# Patterned progression of gut microbiota predisposes preterm infants to necrotizing enterocolitis and late onset sepsis: pilot data from a non-Western population

**Jiayi Liu[1], Jianhua Sun[2], Yuqing Li[3], Yi Feng[4], Liya Pan[5], Zhoulonglong Xie[6], Zhilong Yan[7], Jianhua Zhao[8], and Li Hong[9]**

[1]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[2]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[3]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[4]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[5]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[6]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[7]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**
[8]**Shanghai Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China**
[9]**Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China**

Corresponding author:

Li Hong[9]

Email address: hongli@scmc.com.cn

## ABSTRACT

**Background and Objectives**
Intestinal microbiota dysbiosis might predispose preterm infants to necrotizing enterocolitis(NEC) and late onset sepsis(LOS). In this observational prospective study, we aimed to profile and compare postpartum microbiota progression patterns in non-Western preterm patients with either condition.
**Methods**
We enrolled preterm infants with gestational age less than 33 weeks and birth weight more than 950g, from July 2013 to December 2014. We began fecal sample collection from the the first stool after birth and prospectively collected until discharge. Bacterial V3 V4 region of 16s rRNA genes from each stool sample were amplified, sequenced and analyzed.
**Results**
A total of 192 fecal samples from 24 patiens were studied, of whom four developed NEC, three LOS; the remaining 17 were used as controls. [The post-partum gut microbiota colonization started to diverge among NEC, LOS and their matched control groups, from the second week after birth. Microbiota of the LOS infants was the least diversified (Shannon index=1.66), while that of the control group was the most diversified(Shannon index=0.88, p=0.01). Potentially pathogenic genus Enterococcus (20.86%) and Staphylococcus (8.67%) were prominent in NEC patients and Klebsiella (42.15%) in LOS group. Both two groups addressed lower proportion of Lactococcus (7.98% and 13.76% in NEC and LOS group, respectively) than the control group (3.66%)].
**Conclusions**
Postpartum colonization pattern of gut microbiome might predispose preterm newborns to NEC or LOS, in which reduced diversity of the whole microbiota community and potentially pathogenic genus could have played an essential role in disease progression. Still, more studies are needed to identify etiological strains, underlying mechanisms and correspondent microbial patterns.

# INTRODUCTION

Gut microbiota is a key contributor to human health and the dysbiosis of which are proven to be associated with diseases, such as atherosclerosis(Tang et al., 2017), obesity(Bouter et al., 2017), neuropathy(Sarkar et al., 2016), liver diseases(Tilg et al., 2016), etc. Temporal colonization pattern of the intestinal microbiota during early stages of life also provided evidence of its association with early life events, including Type 1 diabetes(Giongo et al., 2011; Vatanen et al., 2018), asthma(Stokholm et al., 2018) and allergy(Madan et al., 2012; Savage et al., 2018). In light of interindividual variability in gut maturity, innate immunity, birth mode and enviromental exposures, preterm infants are predisposed to complications post-partum intestinal microbiota development

In preterm infants, necrotizing enterocolitis and late onset sepsis

Necrotizing enterocolitis, characterized by rapid progression, high morbidity and mortality, is one of the most devastating gestrointestinal neonatal emergencies, especially in preterm newborns. Previous studies have suggested well how intestinal microbiota pattern is implicated prior to, during and after the course of this condition. Mai et al. reported an increase in the Proteobacteria and a decrease in the Firmicutes phyla during three to seven days prior to NEC onset (Mai et al., 2011). Zhou et al. found a relatively higher abundance of Clostridium and Gamma-Proteobacteria in the proximity of NEC during early and late onset, respectively(Zhou et al., 2015).

late onset sepsis is

Among non-Western population, however, evidence (few studies on?) of microbiota chronological dysbiosis preceding necrotizing enterocolitis or late onset sepsis has been scant so far. Hence, conducting a prospective study among Chinese patients using high-throughput DNA sequencing, we aimed to profile and compare post-postpartum pattern of intestinal microbiota in preterm infants who subsequently developed necrotizing enterocolitis and late onset sepsis, which may be critical in the etiopathogenesis of disease.

# METHODS

## Ethics

This study was approved by the joint committee of ethics of Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University (SCMCIRB-K2013022). Detailed written informed consent was obtained from the parents prior to fecal sample collection.

