Exploration of S-ECC microbiota through a novel developed nutrient enriched microbiological medium, high through-put 16S rRNA sequencing and culturomics (#93239)

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Exploration of S-ECC microbiota through a novel developed nutrient enriched microbiological medium, high through-put 16S rRNA sequencing and culturomics

Yixin Zhang [Equal first author, 1, 2, 3, 4], **Yang Chen** [Equal first author, 5], **Chang Diao** [6], **Haojie Lin** [6], **Jingqi Zhu** [6], **Ruiqing Sun** [5], **Meng Wang** [1], **Yuke Chen** [6], **Hanzhang Zhou** [6], **Zixi Dong** [6], **Xia Bin** [Corresp., 5], **Yixiang Wang** [Corresp. 1]

[1] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China

[2] Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China

[3] National Engineering Laboratory for Digital and Material Technology of Stomatology, Peking University School and Hospital of Stomatology, Beijing, China

[4] Beijing Key Laboratory of Digital Stomatology, Peking University School and Hospital of Stomatology, Beijing, China

[5] Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, China

[6] Education department, Peking University School and Hospital of Stomatology, Beijing, China

Corresponding Authors: Xia Bin, Yixiang Wang
Email address: summerinbeijing@vip.sina.com, kqwangyx@bjmu.edu.cn

Introduction: Severe early childhood caries (S-ECC) is a widespread disease that harms children physically and mentally. Microorganisms are regarded as the dominant etiology of caries, however, S-ECC microbiome remains largely unknown, near 1/4 of them remained uncultivated. To explore S-ECC microbiota, a new bacterial medium, nutrient-enriched microbiological medium (NEMM) was designed in this study.

Methods: Eleven fresh S-ECC dental plaque samples were collected and cultivated in both NEMM and SHI medium (reference medium) for the indicated days under aerobic and anaerobic conditions. Then the cultures were harvested, together with their corresponding clinical S-ECC dental plaque samples, for high through-put 16S rRNA sequencing and culturomics. The single colonies were cultured for further confirmation by sequencing the full length of 16S rRNA gene after bacterial genomic DNA extraction and PCR amplification.

Results: Either NEMM or SHI medium showed a significant decrease in bacterial alpha diversity compared to clinical dental plaque samples by high throughput 16S rRNA sequencing analysis, indicating a larger room for the improvement of both media. NEMM displayed more living bacteria, abundant bacteria species, uncultured bacteria and capacities in carbohydrate transport and metabolism than SHI medium. The dynamic changes in bacterial community composition over time indicated that some bacteria tended to be enriched at specific time points. Culturomics and identification of bacterial species results were further confirmed by high throughput 16S rRNA sequencing results.

Conclusion: We developed a new medium NEMM that could support S-ECC microbiota growth with more living, abundant bacteria species and uncultured bacteria to be conducive to obtaining bacterial clinical isolates via culturomics technology.

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[1] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China.

[2]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China.

[3]National Engineering Laboratory for Digital and Material Technology of Stomatology, Peking University School and Hospital of Stomatology, Beijing, China.

[4]Beijing Key Laboratory of Digital Stomatology, Peking University School and Hospital of Stomatology, Beijing, China.

[5]Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, China.

[6]Education department, Peking University School and Hospital of Stomatology, Beijing, China.

†Equal contribution statement:
Yixin Zhang and Yang Chen have equal contribution to this work and are co-first authors of this paper.

Corresponding Author:
Dr. Bin Xia and Dr. Yixiang Wang are co-corresponding authors and equally contributed to this study.

Bin Xia, DDS, PhD
Address: Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, No. 22, Zhongguancun South Avenue, Haidian District, Beijing, 100081, China.
Email address: summerinbeijing@vip.sina.com

Yixiang Wang, DDS
Address: Central Laboratory, Peking University School and Hospital of Stomatology, No. 22, Zhongguancun South Avenue, Haidian District, Beijing, 100081, China.
Email: kqwangyx@bjmu.edu.cn

Abstract

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Introduction

Diverse microbiomes inhabit the human oral cavity, which is responsible for various infectious diseases, including tooth decay, periodontitis, and endodontic infection. Recent research also linked oral microorganisms with several systemic diseases, including diabetes(Sanz et al. 2018), Alzheimer's disease(Wu et al. 2022), and cardiovascular disease(Sanz et al. 2020). Tooth decay is one of the most prevalent oral diseases globally(Peres et al. 2019). Early childhood caries (Grier et al.) is defined as tooth decay of the primary tooth in children younger than 71 months, which affects 23% of children in the U.S.A. (Force 2021) and more than 60% in China(Hu et al. 2011). Early childhood caries (ECC) not only harms the physical and mental health of children but causes a heavy burden to society as well(Grier et al. 2021; Hemadi et al. 2017). Severe ECC (S-ECC) is an aggressive form of ECC. Based on the definition of S-ECC by the American Academy of Pediatric Dentistry, any sign of smooth-surface caries in a child younger than three years of age, and children aged 3–5 years who have one or more cavitated lesions, caries-caused missing or filled smooth surface in primary anterior teeth or decayed, missing or filled surfaces greater than or equal to four (age of 3), five (age of 4) or six (age of 5) are diagnosed as S-ECC patients(2016).

The etiology of S-ECC is generally accepted as the pathogenic biofilms established as the consequence of complex, dynamic interactions among microorganisms, diet, and the host. The disease process was modulated through the polymicrobial interaction between pathogenic microorganisms and commensals within the highly structured biofilms(Bowen et al. 2018). The latest research assigns that the microorganisms associated with caries are *Bifidobacterium dentium*, *Bifidobacterium adolescentis*, *Streptococcus mutans*, *Scardovia wiggsiae*, *Bifidobacterium longum*, *Selenomounas spp.*, *Prevotella spp.*, *Lactobacillus spp.*(Jenkinson 2011). More than 700 species of microorganisms have been identified in the oral cavity by next-generation sequencing technologies, however, a part of them are uncultivated(Aas et al. 2005; Dewhirst et al. 2010; Paster et al. 2001). The 16S rRNA gene is the most commonly used bacterial taxonomy gene in culture-independent techniques to identify caries pathogenic microorganisms(Pace et al. 1986). However, owing to Koch's postulates (the ground rules to determine whether a given organism can cause a given disease(Fredricks & Relman 1996)), the genetic heterogeneity of bacterial species and the strong influence of the environment on phenotype, species-level identification may not be sufficient(Wade 2013). Hence, intensive studies of pure cultured fastidious bacteria may provide us with a new understanding of the role of microbiology in S-ECC.

Shi and his colleagues developed the SHI medium to sustain a higher diversity of the oral microbial community and more similar microbial profiles to the original saliva-derived oral microflora(Tian et al. 2010b). Using SHI medium, He(He et al. 2015) revealed the TM7 phylum as "microbial dark matter" and its potential pathogenic associations.

Considering that the medium only displayed a certain level of selectivity of microorganisms, thus, in this study, we developed a novel nutrient-enriched microbiological medium (NEMM), to

cultivate more oral bacteria, especially for uncultivated microorganisms, and make the preparation for further investigation of the characterization of the microbiome in S-ECC.

Materials & Methods

Subjects and sample collection

Dental plaque samples were collected from eleven patients with S-ECC. Four boys and seven girls, aged 2.53 to 5.33 years old, were diagnosed that decayed, missing, and filled tooth surface (dmfs) scores were 13.91 ± 3.70 (Table S1). Participants were required to avoid eating, drinking and brushing their teeth for 2 h before collecting samples. Ethical approval of the study was granted by Peking University School of Stomatology (PKUSSIRB-201839140), and written informed consent was obtained from all study participants.

Eleven plaque samples were collected with a sterile curette from the labial smooth surfaces or the decayed cavity. The dental plaque was transferred to 1.5 mL sterile centrifuge tube full of pre-reduced medium (0.15g sodium mercaptoacetate, 0.9 mL fresh 1% calcium chloride, and 100 mL distilled water, filtered for removing bacteria) and taken to the laboratory and processed for culture within 3 min. Samples were dispersed by vortexing for 30 s and diluted tenfold consecutively.

Culture media

All samples were grown in duplicate at 37°C under anaerobic conditions (80% N2, 10% H2, 10% CO2) and normoxic conditions. To mimic the nutritional environment of the dental plaque, a novel culture medium was designed. The basal liquid medium consisted of brain-heart infusion broth 24.5 g/L, agar 15 g/L, dextrose 5 g/L, corn starch 1.5 g/L, casein acid hydrolysate 17.5 g/L, hemin 5 mg/L, VitK 1 mg/L, VitB12 100 μg/L, L-glutamine 0.1 g/L, adenine 10 mg/L, guanine hydrochloride 300 μg/L, p-aminobenzoic acid 130 μg/L, nicotinamide adenine dinucleotide 2.5 mg/L, thiamine pyrophosphate 1 mg/L, ferric nitrate 200 μg/L, cysteine hydrochloride 0.259 g/L, L-cysteine 11 mg/L, and sheep blood 5%. SHI medium was prepared as described before(Tian et al. 2010a).

