Effect of silver fluoride on micro-ecology of plaque from extensive caries of primary teeth - in vitro study

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Baoying Liu

smilinglby@163.com*Corresponding Author*

Jin LIU
The First Affiliated Hospital of Zhengzhou University

Di ZHANG
The First Affiliated Hospital of Zhengzhou University

Zhi lei YANG
The First Affiliated Hospital of Zhengzhou University

Ya ping FENG
The Second Affiliated Hospital of Pingdingshan College

Meng WANG
The Second Affiliated Hospital of Pingdingshan College

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Abstract

Background

The action mechanism of silver diammine fluoride (SDF) on plaque micro-ecology was seldom studied. This study investigated micro-ecological changes in dental plaque on extensive carious cavity of deciduous teeth after topical SDF treatment.

Methods

Deciduous teeth with extensive caries freshly removed from school children were collected in clinic. After initial plaque collection, each cavity was topically treated with 38% SDF in vitro. Repeated plaque collections were done at 24 hours and 1 week post-intervention. Post-intervention micro-ecological changes including microbial diversity, microbial metabolism function as well as inter-microbial connections were analyzed and compared after Pyrosequencing of the DNA from the plaque sample using Illumina MiSeq platform.

Results

After SDF application, microbial diversity decreased ($p>0.05$). Microbial community composition post-intervention was obviously different from that of supragingival and pre-intervention plaque as well as saliva. At 1 week post-intervention, the relative content of *Pseudomonas* , *Fusobacterium* and *Pseudoramibacter* was obviously higher than before, while most of the other bacteria was obviously reduced, although not statistically significant ($P>0.05$). The inter-microbial connections became more complex, with positive connections overcame negative ones. Carbohydrate transportation and metabolic functions in the plaque were significantly reduced at 24 hours and 1 week post-intervention.

Conclusions

SDF has extensive antimicrobial effect on dental plaque, which may reduce carbohydrate metabolism in dental plaque and help promote new balance of the plaque flora.

Background

Untreated deciduous caries affect 573 million children worldwide[1–3], jeopardize their health and increases the burden of medical care[4]. The incidence of permanent tooth decay increases with the

severity of deciduous tooth decay[5]. Fluoride is important effective weapon in fighting dental caries [6, 7], silver diammine fluoride (SDF) won great attention because its super ability in arresting and preventing caries [8–13]. It has been called ‘silver bullet’ for dental caries [14].

Previous working mechanism of SDF in the control of dental caries focused mainly on a few specific cariogenic bacteria [15–20], whereas according to the concept of ‘ecological plaque hypothesis’ [21–24], caries development was due to the loss of micro-ecology balance within the dental plaque. New evidence suggested that SDF has no specific selectivity in inhibiting bacteria [25]. The effect of SDF on micro-ecology of dental plaque should be dug out.

The aim of this study was to explore the effect of SDF on micro-ecology of carious dental biofilm.

Materials And Methods

In vitro study using clinic samples from school children was designed. Ethic approval was gain from the ethics committee of The First Affiliated Hospital of Zhengzhou University (NO. 2018-ky-35). Informed consent from parents or guardians were obtained prior to any clinical examination or sampling.

Subjects

Children aged 6–12 years admitted to Department of Pediatric Dentistry and Department of Oral and Maxillofacial Surgery in The Second Affiliated Hospital of Pingdingshan College in Henan province, during the period from November 2018 to January 2019 were targeted. Children were examined in the clinic and those with at least one deciduous tooth with extensive untreated caries were recruited. Inclusion criteria: (1) children aged 6–12 years; (2) there was at least one deciduous tooth with extensive caries in the mouth, which needs to be extracted. Exclusion criteria: (1) the extensive caries was restored; (2) the surface of the carious lesion was smooth and hard; (3) children with hereditary or systemic diseases; (4) children with long history of medication or have taken antibiotics within the last three months; (5) children received topical fluoride therapy within the last two weeks.

Collection of plaque and saliva before SDF intervention

Dental plaque (PBI) on the tooth surface within the extensive untreated carious cavity, supragingival plaque (PCF) on smooth surface of permanent first molars and resting saliva (S) by spitting method of the recruited children were collected before tooth extraction. Sterilized dental excavators and

sterilized sharp probe were used for collection of dental plaque within the carious cavity and that on sound surfaces respectively. The collected plaque was put into a 1.5 ml centrifuge tube containing 1 ml PBS solution. Saliva was collected using method described by Fang Yang et al. [26], an amount of 2.5-3 ml saliva was collected. All collected samples were immediately stored in a refrigerator at -20℃ before laboratory analysis.

SDF intervention

After extraction, the tooth was carefully cleaned to avoid blood contamination to the carious cavity. An average amount of 10 μl SDF solution drops was topically applied onto dental surface within the extensive untreated carious cavity by using a pipet, avoided touching the surface. The tooth was titled around and assisted with a gentle blow to spread the solution as uniformly as possible on the carious surface. The 38% SDF solution used in this study was prepared in the laboratory of Zhengzhou University according to the method described before [27]. The tooth was left undisturbed for 5 minutes before fully infiltrated into fresh sterilized artificial saliva (Dongguan Yunfei Automation Equipment Technology Co., Ltd., Guangdong, China) separately each and stored in incubator at 37℃. The artificial saliva was replaced every 24 hours and after plaque sampling procedures.