## Patients

Newly born preterm infants with gestational age less than 33 weeks, birth weight over 950g were enrolled from Neonatal Intensive Care Unit at Shanghai Children's Medical Center from July 2013 to December 2014. The exclusion criteria were 1) diagnosed with early-onset sepsis, 2) hepatic diseases, 3) renal impairment (Cr $>88$ $\mu$M), 4) diagnosed with intestinal obstruction, 5) in foreseeable need of cardiovascular or abdominal surgeries (except for male circumcision or PDA ligation), 6) estimated parenteral support to supply over 50% of daily caloric intake for more than four days, 7) given intravenous antibiotics administration (except prophylactic regimen of cefotaxime, piperacillin-tazobactam and/or metronidazole), 8) history of oral antibiotics administration, 9) grossly bloody stools at admission, and 10) over five days old.

NEC cases were defined as infants who met the criteria for Stage II and Stage III NEC disgnosis(Bell et al., 1978), including radiographic intestinal dilation, ileus, pneumatosis intestinalis, and/or absent bowel sounds with or without abdominal tenderness, and/or mild metabolic acidosis and thrombocytopenia. LOS cases was diagnosed if 1)an infant had a positive hemoculture or other suspicious loci of infection after 72 hours of life, with septic signs/symptoms reviewed independently by at least two neonatologists, and had been treated with advanced antibiotics (e.g., Meropenem) after diagnosis. Infants with no infectious complications or sepsis were regarded as controls.

## Sample collection and handling

Fecal samples collection began from neonatal meconium till discharge. Although we intended to collect fecal samples on a daily basis, due to working shifts and flexible clinical scheduling, we set seven days as the maximum interval between two collections from every infant. Every sample was collected within 30 minutes of defecation from patients' diaper with a sterile spatula. The samples were immediately placed in a cryogenic vial on dry ice and stored at 80°C within 30 minutes without additives. All samples were collected and stored before knowing the diagnosis of respective patients.

## DNA extraction and quality control amplification and 16s rRNA gene sequencing

Microbial genomic DNA was isolated from each fecal specimen using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to manufacturer's protocols. The concentration and purity of the DNA were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was checked by 1% agarose gel electrophoresis.

## Broad-range PCR and High-throuput Sequencing of 16s rRNA gene amplicons

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from each sample using bacterialarchaeal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGG GTWTCTAAT-3') using thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were as follows: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s annealing at 55 °C and 45 s elongation at 72 °C, and a final extension at 72 °C for 10 min. The PCR reactions were performed in triplicate, with each 20 $\mu$L mixture containing 4 $\mu$L 5X FastPfu Buffer, 2 $\mu$L 2.5 mM dNTPs, 0.8 $\mu$L of each primer (5 $\mu$M), 0.4 $\mu$L FastPfu Polymerase and 10 ng template DNA. The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocols.

Equimolar amounts of purified amplicons were pooled and pairedend sequenced (2 x 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The reads were de-multiplexed using the Illumina software and separate FASTQ files were generated for each specimen and deposited to the Sequence Read Archive NCBI under the BioProject accession PRJNA470548. Another public archive repository is available at figshare doi: 10.6084/m9.figshare.7205102

## Raw Data Processing

After pyrosequencing, de-multiplexed sequence reads were subjected to quality filtering utilizing Trimmomatic software(Bolger et al., 2014)version????), and were truncated at any site with an Phred score $<20$ over a 50bp-sized window; barcode matching with the primer mismatch from 0 to 2 nucleotides was adopted and reads containing ambiguous characters were removed. After trimming, FLASh(Fast Length Adjustment of Short Read)(**?**), a read pre-processing software, assembled and merged the paired-end

reads from fragments and generated >10 bp overlapped, with the dead match ratio 0.2. Unassembled reads were discarded.