Dental plaque culture

Samples were enriched aerobically and anaerobically using two liquid media SHI and NEMM designed by this study and subcultured in solid media after optimal dilution. The cultures were divided into four groups: (1) aNEMM (samples grew in NEMM anaerobically); (2) aSHI (samples grew in SHI medium anaerobically); (3) nNEMM (samples grew in NEMM under normoxic condition); and (4) nSHI (samples grew in SHI medium under normoxic condition). Consecutive tenfold dilutions of the plaque samples in two media were prepared by PBS and used to inoculate NEMM and SHI-agar plates in triplicate. Plates were incubated and photographed at 0, 1, 3, 6, 9, 15, 21 and 28 days. Meanwhile, pipetting 500 μL suspension cultures at these different time points were collected for 16S rRNA gene sequencing analysis and

bacterial isolation. For clinical isolates, suspension cultures were gradient diluted from 10-1 to 10-7, and then the diluted bacterial solutions were plated on the NEMM- and SHI-agar plates. The clinical isolates were picked up from the plates according to morphology and then identified by sequencing the full length of 16S rRNA gene as described below.

High through-put 16S rRNA gene sequencing

The samples of clinical dental plaque samples and their cultures in NEMM and SHI media at indicated time points were subjected to high through-put 16S rRNA gene sequencing in Biomarker Technologies (Biomarker Technologies, Beijing, China) where the total DNA was isolated, amplified and sequenced according to their standard procedures. In brief, microbial DNA was extracted from the planktonic bacterial suspensions according to the manufacturer's protocols. DNA concentration was assessed by a Nanodrop (Thermo Scientific) and quality was determined by agarose gel electrophoresis. Using 515F 907R barcoded primers, the variable regions 4 and 5 (V4-V5) of bacterial 16S rRNA genes were amplified by PCR. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 M of FastPfu Polymerase and 10 ng of template DNA. The amplicons were then extracted from 2% agarose gels and further purified by using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantified by QuantiFluor -ST (Promega). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform according to the instructions.

Bioinformatics analysis

The bioinformatics analysis was conducted using QIIME. The alpha diversity indices of Chao1, ACE, Shannon, Simpson and phylogenetic diversity (PD whole tree) were calculated using Mothur software (version v.1.30) with coverage over 99%. Beta diversity analysis was performed by Non-Metric Multi-Dimensional Scaling (NMDS) analysis based on the weighted unifrac and unweighted unifrac algorithm. Linear discriminant analysis (LDA) effect size (LEfSe) was conducted to define the biomarkers of these groups. The threshold on the logarithmic LDA score for the distinguishing features was set to 4.0. Microbial functions were predicted using PICRUSt2 (v1.0.0) software following the online protocol and aligned to the Clusters of Orthologous Groups of proteins (COG) database. The profiles of Kyoto Encyclopedia of Genes and Genomes (KEGG) levels were generated based on the KEGG database.

Species identification using full-length 16S rRNA gene amplicon sequence

Single colonies were picked up from NEMM and SHI-agar plates and inoculated into NEMM and SHI liquid media, cultured in aerobically or anaerobically, respectively. DNA from these clinical isolates was extracted using CTAB methods(Zheng et al. 2012). Full-length of 16S rRNA gene was amplified via PCR reaction. 27F (AGAGTTTGATCCTGGCTCAG) and 1492R(GGTTACCTTGTTACGACTT) serves forward and reverse primer, respectively. The full-length of 16S rRNA gene amplicon was sequenced using the Sanger method. The sequence of 16S rRNA gene was blasted in either NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) or

EzBioCloud (https://www.ezbiocloud.net/) to establish taxonomic relationships based on currently recognized prokaryotic reference species. Sequences with homology below 98.65% are defined as potential new strains(Kim et al. 2014).

Statistical analysis

T test was performed on the species abundance data between the groups using Metastats software. The species leading to the composition difference between the two groups of samples were determined by P value or Q value (correcting *P* value). The level of statistical significance (P value or Q value) was set as <0.05.

Results

General diversity of bacterial community between clinical dental plaque samples of patients with S-ECC and their cultured bacteria

To investigate the difference in bacterial community of -SECC among the original dental plaque, and cultured bacteria, we recruited 11 S-ECC patients to collect dental plaque, half for culture and half for 16S rRNA gene high-throughput sequencing directly without culture. Alpha diversity indices of ACE, Chao1, Shannon and Simpson showed clinical dental plaque samples had greater alpha diversity than either all their cultured bacteria (Fig. 1A) or the cultured bacteria under normoxia and anaerobic conditions (Fig. 1B), although Venn diagram showed that the OTU numbers of cultured bacteria under anaerobic and normoxia conditions were higher than that in clinical samples (Fig. 1C). Core flora of clinical S-ECC samples were 14 common features, including two unclassified *Streptococcus*, one unclassified *Granulicatella*, two unclassified *Veillonella*, one unclassified *Fusobacterium*, one unclassified *Aggregatibacter*, one unclassified *Kingella*, one unclassified *Lachnoanaerobaculum*, one unclassified *Lactobacillales*, one unclassified *Abiotrophia*, one unclassified *Rothia*, one *Rothia aeria*, and one *Capnocytophaga endodontalis*(Fig. S1).

To visualize the distribution of samples in coordinate system, Non-Metric Multi-Dimensional Scaling (NMDS) analysis showed that anaerobic cultured bacteria and normoxia cultured bacteria samples had similar culture features. However, they had a markedly difference bacterial structure in comparison with clinical sample group (Fig, 1D).

Percent stacked column displays the percentage of each high-abundance bacteria. In clinical samples, except *Veillonella*, *Leptotrichia* and *Streptococcus*, other bacteria occupied the most of bacteria. No matter under anaerobic and normoxia conditions, the percentages of other bacteria, *Veillonella* and *Leptotrichia* were clearly decreased, instead of the increases in *Desemzia*, *Actietobacterium*, *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *Vagococcus* and *Streptococcus* (Fig.1E). The results indicated there had more bacterial categories in clinical samples than cultures by media. NEMM and SHI media only could cultivate part of clinical bacteria in S-ECC plaques. There is still a lot of room for improvement in the culture medium.

To detect the difference among the groups, ANOVA (analysis of variance) was used to display the relative abundance of bacteria at the genus level. The results showed that both anaerobic and normoxia cultures were beneficial for the growth of *Streptococci* and *Bacillus* including *Bifidobacterium*. As for the relative abundance of most other bacteria, including *Leptotrichia*, *Veillonella*, *Corynebacterium*, *Actinomyces*, *Fusobacterium*, *Neisseria*, *Campocytophaga*, *Prevotella*, *Selenomonas*, *Campylobacter*, *Aggregatibacter*, and so on, was lower than those in clinical samples significantly. It suggested that most of above-mentioned bacteria categories could not successfully culture by NEMM and SHI media (Fig. 1F).

To assess the predicted function of the bacterial community in clinical samples and the cultured bacteria samples, microbial phenotype prediction was evaluated by BugBase followed by the Mann-Whitney U test. The results showed that the decreases in Gram-positive bacteria, facultatively anaerobic bacteria and stress tolerance ability, but the increases in Gram-positive bacteria, anaerobic respiration, biofilms forming and potential pathogenicity in clinical samples compared with cultured samples under aerobic and anaerobic conditions (Fig. 1G). The results indicated that uncultured Gram-negative, anaerobic and biofilm-forming bacteria may contribute to S-ECC development.

NEMM cultivates more abundant bacteria species compared to SHI medium

All cultured microbiota were examined in terms of both species proportion and abundance, with a specific focus on the enrichment of species. Of the organisms presented in the inoculum, all 32 phyla, 87 classes, 260 orders, 501 families, 1107 genera and 1502 bacterial species were captured. We identified 3719, 5605, 3705, and 3460 OTUs in the aNEMM, nNEMM, nSHI, and aSHI groups, respectively. Among them, 608 OTUs were uniform, occupying 4.9% of all the OTUs detected, indicating that a steady facultative anaerobe microorganism composition was found in all four fluid media. The other OTUs were not shared in all groups. Among these variable microbiomes, 2061 OTUs were unique to the aNEMM group, while 1848 OTUs could be detected only in aSHI. Besides, 4010 OTUs could be found only in nNEMM groups, while 2096 OTUs could be found only in nSHI groups.

To compare NEMM and SHI, which are suitable for cultivating specific bacterial types, we further analyzed 16S rRNA gene high-throughput sequencing data. Alpha diversity indices of ACE and Chao1 showed no significant difference between NEMM and SHI under the normoxia condition. However, under anaerobic culture conditions, NEMM had greater alpha diversity indices of ACE and Chao1 than SHI (Fig. 2A). NMDS analysis showed that no matter whether anaerobic or normoxia cultured bacteria samples had similar culture features (Fig. 2B). Venn diagram showed that the OTU numbers of NEMM cultured bacteria under anaerobic and normoxia conditions were higher than that in SHI cultured bacteria under the same conditions (Fig. 2C).

