU was chosen as the repre-

sentative sequence. The representative sequences were aligned with the PyNAST algorithm [20], and the aligned sequences were checked for potentially chimeric sequences using the ChimeraSlayer algorithm. Non-chimeric sequences were assigned to a taxon using the RDP classifier [21] at an 80% confidence cutoff value.

4.6 SCFA analysis with gas chromatography-mass spectrometry (GC-MS)

Frozen fecal samples (approximately 50 mg) were diluted 10-fold with H_2O, and soluble substances were extracted three times by intense mixing for 1 min followed by storage for 5 min in an icebox. After extraction, the precipitate was removed by centrifugation (10,000 × *g* at 4℃ for 5 min). Fecal extracts were spiked with 10 μL of 100 μM crotonic acid as an internal standard, and were deproteinized with 10% (v/v) of a 20% (w/v) 5-sulfosalichylic acid solution. Then, the samples were centrifuged at 15,000 × *g* at 4℃ for 10 min, and the supernatant were stored at −80℃ until analysis. Two hundred microliters of deproteinized fecal extracts were thawed on ice, and transferred into a new 2-mL tube. After acidification with 10 μL of hydrochloric acid (37%), SCFAs were extracted with 1 mL of diethyl ether by mixing for 15 min. After the mixing step, the two phases were separated by centrifugation (15,000 × *g* at 4℃ for 5 min), and the upper organic layer containing SCFAs was transferred into a new tube. The extraction procedure was performed in two cycles: 500 μL of each organic layer was placed in a glass vial for GC-MS, added to 50 μL of N-tert-butyldimethylsilyl-N-methyltrifluoracetamide (MTBSTFA; Sigma-Aldrich Co., St. Louis, MO, USA) as derivatization reagent, and kept at room temperature for 24 h in the dark. Two microliters of the derivatized sample was injected with a 5:1 split into a gas chromatograph coupled with a mass spectrometer detector (GCMS-QP2010, Shimadzu Co., Kyoto, Japan). The analysis was carried out on a ZB-5 capillary column (60 m × 0.25 mm, 0.25-μm film thickness, Shimadzu GLC Ltd., Tokyo, Japan). Helium was used as a carrier gas. The temperatures of the injector and source were 250℃ and 200℃, respectively. The GC oven was programmed as follows: initial temperature 55℃, increased to 70℃ at a rate of 10℃/min, increased to 280℃ at a rate of 20℃/min, and finally held for 3 min. Quantification was performed using selected ion monitoring as follows: m/z 117 for acetic acid, m/z 131 for propionic acid, m/z 145 for butyric acid, m/z 159 for valeric acid, m/z 173 for crotonic acid, m/z 261 for lactic acid, and m/z 289 for succinic acid. The extraction rate and the derivatization rate were standardized using crotonic acid, and the quantification was performed using an external calibration curve.

4.7 Fecal pH measurement

Fecal samples were dissolved 10-fold with Milli-Q water and the pH was measured directly using a pH meter (Twin pH AS-212, Horiba, Ltd., Kyoto, Japan).

4.8 Determination of sIgA contents

Fecal sIgA was measured using a commercially available sandwich ELISA kit, for sIgA (Immundiagnostik AG, Bensheim, Germany). The reaction was performed using 50,000-fold diluted fecal extracts after preliminary measurements. The reaction solution was quantified spectrophotometrically at 450 nm on a TECAN GENios spectrophotometer (Tecan Trading AG, Switzerland).

4.9 NLR

The NLR was calculated as the ratio of neutrophils to lymphocytes obtained from the same auto-

mated blood sample tests taken upon inclusion in the study.

4.10 Statistical analysis

The alteration in fecal microbiota and the changes in serum NLR and CRP between the post-CPB (Day 1–2) sample and pre-CPB sample, and among the post-CPB (Day 3–4), Last (upon hospital discharge) sample, and post-CPB (Day 1–2) samples were evaluated with the paired t-test, using StatMate VI statistical software (Atoms, Tokyo, Japan).

Conflicts of interest

There were no identified associations between the study conclusion and sponsorship under COI.

Kyodo Milk Industry Co., Ltd. has no consideration for interest by using the results from our study because they can have exploratory study on our paper that is "Preliminary study of neuro-immune cross-talk by gut microbiota for perioperative management in infants with congenital heart disease" as co-authors under using specific technology by themselves.

Statement of financial support

This study was supported by a grant for medical research (2015) from the Sakakibara Heart Institute Foundation.

Ethics committee review

This study was approved by the ethics committee for clinical investigation of Sakakibara Heart Institute (approval number 15-021. 23; approved on July 2015, Tokyo, Japan).

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(Submitted February 24, 2016)
(Accepted March 28, 2016)
(Published April 12, 2016)

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