To fairly compare all the samples at the same sequencing depth, the "sub.sample" command of mothur program(version1.30.1)(Schloss et al., 2009) was used for normalization to the smallest sample size. UCHIME Algorithm detected chimeric sequences, removed chimera to obtain effective reads, which were then sorted by cluster size and processed using Operational Taxonomic Units(OTUs) were clustered with 97% similarity cutoff using USEARCH v7(UPARSE version 7.1). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm(Wang et al., 2007) against the Silva (SSU128)(Quast et al., 2012) 16S rRNA database using confidence threshold of 70%. Each sequence was assigned the taxonomy by QIIME(Caporaso et al., 2010). The representative sequences were allocated phylogenetically down to the domain, phylum, class, order, family, and genus levels. The relative abundance of a given taxonomic group was calculated as a percentage of the sequences number belonging to that group devided by the total number of obtained sequences.

Alpha diversty analysis, including Shannon index and Observed species richness (sobs), were obtained using the "summary.single" command of mothur program(version1.30.1).

### Bacterial Species Classification Reference Set Creation

### Statistical and Bioinformatics Analysis

#### *Demographics and Clinical Sample comparisons*

Non-parametric tests, including Kruskal-Wallis test and Wilcoxon rank-sum test were used to compare gestational age, birth weight, age when the patients were diagnosed as NEC or LOS and length of stay at $\alpha$ level of 0.05 among three groups. All statistical test not involving microbiome 16s rRNA sequencing data was performed using *"stats"* package using R(v.3.5.1).

#### *Microbiota and Bioinformatics Analyses*

#### *Figures*

Figures were generated with the *"ggpubr"*(Kassambara, 2017) and *"ggplot2"*(Wickham, 2016) packages using R(v.3.5.1)

## RESULTS

### Patients and samples characteristics

After raw data quality control, 7,472,400 V3-V4 tags of 16s rRNA gene were produced, with an average length of 448 bp.(Table S1)

## DISCUSSION

Previous studies have

## CONCLUSIONS

Necrotizing enterocolitis, a worldwidely concern that threatern


## ACKNOWLEDGMENTS

We appreciate the support from enrolled patients, their families, and all staffs at Shanghai Children's Medical Center.


## SOME LaTeX EXAMPLES

Use section and subsection commands to organize your document. LaTeX handles all the formatting and numbering automatically. Use ref and label commands for cross-references.

### Figures and Tables

Use the table and tabular commands for basic tables — see Table 1, for example. You can upload a figure (JPEG, PNG or PDF) using the project menu. To include it in your document, use the includegraphics command as in the code for Figure 1 below.

**Figure 1.** An example image.

| Item | Quantity |
|---|---|
| Widgets | 42 |
| Gadgets | 13 |

**Table 1.** An example table.

## Citations

LaTeX formats citations and references automatically using the bibliography records in your .bib file, which you can edit via the project menu. Use the cite command for an inline citation, like **?**, and the citep command for a citation in parentheses (**?**).

## Mathematics

LaTeX is great at typesetting mathematics. Let $X_1, X_2, \ldots, X_n$ be a sequence of independent and identically distributed random variables with $\mathrm{E}[X_i] = \mu$ and $\mathrm{Var}[X_i] = \sigma^2 < \infty$, and let

$$S_n = \frac{X_1 + X_2 + \cdots + X_n}{n} = \frac{1}{n}\sum_{i}^{n} X_i$$

denote their mean. Then as $n$ approaches infinity, the random variables $\sqrt{n}(S_n - \mu)$ converge in distribution to a normal $\mathcal{N}(0, \sigma^2)$.

## Lists

You can make lists with automatic numbering ...

1. Like this,
2. and like this.

... or bullet points ...

- Like this,
- and like this.

... or with words and descriptions ...

**Word** Definition

**Concept** Explanation

**Idea** Text

We hope you find write$\LaTeX$ useful for your PeerJ submission, and please let us know if you have any feedback. Further examples with dummy text are included in the following pages.