Differential predominant bacteria between NEMM and SHI were analyzed in the relative abundance of bacteria at the genus level by ANOVA. Of the 19 most predominant bacterial genera found in NEMM and SHI media, *Ligilactobacillus, Limosilactobacillus*, *Lactobacillus,* and *Desemzia*, showed the highest abundance levels. Based on the relative abundance of cultured bacteria, NEMM was suitable for cultivating bacteria including *Lactobacillus, Desemzia*, *Veillonella, Psychrobacterium,* and *Leptotrichia,* while SHI was good for culturing *Ligilctobacillus*, *Limosilactobacillus*, *Bacillus*, *Neisseria*, *Leuconostoc, Cryptobacterium*, *Aggegatibacterium*, and *Kingella* (Fig. 2D).

Bugbase was used to predict the function of bacterial community in NEMM and SHI under anaerobic and normoxia culture conditions. The results showed that the microbiota developed in NEMM were predicted stronger anaerobic ability, whether under anaerobic conditions or normoxic conditions. Under aerobic culture conditions, there were no significant differences in Gram-positive bacteria among the four groups (aNEMM, aSHI, nNEMM and nSHI). However, under anaerobic conditions, NEMM supported the growth of more gram-negative bacteria and less gram-positive bacteria (Fig. 2E). Dental plaque samples cultured in NEMM were less likely to be stress tolerance and more likely to be potential pathogens and form biofilms. Besides, it had weaker facultatively anaerobic ability when under anaerobic conditions (Fig.2F).

To assess the effects of the two media on the predicted gene categories (COGs), we compared the predicted COGs between aNEMM and aSHI, nNEMM and nSHI (Fig. 2G). NEMM showed more ability in carbohydrate transport and metabolism than SHI medium under both anaerobic conditions and normoxia conditions. Besides, those in NEMM displayed an increased relative abundance of protein functions linked to defense metabolism, inorganic ion transport and metabolism under anaerobic conditions. Furthermore, pathways of membrane transport and cellular community-prokaryotes were associated with aNEMM group (Fig. 2H).

NEMM and SHI media were suitable for culturing specific bacteria respectively

The predominant bacteria were largely consistent among the four groups (aNEMM, aSHI, nNEMM, nSHI), but different relative abundances could be observed (Fig. S2). More genus, species and uncultured bacteria were captured in aNEMM groups at day 1(Table S2) under anaerobical condition compared with those in aSHI Day1 group. For aerobic conditions, the difference between NEMM and SHI media were clearer (Table 1). Table S3 showed that genus, species and uncultured bacteria detected in nNEMM were higher compared to nSHI at each time point.

Principal component analysis of the development of the oral microbiome showed distinct shifts in the composition of the microbiome. We could see the changes in bacterial community structure over time in Figure 3A and Figure 3B. The proportions of *Streptococcus* were reduced along with time in the four groups. In this study, the NEMM as a growth and colonization substrate resulted

in a dynamic microbial succession, characterized by an initial proportional reduction in *Veillonella*, corresponding to an increase of *Lactobacillus* and *unclassified_Bacilli* proportions under anaerobic conditions. *Bifidobacterium* reached the highest percentage on the 15th day and then decreased in aNEMM group. In aSHI group, the proportions of *Lactobacillus* and *unclassified_Bacilli* increased gradually before the 15th day. While *Ligilactobacillus* increased rapidly from day one to day three in both aSHI and nSHI groups. In nNEMM group, *Ligilactobacillus, unclassified_Bacilli, Lactobacillus, Lacticaseibacillus* and *Lactiplantibacillus* showed the time-dependent accumulation, while *Acinetobacter* exhibited the opposite tendency.

The taxa that most likely explains the differences between NEMM and SHI media from different time points defined by LEfSe. Figure 3C and 3D showed cladograms representing the potential biomarkers of different groups. At the genus level, *Veillonella* was significantly enrichment in the aNEMM cultured Day1 group (aNEMM-Day1), while *Streptococcus*, *Gardiobacterium* and *Granulicatella* exhibited relatively higher abundance in the aSHI-Day1 group. *Bifidobacterium*, *Akkermansia* and *Desemzia* were significantly more abundant in the aNEMM-Day15, aNEMM-Day21 and aNEMM-Day28 groups, respectively. While *Ligilactobacillus*, *unclassified_Bacilli* and *Lacticaseibacillus* were most abundant in aSHI-Day6, aSHI-Day21 and aSHI-Day28 groups, respectively. For samples grown aerobically, significant bacterial differences were also detected, with *Veillonella*, *Desemzia* and *Lacticaseibacillus* remarkably enriched in the nNEMM-Day1, nNEMM-Day9 and nNEMM-Day28 groups at the genus level, while *Streptococcus* exhibited higher abundance in the nSHI-Day1 group (Fig.3E and 3F, LDA > 4, $P < 0.05$).

To study the difference in microbial community abundance between two groups of samples, Metastats software was used to conduct *t*-test on genus richness data between groups (Fig. 3G). At genus level, *Actinobacillus*, *Pseudoxanthomonas* and *Bilophila* were significantly enriched in aNEMM biofilms. Unclassified Clade Ⅲ, NS3a marine group and unclassified *Saprospiraceae* were more abundant in nNEMM suspension. The same tendency can also be seen in Figure S2. From the taxa tree, aNEMM was suitable for culturing *Anaerolinea*, *Aerococcus*, *Lactobacillus* and *Negativicutes*. *Planococcus*, *Desemzia* and *Psychrobacter* were enriched in NEMM no matter under normoxia or anaerobic condition. *Geobacillus*, *Lactococcus*, *Clostridiaceae*, *Hyphomicrobium*, *Pseudolabrys*, *Paracoccus*, *Rhodobacter*, *Rickettsiaceae*, *Sphingobium*, *Legionella*, *Moraxella*, *Luteimonas*, *Lysobacter* were suitable for growth in nNEMM. *Bacillus*, *Pediococcus*, *Brevundimonas*, *Caulobacter*, *Phenylobacterium*, *Sphingomonas* and *Pseudoxanthomonas* were suitable for growing in aSHI. *Blastomonas* were enriched in nSHI. *Abiotrophia* grew well in SHI under both normoxia and anaerobic conditions (Fig. S2).

Microbiota developed in NEMM and SHI media, under anaerobic and normoxia conditions exhibited distinct community structures. The special microbial profiles of the suspensions, cultured in NEMM medium and SHI medium, were listed in Table 2. At the genus level, *Anaerolinea*, *Planococcaceae*, *Acerococcus*, *Desemzia*, *Sphongopyxia*, *Psychrobacter* and some not assigned

were significantly enriched in aNEMM. *Planococcaceae, Geobacillus, Facklamia, Desemzia, Lactococcus, Blastomonas, Sphongopyxia, Legionella, Psychrobacter, Luteimonas, Lysobacter and Pseudoxanthomonas* were more abundant in nNEMM conditions. The aSHI condition was suitable for *Pseudograclibacillus*, *Sphingomonas* and *Pseudoxanthomonas*, while *Lactococcus, Blastomonas, Sphingomonas, Legionella, Luteimonas, Lysobacter* and *Pseudoxanthomonas* were more abundant in nSHI condition.

NEMM helps to culture previously uncultivated oral bacteria

NEMM supported the growth of many oral species that have not been cultured so far. All 128 previously uncultivated species were detected by 16s rRNA gene sequencing using SILVA as the reference database.

To study the difference in microbial community abundance between groups of samples, Metastats analysis was used to conduct *t*-test on species richness data between groups. Uncultured bacteria including *methanogenic_archaeon, Truepera_sp., Acidobacterium_sp., Desulfovibrionaceae_bacterium, Nitrosomonadaceae_bacterium* could only be detected in aNEMM group. Comparing relative abundance of these uncultured species between aNEMM and aSHI media, uncultured bacteria including *forest_soil_bacterium, Clostridiales_bacterium, Desulfuromonadales_bacterium, Firmicutes_bacterium* and *Alphaproteobacteria_bacterium* were observed higher in aNEMM group (Table S4). Discrepancies were also detected between the nNEMM and nSHI groups, with higher abundances of uncultured bacteria including *Desulfuromonadales_bacterium, Bacteroidetes_bacterium, Clostridium_sp., Mollicutes_bacterium, Bacteroidales_bacterium, Holophaga_sp.* and *Acidobacteria_bacterium* in the nNEMM group. Besides, uncultured bacteria including *methanogenic_archaeon, Sphingobacteriales_bacterium, Aminicenantes_bacterium, marine_bacterium, forest_soil_bacterium, Acidobacterium_sp., Xanthomonadaceae_bacterium, Sorangiineae_bacterium, marine_microorganism* and *candidate_division_SBR1093_bacterium* were only observed in the nNEMM group ($P < 0.05$) (Table S5).

Plaque samples were inoculated into the two media and incubated under normoxia and anaerobic, respectively, for 28 days (Fig. S4). After this process, the slow-growing colonies were subcultured and identified to isolate previous uncultivated phylotypes. Through culturomics, uncultivated bacterium belonging to the *Prevotella*, *Actinomyces*, *Pseudomonas, Granulicatella, Lactobacillus*, *Campylobacter*, *Streptococcus* and *Bacillus* genera were identified in the biofilm growth media. The oral taxa identified on the four conditions were shown in Fig. S5, displaying that both NEMM and SHI media could culture the uncultivated bacterium in aerobic and anaerobic conditions.

Also, it turned out that *Achromobacter spanius*, *Lactobacillus delbrueckii subsp* and *Actinomyces naeslundii* in aNEMM, *Pseudomonas sp*. and *Veillonella parvula* in aSHI, *Brevibacillus agri* in

nNEMM, *Leuconostoc lactis*, *Chryseobacterium indologenes*, *Cardiobacterium hominis* and *Kingella bonacorsii* in nSHI may tend to be enriched in each growth conditions.

Discussion

Due to different nutritional requirements, factors produced by other microbes, strict inter-species interactions, slow growth, competition/inhibition and dormancy in diversified bacterial species(Overmann et al. 2017; Tidjani Alou et al. 2020), developing a medium for cultivating microbiota, especially oral microbial flora, has always been a challenge.

In previous studies, SHI medium has been widely used for oral bacteria research, but it was originally designed to support different sub-populations within the original oral microbial community derived from pooled saliva(Edlund et al. 2013; Tian et al. 2010a). Specific nutrient or chemical requirements may be necessary for the growth of some fastidious bacteria. The studies functional alteration association with S-ECC indicated that carbohydrate metabolism, amino acid metabolism, and metabolism of cofactors and vitamins are the major pathways of KEGG in caries and healthy groups(Tang et al. 2022; Wang et al. 2019). Thus, we designed a novel bacterial medium attempting to culture the fastidious bacteria, especially for dental plaque in a diseased state of S-ECC.

In the metabolism of S-ECC, several pathways involving carbohydrate metabolism were deferentially regulated(Tang et al. 2022; Wang et al. 2019). Dextrose and corn starch served as carbon sources. NAD, TPP, and L-glutamine play key roles in glycolysis. As a cofactor in over 300 redox reactions, NAD (NAD(+)) and its reduced form (NADH) play a critical role in cellular metabolism due to their function (Zhou et al. 2011). TPP, an active form of thiamine (Vitamin B1), is an essential molecule for all living organisms(Goyer 2017). TPP acts as a co-factor for key enzymes of glycolysis such as pyruvate dehydrogenase, transketolase, and pyruvate decarboxylase(Llavero-Pasquina et al. 2022; Strobbe & Van Der Straeten 2018). Many bacteria rely on the TPP to lose the ability to produce thiamine de novo(Llavero-Pasquina et al. 2022).

Cofactors and vitamins are vital for bacteria growth and function as well. Glutamine comprises more than 50% of the body's free amino acid pool and is a precursor for the synthesis of nucleic acids and glutathione(Heys & Ashkanani 1999). Glutamine might initiate the signaling pathways related to the metabolism of nitrogenous compounds in bacteria(Dai et al. 2013). A study demonstrated that L-glutamine supplements in basal salt medium with carbon and nitrogen sources along with requisite inoculum size yielded the best quantities(Patel et al. 2021).

The studies showed that cobalamin (vitamin B12) has regulatory involvement in gene expression, microbial folate, ubiquinone, and methionine processes(Romine et al. 2017; Rosnow et al. 2018), suggesting a requirement for this co-factor. Cobalamin was demonstrated to be the essential

nutrilite(Lochhead & Thexton 1951), because of lacking the ability to biosynthesize cobalamin for most bacteria(Lu et al. 2020). Recent research reported that Porphyromonas gingivalis growth relies on Vitamin B12(Saiki et al. 2022).

Adenine and guanine have various functions in bacteria. Kiyoshi(Kiyoshi et al. 2017) et al. adds adenine to culture Clostridium saccharoperbutylacetonicum strain N1-4 at 37 ℃. The addition of adenine exhibits the maintenance of cell viability originating from the maintenance of ATP levels. Torino(Torino et al. 2005) et al. compared the growth and EPS production by Lact. helveticus ATCC 15807 in the different media. This study found that the addition of p-amino benzoic acid did not affect either on growth or the EPS production, while guanine displayed a dual effect, a decrease in cell growth and an increase in EPS formation. The addition of adenine enhanced both the cell growth and the EPS production.

NEMM medium aims to target the bacterial metabolism which can be used in service of their isolation. Bacteria must obtain nutrients from their surrounding environment to survive. Carbohydrates are the primary energy and carbon sources for most bacteria during growth in laboratory media. In bacteria, proteins in the outer membrane surrounding the cell actively transport carbohydrates and trace nutrients like iron into the cell's interior. In this study, NEMM met the needs of previous studies' findings that multiple pathways involving carbohydrate metabolisms were obviously up-regulated in the S-ECC group compared to caries free group(Tang et al. 2022; Wang et al. 2019). As such, the upregulation of carbohydrate transport and metabolism in NEMM might reflect more bacteria with higher cariogenic potential.

To date, although nearly 1/4 of bacteria are still uncultured in the oral cavity, researchers have known the existence of these uncultured bacteria through next-generation sequencing or third-generation sequencing technology, and therefore a better understanding of the human oral microbiome, mostly through amplicon sequencing and whole-genome metagenomics. However, some species were likely present at a low abundance in the inoculum and were therefore not detected via sequencing. Since then, many studies have combined high-throughput culture with metagenomics and reduced the dark matter associated with metagenomics through the isolation and sequencing of previously uncultured species(Lagier et al. 2015; Lagier et al. 2012). Therefore, we combined 16S rRNA gene sequencing and culturomics to prove that NEMM can support the development of more bacteria from S-ECC dental plaque and help to cultivate uncultured or targeted bacteria in this study.

Veillonella, which belongs to Gram-negative obligate anaerobic coccus, is recognized to play an important role in the S-ECC-causing process and identified as a candidate biomarker in S-ECC with low abundance or without S. mutans(Gross et al. 2012; Zhang et al. 2022). The potential biomarkers of different groups were presented by LEfSe. Among these significantly different genera, Veillonella was one of the most specific biomarkers in aNEMM group. Besides, we

exhibited the changing process of bacterial community structure through 16S rRNA gene sequencing and culturomics. It indicated a better capture ability for Veillonella before the first day in aNEMM group. Growth development and bacterial activity are important aspects that need to be addressed. It will be helpful for isolating targeted strains at proper time points. The relative abundance of bacterial genus shifted in biofilms over 28 days of growth in NEMM and SHI medium. It is likely that inhibition of metabolism, due to low pH and possibly carbohydrate limitation, starts to impact growth of some community members and leads to a shift in the community structure. Desemzia and Vagococcus, who were uncommon in the oral bacteria community, were found enriched in NEMM and SHI medium. Actually, there has been some articles that reported their existence in the oral cavity such as Vagococcus fluvialis(Al-Ahmad et al. 2008; Schirrmeister et al. 2009). We can rule out the possibility of medium contamination from Figure S5, so we speculate some of them may come from the food eaten such as milk or food additives (Wullschleger et al. 2018).

Conclusions

In conclusion, NEMM medium was superior in yielding more biomass when used to cultivate dental plaque-derived biofilm. 16s rRNA gene sequencing results strongly supported that NEMM medium was able to maintain an in vitro oral microbial community with high diversity. It also supported the growth of many oral species that have not been cultured so far, which may be helpful in capturing more pathogenic bacteria and will contribute to real strains for research via culturomics.

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References

2016. Policy on Early Childhood Caries (ECC): Classifications, Consequences, and Preventive Strategies. *Pediatric Dentistry* 38:52-54.

Aas JA, Paster BJ, Stokes LN, Olsen I, and Dewhirst FE. 2005. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 43:5721-5732. 10.1128/jcm.43.11.5721-5732.2005

Al-Ahmad A, Pelz K, Schirrmeister JF, Hellwig E, and Pukall R. 2008. Characterization of the first oral vagococcus isolate from a root-filled tooth with periradicular lesions. *Current Microbiology* 57:235-238. 10.1007/s00284-008-9182-0

Bowen WH, Burne RA, Wu H, and Koo H. 2018. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. *Trends Microbiol* 26:229-242. 10.1016/j.tim.2017.09.008

Dai ZL, Li XL, Xi PB, Zhang J, Wu G, and Zhu WY. 2013. L-Glutamine regulates amino acid utilization by intestinal bacteria. *Amino Acids* 45:501-512. 10.1007/s00726-012-1264-4

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, and Wade WG. 2010. The human oral microbiome. *J Bacteriol* 192:5002-5017. 10.1128/jb.00542-10

Edlund A, Yang Y, Hall AP, Guo L, Lux R, He X, Nelson KE, Nealson KH, Yooseph S, Shi W, and McLean JS. 2013. An in vitro biofilm model system maintaining a highly reproducible species and metabolic diversity approaching that of the human oral microbiome. *Microbiome* 1:25. 10.1186/2049-2618-1-25

Force UPST. 2021. Screening and Interventions to Prevent Dental Caries in Children Younger Than 5 Years: US Preventive Services Task Force Recommendation Statement. *JAMA* 326:2172-2178. 10.1001/jama.2021.20007 %J JAMA

Fredricks DN, and Relman DA. 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical Microbiology Reviews* 9:18-33.