# METHODS

Quisque ullamcorper placerat ipsum. Cras nibh. Morbi vel justo vitae lacus tincidunt ultrices. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. In hac habitasse platea dictumst. Integer tempus convallis augue. Etiam facilisis. Nunc elementum fermentum wisi. Aenean placerat. Ut imperdiet, enim sed gravida sollicitudin, felis odio placerat quam, ac pulvinar elit purus eget enim. Nunc vitae tortor. Proin tempus nibh sit amet nisl. Vivamus quis tortor vitae risus porta vehicula.

$$\cos^3\theta = \frac{1}{4}\cos\theta + \frac{3}{4}\cos 3\theta \tag{1}$$

Fusce mauris. Vestibulum luctus nibh at lectus. Sed bibendum, nulla a faucibus semper, leo velit ultricies tellus, ac venenatis arcu wisi vel nisl. Vestibulum diam. Aliquam pellentesque, augue quis sagittis posuere, turpis lacus congue quam, in hendrerit risus eros eget felis. Maecenas eget erat in sapien mattis porttitor. Vestibulum porttitor. Nulla facilisi. Sed a turpis eu lacus commodo facilisis. Morbi fringilla, wisi in dignissim interdum, justo lectus sagittis dui, et vehicula libero dui cursus dui. Mauris tempor ligula sed lacus. Duis cursus enim ut augue. Cras ac magna. Cras nulla. Nulla egestas. Curabitur a leo. Quisque egestas wisi eget nunc. Nam feugiat lacus vel est. Curabitur consectetuer.

## Subsection

Suspendisse vel felis. Ut lorem lorem, interdum eu, tincidunt sit amet, laoreet vitae, arcu. Aenean faucibus pede eu ante. Praesent enim elit, rutrum at, molestie non, nonummy vel, nisl. Ut lectus eros, malesuada sit amet, fermentum eu, sodales cursus, magna. Donec eu purus. Quisque vehicula, urna sed ultricies auctor, pede lorem egestas dui, et convallis elit erat sed nulla. Donec luctus. Curabitur et nunc. Aliquam dolor odio, commodo pretium, ultricies non, pharetra in, velit. Integer arcu est, nonummy in, fermentum faucibus, egestas vel, odio.

**Paragraph** Sed commodo posuere pede. Mauris ut est. Ut quis purus. Sed ac odio. Sed vehicula hendrerit sem. Duis non odio. Morbi ut dui. Sed accumsan risus eget odio. In hac habitasse platea dictumst. Pellentesque non elit. Fusce sed justo eu urna porta tincidunt. Mauris felis odio, sollicitudin sed, volutpat a, ornare ac, erat. Morbi quis dolor. Donec pellentesque, erat ac sagittis semper, nunc dui lobortis purus, quis congue purus metus ultricies tellus. Proin et quam. Class aptent taciti sociosqu ad litora torquent per conubia nostra, per inceptos hymenaeos. Praesent sapien turpis, fermentum vel, eleifend faucibus, vehicula eu, lacus.

**Paragraph** Pellentesque habitant morbi tristique senectus et netus et malesuada fames ac turpis egestas. Donec odio elit, dictum in, hendrerit sit amet, egestas sed, leo. Praesent feugiat sapien aliquet odio. Integer vitae justo. Aliquam vestibulum fringilla lorem. Sed neque lectus, consectetuer at, consectetuer sed, eleifend ac, lectus. Nulla facilisi. Pellentesque eget lectus. Proin eu metus. Sed porttitor. In hac habitasse platea dictumst. Suspendisse eu lectus. Ut mi mi, lacinia sit amet, placerat et, mollis vitae, dui. Sed ante tellus, tristique ut, iaculis eu, malesuada ac, dui. Mauris nibh leo, facilisis non, adipiscing quis, ultrices a, dui.

## Subsection

Morbi luctus, wisi viverra faucibus pretium, nibh est placerat odio, nec commodo wisi enim eget quam. Quisque libero justo, consectetuer a, feugiat vitae, porttitor eu, libero. Suspendisse sed mauris vitae elit sollicitudin malesuada. Maecenas ultricies eros sit amet ante. Ut venenatis velit. Maecenas sed mi eget dui varius euismod. Phasellus aliquet volutpat odio. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Pellentesque sit amet pede ac sem eleifend consectetuer. Nullam elementum, urna vel imperdiet sodales, elit ipsum pharetra ligula, ac pretium ante justo a nulla. Curabitur tristique arcu eu metus. Vestibulum lectus. Proin mauris. Proin eu nunc eu urna hendrerit faucibus.

Aliquam auctor, pede consequat laoreet varius, eros tellus scelerisque quam, pellentesque hendrerit ipsum dolor sed augue. Nulla nec lacus.