Goyer A. 2017. Thiamin biofortification of crops. *Curr Opin Biotechnol* 44:1-7. 10.1016/j.copbio.2016.09.005

Grier A, Myers JA, O'Connor TG, Quivey RG, Gill SR, and Kopycka-Kedzierawski DT. 2021. Oral Microbiota Composition Predicts Early Childhood Caries Onset. *J Dent Res* 100:599-607. 10.1177/0022034520979926

Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, and Griffen AL. 2012. Beyond Streptococcus mutans: dental caries onset linked to multiple species by 16S rRNA community analysis. *PloS One* 7:e47722. 10.1371/journal.pone.0047722

He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, Dorrestein PC, Esquenazi E, Hunter RC, Cheng G, Nelson KE, Lux R, and Shi W. 2015. Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. *Proc Natl Acad Sci U S A* 112:244-249. 10.1073/pnas.1419038112

Hemadi AS, Huang R, Zhou Y, and Zou J. 2017. Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment. *Int J Oral Sci* 9:e1. 10.1038/ijos.2017.35

Heys SD, and Ashkanani F. 1999. Glutamine. *Br J Surg* 86:289-290. 10.1046/j.1365-2168.1999.01060.x

Hu DY, Hong X, and Li X. 2011. Oral health in China--trends and challenges. *Int J Oral Sci* 3:7-12. 10.4248/ijos11006

Jenkinson HF. 2011. Beyond the oral microbiome. *Environ Microbiol* 13:3077-3087. 10.1111/j.1462-2920.2011.02573.x

Kim M, Oh H-S, Park S-C, and Chun J. 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology* 64:346-351. 10.1099/ijs.0.059774-0

Kiyoshi K, Kawashima S, Nobuki K, Kadokura T, Nakazato A, Suzuki KI, and Nakayama S. 2017. Adenine Addition Restores Cell Viability and Butanol Production in Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564) Cultivated at 37°C. *Appl Environ Microbiol* 83. 10.1128/aem.02960-16

Lagier J-C, Hugon P, Khelaifia S, Fournier P-E, La Scola B, and Raoult D. 2015. The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clinical Microbiology Reviews* 28:237-264. 10.1128/CMR.00014-14

Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, Bittar F, Fournous G, Gimenez G, Maraninchi M, Trape JF, Koonin EV, La Scola B, and Raoult D. 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection : the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 18:1185-1193. 10.1111/1469-0691.12023

Llavero-Pasquina M, Geisler K, Holzer A, Mehrshahi P, Mendoza-Ochoa GI, Newsad SA, Davey MP, and Smith AG. 2022. Thiamine metabolism genes in diatoms are not regulated by thiamine despite the presence of predicted riboswitches. *New Phytol* 235:1853-1867. 10.1111/nph.18296

Lochhead AG, and Thexton RH. 1951. Vitamin B12 as a growth factor for soil bacteria. *Nature* 167:1034. 10.1038/1671034a0

Lu X, Heal KR, Ingalls AE, Doxey AC, and Neufeld JD. 2020. Metagenomic and chemical characterization of soil cobalamin production. *Isme j* 14:53-66. 10.1038/s41396-019-0502-0

Overmann J, Abt B, and Sikorski J. 2017. Present and Future of Culturing Bacteria. *Annual Review of Microbiology* 71:711-730. 10.1146/annurev-micro-090816-093449

Pace NR, Olsen GJ, and Woese CR. 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. *Cell* 45:325-326. 10.1016/0092-8674(86)90315-6

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, and Dewhirst FE. 2001. Bacterial diversity in human subgingival plaque. *J Bacteriol* 183:3770-3783. 10.1128/jb.183.12.3770-3783.2001

Patel NY, Baria DM, Yagnik SM, Rajput KN, Panchal RR, and Raval VH. 2021. Bio-prospecting the future in perspective of amidohydrolase L-glutaminase from marine habitats. *Applied Microbiology and Biotechnology* 105:5325-5340. 10.1007/s00253-021-11416-6

Peres MA, Macpherson LMD, Weyant RJ, Daly B, Venturelli R, Mathur MR, Listl S, Celeste RK, Guarnizo-Herreño CC, Kearns C, Benzian H, Allison P, and Watt RG. 2019. Oral diseases: a global public health challenge. *Lancet (London, England)* 394:249-260. 10.1016/S0140-6736(19)31146-8

Romine MF, Rodionov DA, Maezato Y, Anderson LN, Nandhikonda P, Rodionova IA, Carre A, Li X, Xu C, Clauss TR, Kim YM, Metz TO, and Wright AT. 2017. Elucidation of roles for vitamin B(12) in regulation of folate, ubiquinone, and methionine metabolism. *Proc Natl Acad Sci U S A* 114:E1205-e1214. 10.1073/pnas.1612360114

Rosnow JJ, Hwang S, Killinger BJ, Kim YM, Moore RJ, Lindemann SR, Maupin-Furlow JA, and Wright AT. 2018. A Cobalamin Activity-Based Probe Enables Microbial Cell Growth and Finds New Cobalamin-Protein Interactions across Domains. *Appl Environ Microbiol* 84. 10.1128/aem.00955-18

Saiki K, Urano-Tashiro Y, Yamanaka Y, and Takahashi Y. 2022. Calcium ions and vitamin B(12) are growth factors for Porphyromonas gingivalis. *J Oral Biosci*. 10.1016/j.job.2022.09.001

Sanz M, Ceriello A, Buysschaert M, Chapple I, Demmer RT, Graziani F, Herrera D, Jepsen S, Lione L, Madianos P, Mathur M, Montanya E, Shapira L, Tonetti M, and Vegh D. 2018. Scientific evidence on the links between periodontal diseases and diabetes: Consensus report and guidelines of the joint workshop on periodontal diseases and diabetes by the International Diabetes Federation and the European Federation of Periodontology. *Journal of Clinical Periodontology* 45:138-149. 10.1111/jcpe.12808

Sanz M, Marco Del Castillo A, Jepsen S, Gonzalez-Juanatey JR, D'Aiuto F, Bouchard P, Chapple I, Dietrich T, Gotsman I, Graziani F, Herrera D, Loos B, Madianos P, Michel J-B, Perel P, Pieske B, Shapira L, Shechter M, Tonetti M, Vlachopoulos C, and Wimmer G. 2020. Periodontitis and cardiovascular diseases: Consensus report. *Journal of Clinical Periodontology* 47:268-288. 10.1111/jcpe.13189

Schirrmeister JF, Liebenow A-L, Pelz K, Wittmer A, Serr A, Hellwig E, and Al-Ahmad A. 2009. New bacterial compositions in root-filled teeth with periradicular lesions. *Journal of Endodontics* 35:169-174. 10.1016/j.joen.2008.10.024

Strobbe S, and Van Der Straeten D. 2018. Toward Eradication of B-Vitamin Deficiencies: Considerations for Crop Biofortification. *Front Plant Sci* 9:443. 10.3389/fpls.2018.00443

Tang Z, Xu W, Zhou Z, Qiao Y, Zheng S, and Rong W. 2022. Taxonomic and functional alterations in the salivary microbiota of children with and without severe early childhood caries (S-ECC) at the age of 3. *PeerJ* 10:e13529. 10.7717/peerj.13529

Tian Y, He X, Torralba M, Yooseph S, Nelson KE, Lux R, McLean JS, Yu G, and Shi W. 2010a. Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. *Molecular Oral Microbiology* 25:357-367. 10.1111/j.2041-1014.2010.00585.x

Tian Y, He X, Torralba M, Yooseph S, Nelson KE, Lux R, McLean JS, Yu G, and Shi W. 2010b. Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. *Mol Oral Microbiol* 25:357-367. 10.1111/j.2041-1014.2010.00585.x

Tidjani Alou M, Naud S, Khelaifia S, Bonnet M, Lagier J-C, and Raoult D. 2020. State of the Art in the Culture of the Human Microbiota: New Interests and Strategies. *Clinical Microbiology Reviews* 34. 10.1128/CMR.00129-19

Torino MI, Hébert EM, Mozzi F, and Font de Valdez G. 2005. Growth and exopolysaccharide production by Lactobacillus helveticus ATCC 15807 in an adenine-supplemented chemically defined medium. *J Appl Microbiol* 99:1123-1129. 10.1111/j.1365-2672.2005.02701.x

Wade WG. 2013. The oral microbiome in health and disease. *Pharmacol Res* 69:137-143. 10.1016/j.phrs.2012.11.006

Wang Y, Wang S, Wu C, Chen X, Duan Z, Xu Q, Jiang W, Xu L, Wang T, Su L, Wang Y, Chen Y, Zhang J, Huang Y, Tong S, Zhou C, Deng S, and Qin N. 2019. Oral Microbiome Alterations Associated with Early Childhood Caries Highlight the Importance of Carbohydrate Metabolic Activities. *mSystems* 4. 10.1128/mSystems.00450-19

Wu H, Qiu W, Zhu X, Li X, Xie Z, Carreras I, Dedeoglu A, Van Dyke T, Han YW, Karimbux N, Tu Q, Cheng L, and Chen J. 2022. The Periodontal Pathogen Exacerbates Alzheimer's Pathogenesis Specific Pathways. *Frontiers In Aging Neuroscience* 14:912709. 10.3389/fnagi.2022.912709

Wullschleger S, Jans C, Seifert C, Baumgartner S, Lacroix C, Bonfoh B, Stevens MJA, and Meile L. 2018. Vagococcus teuberi sp. nov., isolated from the Malian artisanal sour milk fènè. *Systematic and Applied Microbiology* 41:65-72. 10.1016/j.syapm.2017.11.003

Zhang Y, Fang J, Yang J, Gao X, Dong L, Zheng X, Sun L, Xia B, Zhao N, Ma Z, and Wang Y. 2022. associated bacteria in dental plaque of severe early childhood caries. *Journal of Oral Microbiology* 14:2046309. 10.1080/20002297.2022.2046309

Zheng L, Gao N, and Deng Y. 2012. Evaluation of DNA extraction methods for the analysis of microbial community in biological activated carbon. *Environmental Technology* 33:437-444.

Zhou Y, Wang L, Yang F, Lin X, Zhang S, and Zhao ZK. 2011. Determining the extremes of the cellular NAD(H) level by using an Escherichia coli NAD(+)-auxotrophic mutant. *Appl Environ Microbiol* 77:6133-6140. 10.1128/aem.00630-11

Figure 1

Figure 1. General bacterial community structure analysis among aerobic culture, anaerobic culture and clinical.

(A) Alpha diversity was measured with ACE index, Chao1 index, Shannon index and Simpson index between clinical samples and cultured bacteria. (B) Alpha diversity was measured with ACE index, Chao1 index, Shannon index and Simpson index between clinical sample group and cultured bacteria group among clinical sample group, anaerobic cultured bacteria group and normoxia bacteria group. (C) Venn diagram displays the number of common and unique characteristics among the groups. Different colors represent different groups. The overlaps represent the common taxa between groups, and the non-overlapping portions represent unique taxa in each group. (D) The distributions of the predominant bacteria at the genus level. (E) NMDS was measured with weighted UniFrac. Each sample is represented by a dot. (F) The relative abundance of differential predominant bacteria among aerobic culture, anaerobic culture and clinical groups analyzed at genus level by ANOVA. (G) BugBase analysis among different groups. The three lines from the bottom up are the lower quartile, the mean and the upper quartile in BugBase analysis.

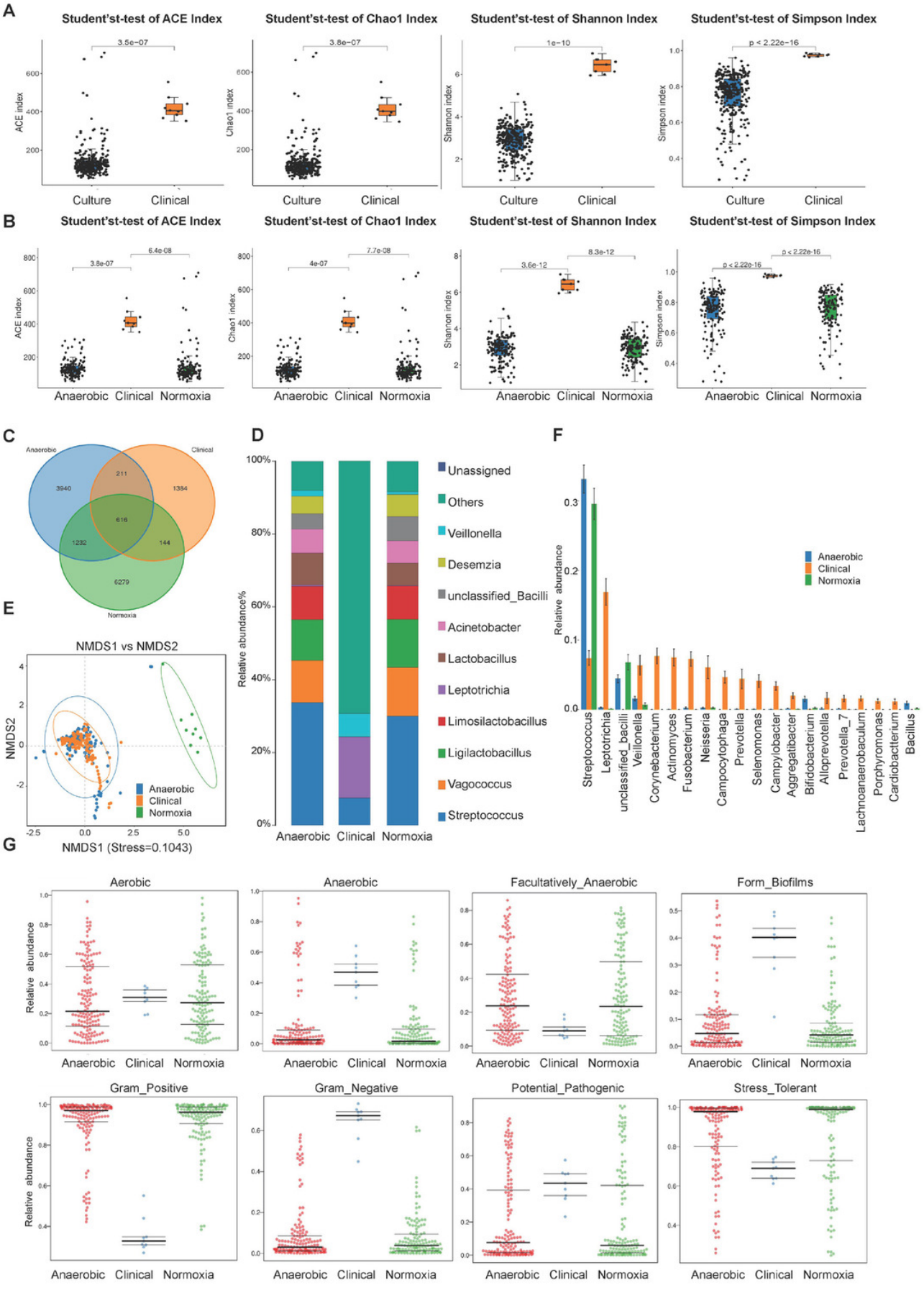
A
Student'st-test of ACE Index
Student'st-test of Chao1 Index
Student'st-test of Shannon Index
Student'st-test of Simpson Index
3.5e-07
3.8e-07
1e-10
p < 2.22e-16
ACE index
Chao1 index
Shannon index
Simpson index
Culture
Clinical
B
Student'st-test of ACE Index
Student'st-test of Chao1 Index
Student'st-test of Shannon Index
Student'st-test of Simpson Index
3.8e-07
6.4e-08
4e-07
7.7e-08
3.6e-12
8.3e-12
p < 2.22e-16
p < 2.22e-16
Anaerobic
Clinical
Normoxia
C
Anaerobic
Clinical
Normoxia
3940
211
1384
616
1232
144
6279
D
Relative abundance%
Unassigned
Others
Veillonella
Desemzia
unclassified_Bacilli
Acinetobacter
Lactobacillus
Leptotrichia
Limosilactobacillus
Ligilactobacillus
Vagococcus
Streptococcus
E
NMDS1 vs NMDS2
NMDS2
NMDS1 (Stress=0.1043)
F
Relative abundance
Streptococcus
Leptotrichia
unclassified_bacilli
Veillonella
Corynebacterium
Actinomyces
Fusobacterium
Neisseria
Campocytophaga
Prevotella
Selenomonas
Campylobacter
Aggregatibacter
Bifidobacterium
Alloprevotella
Prevotella_7
Lachnoanaerobaculum
Porphyromonas
Cardiobacterium
Bacillus
G
Aerobic
Anaerobic
Facultatively_Anaerobic
Form_Biofilms
Gram_Positive
Gram_Negative
Potential_Pathogenic
Stress_Tolerant
Relative abundance

Figure 2

Figure 2. General bacterial community structure analysis between NEMM and SHI media during aerobic (normoxia) and anaerobic culture.