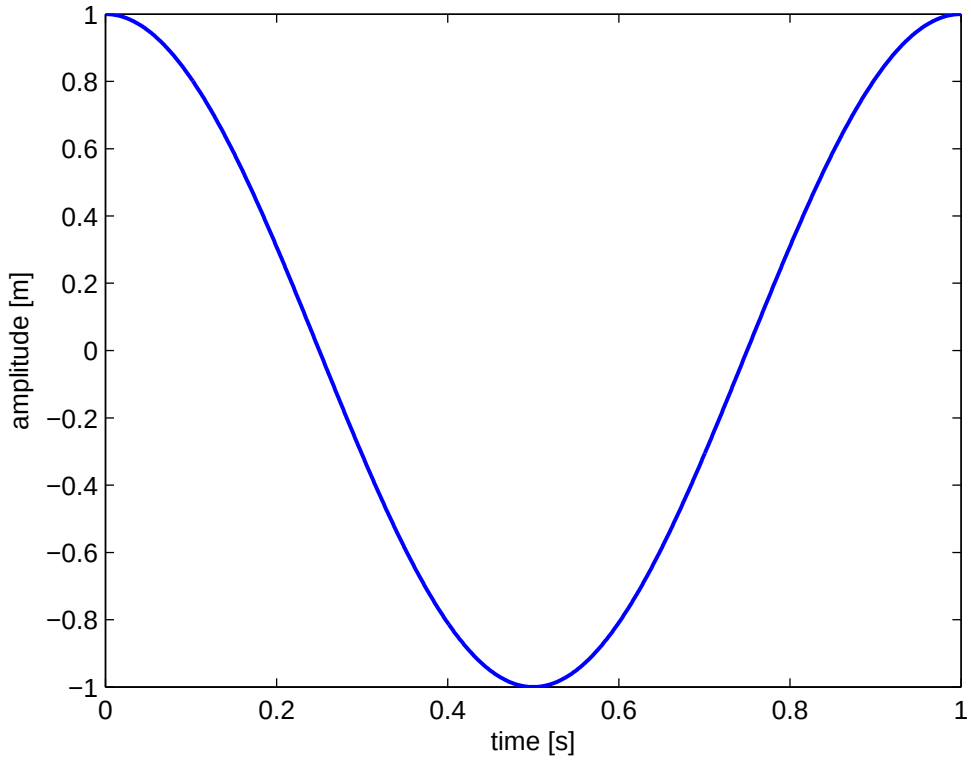


**Figure 2.** In-text Picture

Reference to Figure 2.

## RESULTS AND DISCUSSION

Suspendisse vitae elit. Aliquam arcu neque, ornare in, ullamcorper quis, commodo eu, libero. Fusce sagittis erat at erat tristique mollis. Maecenas sapien libero, molestie et, lobortis in, sodales eget, dui. Morbi ultrices rutrum lorem. Nam elementum ullamcorper leo. Morbi dui. Aliquam sagittis. Nunc placerat. Pellentesque tristique sodales est. Maecenas imperdiet lacinia velit. Cras non urna. Morbi eros pede, suscipit ac, varius vel, egestas non, eros. Praesent malesuada, diam id pretium elementum, eros sem dictum tortor, vel consectetuer odio sem sed wisi.

### Subsection

Sed feugiat. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Ut pellentesque augue sed urna. Vestibulum diam eros, fringilla et, consectetuer eu, nonummy id, sapien. Nullam at lectus. In sagittis ultrices mauris. Curabitur malesuada erat sit amet massa. Fusce blandit. Aliquam erat volutpat. Aliquam euismod. Aenean vel lectus. Nunc imperdiet justo nec dolor.

#### *Subsubsection*

Etiam euismod. Fusce facilisis lacinia dui. Suspendisse potenti. In mi erat, cursus id, nonummy sed, ullamcorper eget, sapien. Praesent pretium, magna in eleifend egestas, pede pede pretium lorem, quis consectetuer tortor sapien facilisis magna. Mauris quis magna varius nulla scelerisque imperdiet. Aliquam non quam. Aliquam porttitor quam a lacus. Praesent vel arcu ut tortor cursus volutpat. In vitae pede quis diam bibendum placerat. Fusce elementum convallis neque. Sed dolor orci, scelerisque ac, dapibus nec, ultricies ut, mi. Duis nec dui quis leo sagittis commodo.