(A) Alpha diversity was measured with ACE index and Chao1 index. (B) NMDS was measured with Unweighted UniFrac. Each sample is represented by a dot. (C) Venn diagram displays the number of common and unique characteristics among the groups. (D) The relative abundance of differential predominant bacteria between NEMM and SHI analyzed at genus level by ANOVA. Function prediction by PICRUSt2. (E&F)BugBase analysis among different groups The difference analysis of COG function (G) and KEGG pathway (H) show the abundance ratio of different functions between groups, whose middle figures show the difference ratio of functional abundance within the 95% confidence interval.

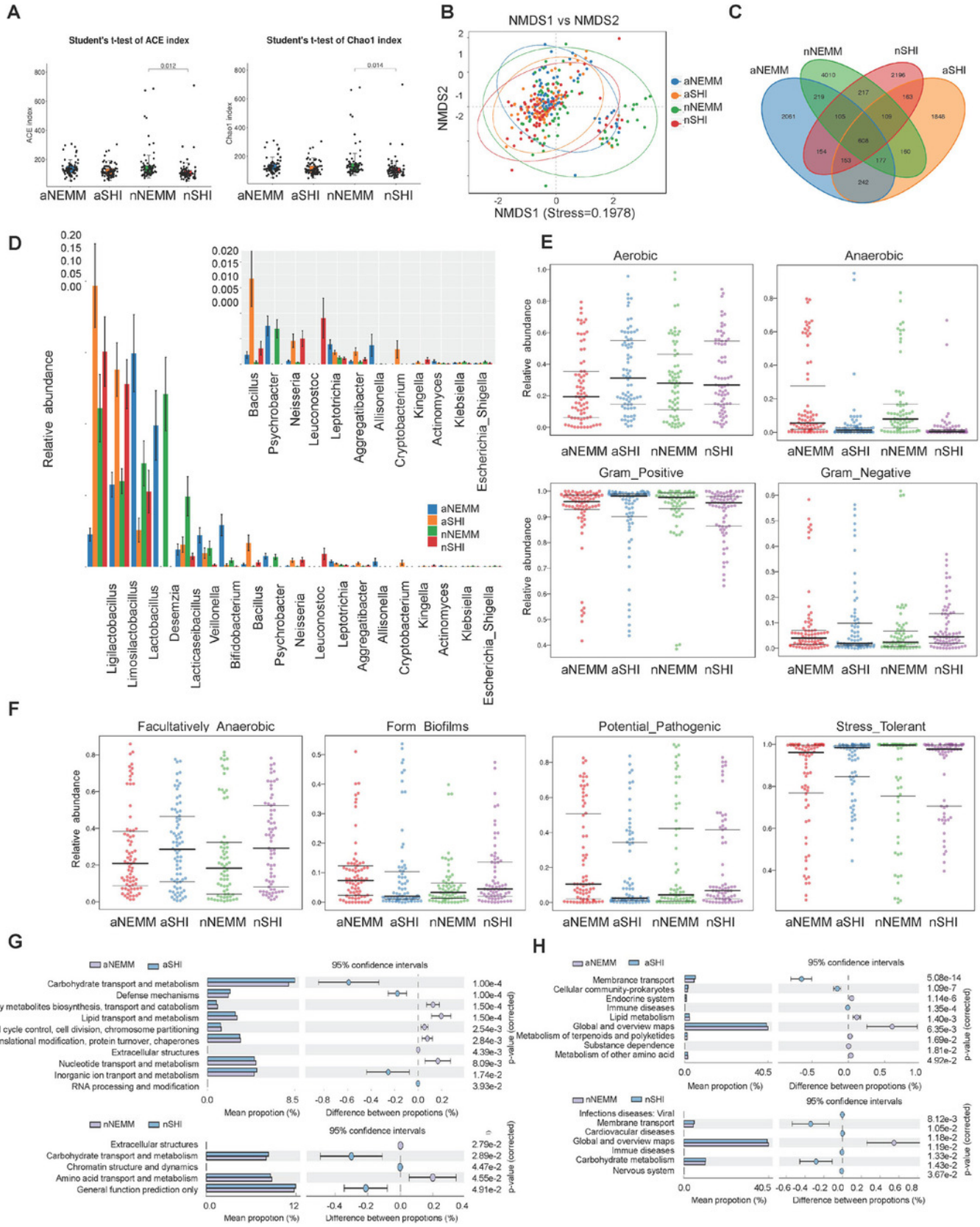
A
Student's t-test of ACE index
Student's t-test of Chao1 index
aNEMM aSHI nNEMM nSHI
B
NMDS1 vs NMDS2
NMDS1 (Stress=0.1978)
NMDS2
C
aNEMM
nNEMM
nSHI
aSHI
D
Relative abundance
Ligilactobacillus
Limosilactobacillus
Lactobacillus
Desemzia
Lacticaseibacillus
Veillonella
Bifidobacterium
Bacillus
Psychrobacter
Neisseria
Leuconostoc
Leptotrichia
Aggregatibacter
Allisonella
Cryptobacterium
Kingella
Actinomyces
Klebsiella
Escherichia_Shigella
E
Aerobic
Anaerobic
Gram_Positive
Gram_Negative
F
Facultatively Anaerobic
Form Biofilms
Potential_Pathogenic
Stress_Tolerant
G
95% confidence intervals
Mean propotion (%)
Difference between propotions (%)
p-value (corrected)
Carbohydrate transport and metabolism
Defense mechanisms
Secondary metabolites biosynthesis, transport and catabolism
Lipid transport and metabolism
Cell cycle control, cell division, chromosome partitioning
Posttranslational modification, protein turnover, chaperones
Extracellular structures
Nucleotide transport and metabolism
Inorganic ion tranport and metabolism
RNA processing and modification
Chromatin structure and dynamics
Amino acid transport and metabolism
General function prediction only
H
Membrance transport
Cellular community-prokaryotes
Endocrine system
Immune diseases
Lipid metabolism
Global and overview maps
Metabolism of terpenoids and polyketides
Substance dependence
Metabolism of other amino acid
Infections diseases: Viral
Membrane transport
Cardiovascular diseases
Global and overview maps
Immune diseases
Carbohydrate metabolism
Nervous system

Figure 3

Figure 3. Bacterial genus change between NEMM medium and SHI medium over time under anaerobic condition

(A) and normoxia condition (B). Cladogram for taxonomic representation of significant differences among groups. The colored nodes from the inner to the outer circles represent taxa from the phylum to genus level. The significantly different taxa are signified by different colors representing the four groups (C&D). Histogram of the LDA scores for deferentially abundant features among groups. The threshold on the logarithmic LDA score for discriminative features was set to 4.0 (E&F). (G) Metastats statistical analysis at genus level.