### *Subsubsection*

Etiam ac leo a risus tristique nonummy. Donec dignissim tincidunt nulla. Vestibulum rhoncus molestie odio. Sed lobortis, justo et pretium lobortis, mauris turpis condimentum augue, nec ultricies nibh arcu pretium enim. Nunc purus neque, placerat id, imperdiet sed, pellentesque nec, nisl. Vestibulum imperdiet neque non sem accumsan laoreet. In hac habitasse platea dictumst. Etiam condimentum facilisis libero. Suspendisse in elit quis nisl aliquam dapibus. Pellentesque auctor sapien. Sed egestas sapien nec lectus. Pellentesque vel dui vel neque bibendum viverra. Aliquam porttitor nisl nec pede. Proin mattis libero vel turpis. Donec rutrum mauris et libero. Proin euismod porta felis. Nam lobortis, metus quis elementum commodo, nunc lectus elementum mauris, eget vulputate ligula tellus eu neque. Vivamus eu dolor.

## Subsection

Nulla in ipsum. Praesent eros nulla, congue vitae, euismod ut, commodo a, wisi. Pellentesque habitant morbi tristique senectus et netus et malesuada fames ac turpis egestas. Aenean nonummy magna non leo. Sed felis erat, ullamcorper in, dictum non, ultricies ut, lectus. Proin vel arcu a odio lobortis euismod. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Proin ut est. Aliquam odio. Pellentesque massa turpis, cursus eu, euismod nec, tempor congue, nulla. Duis viverra gravida mauris. Cras tincidunt. Curabitur eros ligula, varius ut, pulvinar in, cursus faucibus, augue.

Nulla mattis luctus nulla. Duis commodo velit at leo. Aliquam vulputate magna et leo. Nam vestibulum ullamcorper leo. Vestibulum condimentum rutrum mauris. Donec id mauris. Morbi molestie justo et pede. Vivamus eget turpis sed nisl cursus tempor. Curabitur mollis sapien condimentum nunc. In wisi nisl, malesuada at, dignissim sit amet, lobortis in, odio. Aenean consequat arcu a ante. Pellentesque porta elit sit amet orci. Etiam at turpis nec elit ultricies imperdiet. Nulla facilisi. In hac habitasse platea dictumst. Suspendisse viverra aliquam risus. Nullam pede justo, molestie nonummy, scelerisque eu, facilisis vel, arcu.

Curabitur tellus magna, porttitor a, commodo a, commodo in, tortor. Donec interdum. Praesent scelerisque. Maecenas posuere sodales odio. Vivamus metus lacus, varius quis, imperdiet quis, rhoncus a, turpis. Etiam ligula arcu, elementum a, venenatis quis, sollicitudin sed, metus. Donec nunc pede, tincidunt in, venenatis vitae, faucibus vel, nibh. Pellentesque wisi. Nullam malesuada. Morbi ut tellus ut pede tincidunt porta. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Etiam congue neque id dolor.

Donec et nisl at wisi luctus bibendum. Nam interdum tellus ac libero. Sed sem justo, laoreet vitae, fringilla at, adipiscing ut, nibh. Maecenas non sem quis tortor eleifend fermentum. Etiam id tortor ac mauris porta vulputate. Integer porta neque vitae massa. Maecenas tempus libero a libero posuere dictum. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Aenean quis mauris sed elit commodo placerat. Class aptent taciti sociosqu ad litora torquent per conubia nostra, per inceptos hymenaeos. Vivamus rhoncus tincidunt libero. Etiam elementum pretium justo. Vivamus est. Morbi a tellus eget pede tristique commodo. Nulla nisl. Vestibulum sed nisl eu sapien cursus rutrum.

Nulla non mauris vitae wisi posuere convallis. Sed eu nulla nec eros scelerisque pharetra. Nullam varius. Etiam dignissim elementum metus. Vestibulum faucibus, metus sit amet mattis rhoncus, sapien dui laoreet odio, nec ultricies nibh augue a enim. Fusce in ligula. Quisque at magna et nulla commodo consequat. Proin accumsan imperdiet sem. Nunc porta. Donec feugiat mi at justo. Phasellus facilisis ipsum quis ante. In ac elit eget ipsum pharetra faucibus. Maecenas viverra nulla in massa.