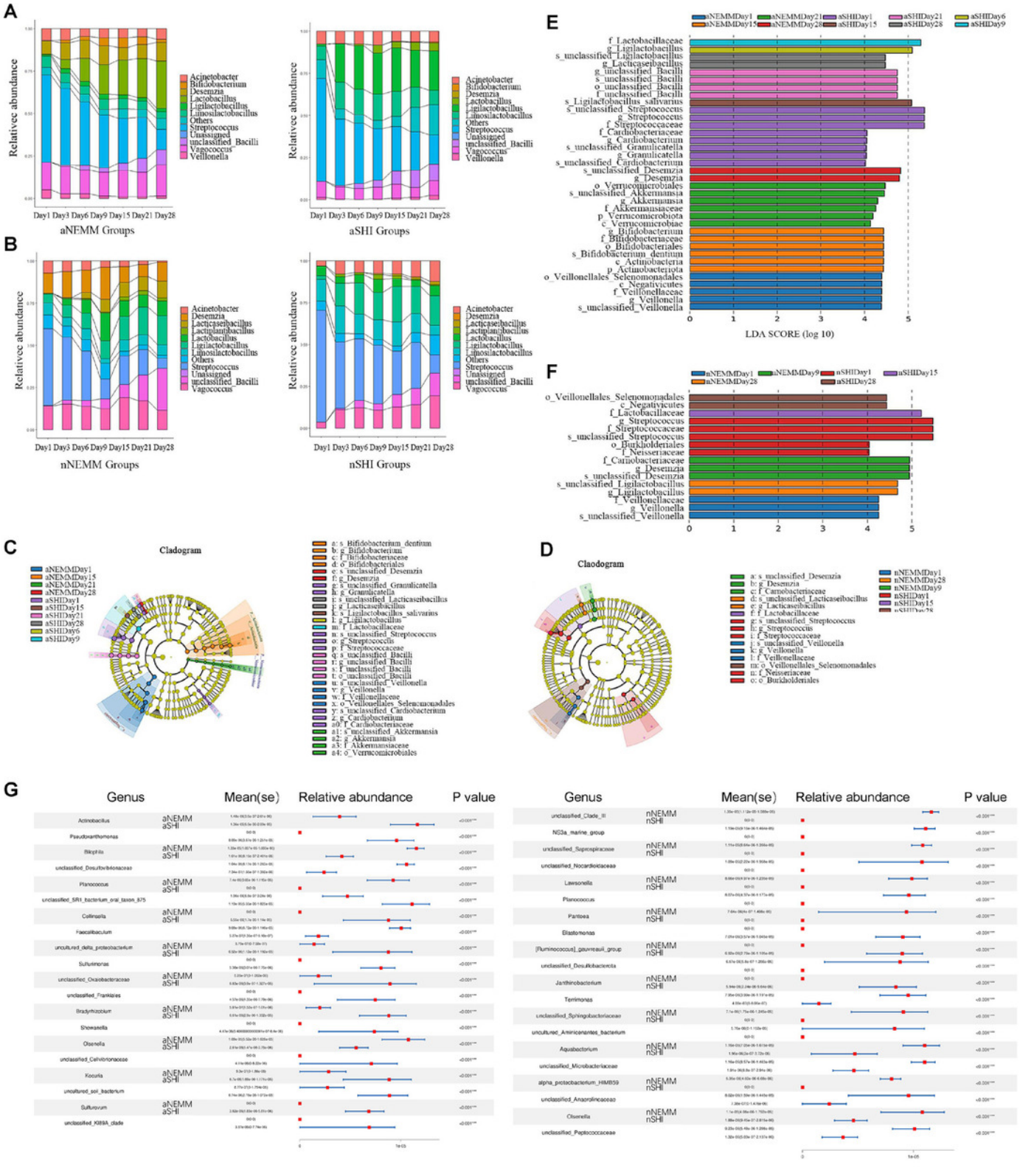
A
Relative abundance
Acinetobacter
Bifidobacterium
Desemzia
Lactobacillus
Ligilactobacillus
Limosilactobacillus
Others
Streptococcus
Unassigned
unclassified_Bacilli
Vagococcus
Veillonella
Day1 Day3 Day6 Day9 Day15 Day21 Day28
aNEMM Groups
aSHI Groups
B
nNEMM Groups
nSHI Groups
E
aNEMMDay1
aNEMMDay15
aNEMMDay21
aNEMMDay28
aSHIDay1
aSHIDay15
aSHIDay21
aSHIDay28
aSHIDay6
aSHIDay9
f_Lactobacillaceae
g_Ligilactobacillus
s_unclassified_Ligilactobacillus
g_Lacticaseibacillus
g_unclassified_Bacilli
s_unclassified_Bacilli
o_unclassified_Bacilli
f_unclassified_Bacilli
s_Ligilactobacillus_salivarius
s_unclassified_Streptococcus
g_Streptococcus
f_Streptococcaceae
f_Cardiobacteriaceae
g_Cardiobacterium
s_unclassified_Granulicatella
g_Granulicatella
s_unclassified_Cardiobacterium
s_unclassified_Desemzia
g_Desemzia
o_Verrucomicrobiales
s_unclassified_Akkermansia
g_Akkermansia
f_Akkermansiaceae
p_Verrucomicrobiota
c_Verrucomicrobiae
g_Bifidobacterium
f_Bifidobacteriaceae
o_Bifidobacteriales
s_Bifidobacterium_dentium
c_Actinobacteria
p_Actinobacteriota
o_Veillonellales_Selenomonadales
c_Negativicutes
f_Veillonellaceae
g_Veillonella
s_unclassified_Veillonella
LDA SCORE (log 10)
F
o_Veillonellales_Selenomonadales
c_Negativicutes
f_Lactobacillaceae
g_Streptococcus
f_Streptococcaceae
s_unclassified_Streptococcus
o_Burkholderiales
f_Neisseriaceae
f_Carnobacteriaceae
g_Desemzia
s_unclassified_Desemzia
s_unclassified_Ligilactobacillus
g_Ligilactobacillus
f_Veillonellaceae
g_Veillonella
s_unclassified_Veillonella
C
Cladogram
a: s_Bifidobacterium_dentium
b: g_Bifidobacterium
c: f_Bifidobacteriaceae
d: o_Bifidobacteriales
e: s_unclassified_Desemzia
f: g_Desemzia
g: s_unclassified_Granulicatella
h: g_Granulicatella
i: s_unclassified_Lacticaseibacillus
j: g_Lacticaseibacillus
k: s_Ligilactobacillus_salivarius
l: g_Ligilactobacillus
m: f_Lactobacillaceae
n: s_unclassified_Streptococcus
o: g_Streptococcus
p: f_Streptococcaceae
q: s_unclassified_Bacilli
r: g_unclassified_Bacilli
s: f_unclassified_Bacilli
t: o_unclassified_Bacilli
u: s_unclassified_Veillonella
v: g_Veillonella
w: f_Veillonellaceae
x: o_Veillonellales_Selenomonadales
y: s_unclassified_Cardiobacterium
z: g_Cardiobacterium
a0: f_Cardiobacteriaceae
a1: s_unclassified_Akkermansia
a2: g_Akkermansia
a3: f_Akkermansiaceae
a4: o_Vermicomicrobiales
D
Claodogram
a: s_unclassified_Desemzia
b: g_Desemzia
c: f_Carnobacteriaceae
d: s_unclassified_Lacticaseibacillus
e: g_Lacticaseibacillus
f: f_Lactobacillaceae
g: s_unclassified_Streptococcus
h: g_Streptococcus
i: f_Streptococcaceae
j: s_unclassified_Veillonella
k: g_Veillonella
l: f_Veillonellaceae
m: o_Veillonellales_Selenomonadales
n: f_Neisseriaceae
o: o_Burkholderiales
nNEMMDay1
nNEMMDay28
nNEMMDay9
nSHIDay1
nSHIDay15
G
Genus
Mean(se)
Relative abundance
P value
Actinobacillus
Pseudoxanthomonas
Bilophila
unclassified_Desulfovibrionaceae
Planococcus
unclassified_SR1_bacterium_oral_taxon_875
Collinsella
Faecalibaculum
uncultured_delta_proteobacterium
Sulfurimonas
unclassified_Oxalobacteraceae
unclassified_Frankiales
Bradyrhizobium
Shewanella
Olsenella
unclassified_Cellvibrionaceae
Kocuria
uncultured_soil_bacterium
Sulfurovum
unclassified_K189A_clade
aNEMM
aSHI
unclassified_Clade_III
NS3a_marine_group
unclassified_Sporosphaeraceae
unclassified_Nocardioidaceae
Lawsonella
Planococcus
Pantoea
Blastomonas
[Ruminococcus]_gauvreauii_group
unclassified_Desulfobacterota
Janthinobacterium
Terrimonas
unclassified_Sphingobacteriaceae
uncultured_Aminicenantes_bacterium
Aquabacterium
unclassified_Microbacteriaceae
alpha_proteobacterium_HIMB59
unclassified_Anaerolineaceae
Olsenella
unclassified_Peptococcaceae
nNEMM
nSHI

Table 1(on next page)

Table 1. Statistical analysis of difference between NEMM and SHI media by analyzing genus, species and uncultured bacteria (at species level) captured at different time points.

	aNEMM vs aSHI			nNEMM vs nSHI		
	mean ± SD (aNEMM)	mean ± SD (aSHI)	*P* value	mean ± SD (nNEMM)	mean ± SD (nSHI)	*P* value
Genus	268.43± 15.76	253.57± 47.13	0.5	369.86±61.63	255.57±65.01	0.0469*
Species	345.86± 14.97	327.71± 55.23	0.5781	453.43±83.62	317.86±80.56	0.0781
Uncultured bacteria	18.29± 2.60	19.00± 6.97	0.875	30.14±8.06	17.71±7.26	0.0469*

The nonparametric Wilcoxon test was performed to evaluate differences. *$P < 0.05$. SD, standard deviation.

1

Table 2(on next page)

Table 2

NEMM and SHI media are suitable for culturing specific bacteria, respectively.

Bacteria type	aNEMM	aSHI	nNEMM	nSHI
Anaerolinea	√			
Planococcaceae	√		√	
Pseudograclibacillu		√		
Geobacillus			√	
Acerococcus	√			
Facklamia			√	
Desemzia	√		√	
lactococcus			√	√
Blastomonas			√	√ (dominant)
Sphingomonas		√		√
Sphongopyxia	√		√	
Legionella			√ (dominant)	√
Psychrobacter	√		√	
Luteimonas			√ (dominant)	√
Lysobacter			√ (dominant)	√
Pseudoxanthomonas		√ (dominant)	√	√
Not assigned	√		√ (dominant)	√

Anaerolinea, *Planococcaceae*, *Acerococcus*, *Desemzia*, *Sphongopyxia*, *Psychrobacter* and some not assigned were significantly enriched in aNEMM. *Planococcaceae, Geobacillus, Facklamia, Desemzia, Lactococcus, Blastomonas, Sphongopyxia, Legionella, Psychrobacter, Luteimonas, Lysobacter and Pseudoxanthomonas* were more abundant in nNEMM conditions. The aSHI condition was suitable for *Pseudograclibacillus*, *Sphingomonas* and *Pseudoxanthomonas*, while *Lactococcus, Blastomonas, Sphingomonas, Legionella, Luteimonas, Lysobacter* and *Pseudoxanthomonas* were more abundant in nSHI condition. Note that √ means significantly enriched.