Nulla ac nisl. Nullam urna nulla, ullamcorper in, interdum sit amet, gravida ut, risus. Aenean ac enim. In luctus. Phasellus eu quam vitae turpis viverra pellentesque. Duis feugiat felis ut enim. Phasellus pharetra, sem id porttitor sodales, magna nunc aliquet nibh, nec blandit nisl mauris at pede. Suspendisse risus risus, lobortis eget, semper at, imperdiet sit amet, quam. Quisque scelerisque dapibus nibh. Nam enim. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Nunc ut metus. Ut metus justo, auctor at, ultrices eu, sagittis ut, purus. Aliquam aliquam.

# ACKNOWLEDGMENTS

So long and thanks for all the fish.

# REFERENCES

Bell, M. J., Ternberg, J. L., Feigin, R. D., Keating, J. P., Marshall, R., Barton, L., and Brotherton, T. (1978). Neonatal necrotizing enterocolitis. therapeutic decisions based upon clinical staging. *Annals of surgery*, 187(1):1.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics*, 30(15):2114–2120.

Bouter, K. E., van Raalte, D. H., Groen, A. K., and Nieuwdorp, M. (2017). Role of the gut microbiome in the pathogenesis of obesity and obesity-related metabolic dysfunction. *Gastroenterology*, 152(7):1671–1678.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nature methods*, 7(5):335.

Giongo, A., Gano, K. A., Crabb, D. B., Mukherjee, N., Novelo, L. L., Casella, G., Drew, J. C., Ilonen, J., Knip, M., Hyöty, H., et al. (2011). Toward defining the autoimmune microbiome for type 1 diabetes. *The ISME journal*, 5(1):82.

Kassambara, A. (2017). ggpubr:"ggplot2" based publication ready plots. r package version 0.1. 6.

Madan, J. C., Farzan, S. F., Hibberd, P. L., and Karagas, M. R. (2012). Normal neonatal microbiome variation in relation to environmental factors, infection and allergy. *Current opinion in pediatrics*, 24(6):753.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. (2012). The silva ribosomal rna gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1):D590–D596.

Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., and Burnet, P. W. (2016). Psychobiotics and the manipulation of bacteria–gut–brain signals. *Trends in neurosciences*, 39(11):763–781.

Savage, J. H., Lee-Sarwar, K. A., Sordillo, J., Bunyavanich, S., Zhou, Y., O'connor, G., Sandel, M., Bacharier, L. B., Zeiger, R., Sodergren, E., et al. (2018). A prospective microbiome-wide association study of food sensitization and food allergy in early childhood. *Allergy*, 73(1):145–152.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology*, 75(23):7537–7541.

Stokholm, J., Blaser, M. J., Thorsen, J., Rasmussen, M. A., Waage, J., Vinding, R. K., Schoos, A.-M. M., Kunøe, A., Fink, N. R., Chawes, B. L., et al. (2018). Maturation of the gut microbiome and risk of asthma in childhood. *Nature communications*, 9(1):141.

Tang, W. W., Kitai, T., and Hazen, S. L. (2017). Gut microbiota in cardiovascular health and disease. *Circulation research*, 120(7):1183–1196.

Tilg, H., Cani, P. D., and Mayer, E. A. (2016). Gut microbiome and liver diseases. *Gut*, 65(12):2035–2044.

Vatanen, T., Franzosa, E. A., Schwager, R., Tripathi, S., Arthur, T. D., Vehik, K., Lernmark, Å., Hagopian, W. A., Rewers, M. J., She, J.-X., et al. (2018). The human gut microbiome in early-onset type 1 diabetes from the teddy study. *Nature*, 562(7728):589.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive bayesian classifier for rapid assignment of rrna sequences into the new bacterial taxonomy. *Applied and environmental microbiology*, 73(16):5261–5267.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Zhou, Y., Shan, G., Sodergren, E., Weinstock, G., Walker, W. A., and Gregory, K. E. (2015). Longitudinal analysis of the premature infant intestinal microbiome prior to necrotizing enterocolitis: a case-control study. *PloS one*, 10(3):e0118632.