Collection of plaque after SDF intervention

At the time of 24 hours (PAI-24 h) and 1 week (PAI-1w) after SDF intervention, Dental plaque within the extensive untreated carious cavity was collected and stored again according to the same method as mentioned above.

Laboratory processing and analysis

Samples were send out to Shanghai Majorbio Bio-pharm Technology Co. Ltd., China for laboratory procedures including DNA extraction, PCR amplification, 16S rDNA sequencing using the Illumina Miseq platform and sequencing data processing. Laboratory process was conducted according to standard operating procedures[28].

Statistical analysis

Sequenced dilution curves, Alpha diversity, and microbial species counts are calculated and analyzed on the free Majorbio online platform (www.majorbio.com). Description was given to qualitative data, t test was used for pairwise comparison of sample Alpha diversity between groups, and Kruskal-Wallis

rank sum test was used for comparison of sample species composition. Network analysis was used to analyze the relationship between bacteria genera, and PICRUSt software (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to analyze the functional characteristics of microorganisms in each group. Statistically significant level was set at $\alpha=0.05$.

Results

In total, five children were recruited. Each donated one extracted deciduous molar with extensive untreated carious cavity. A total of 15 plaque samples from carious cavity, 5 saliva samples and 5 supragingival plaque samples were collected. All samples passed qualification test for further DNA sequencing.

DNA sequencing information

A total of 1,280,788 effective sequences were detected (an average of 51 232 sequences per sample), with an average sequence length of 427 bp. The dilution curve (Fig. 1) tends to be flat, indicating reasonable and representative sequencing data.

Figure 1 dilution curve of sample. The samples were grouped as following: saliva samples (S), supragingival plaque of sound teeth (PCF), plaque from carious cavity before SDF intervention (PBI), plaque from carious cavity 24 hours after SDF intervention (PAI_24 h), and plaque from carious cavity 1 week after SDF intervention (PAI_1w).

Alpha diversity

As shown in Fig. 2, in general, microbial diversity of plaque sample from carious cavity as indicated by Shannon index decreased over time after the application of SDF, but no statistically significant difference was found ($P > 0.05$). The microbial diversity of PAI-1w were statistically significantly lower than that of saliva samples ($0.01 < P \leq 0.05$).

Microbial community composition and similarity analysis

The microbial community composition of each group of sample was shown in bar chart (Fig. 3a). The microbial community composition of PAI-24 h and PAI-1w samples were obviously different from that of PBI group as well as those of saliva and PCF samples. Principal co-ordinates analysis (PCoA) (Fig. 3b) showed different degrees of overlap (similarity) among the PBI, PAI-24 h and PAI-1w samples, and small overlap between PCF and saliva samples, however, no overlap between the former three

and the latter two group of samples. The longer the time after intervention, the smaller the overlap among the samples.

Figure 3 Community bar plot analysis and PCoA diagram. a is the Community barplot, the abscissa is the sample name, and the ordinate is the proportion of species in the sample. b is the PCoA diagram on OUT level. The X-axis and Y-axis represent the two selected primary axes, and the percentage represents the explanatory value of the primary axis to the difference in sample composition, the scale of the X-axis and Y-axis is the relative distance, which is meaningless; Dots of different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition of the two samples is.

Results of community composition diversity test at the genus level was shown in Fig. 4. The results showed that the relative contents of Pseudomonas, Fusobacterium and Pseudoramibacter were obviously higher in PAI-1w samples compared to PBI samples, but no statistically significant difference was found ($P > 0.05$). After SDF intervention, the genus of Olsenella, Streptococcus, Bifidobacterium, Prevotella_7, Lactobacillus, Actinomyces, Leptotrichia, Selenomonas_3 and Veillonella showed decreasing trend in different degrees, but no statistically significant difference was found ($P > 0.05$).

Network analysis and function prediction

The network diagram of species correlation (Fig. 5 dotted line) shows that after SDF intervention, the intergeneric connections became more complex and tight, especially at 1 week after intervention. The positive connections between species in the plaque sample increased compared with that of plaque before intervention, and the positive connections were found overwhelm the negative ones at each time point.

Analysis of the microbial functional characteristics based on the COG (cluster of Orthologous Groups) database was carried out (Fig. 5 bar chart). It showed that the carbohydrate transportation and metabolic functions (shown in yellow in Fig. 5 bar chart, G) were significantly ($p < 0.05$) decreased 1 day and 1 week after SDF intervention. The values of relevant functional parameters for PBI, PAI-24 h and PAI-1w samples were 0.080, 0.068 and 0.064, respectively. The value for Saliva and PCF samples were 0.067 and 0.066, respectively. The replication as well as recombination and repair

functions (shown in pink in bar chart in Fig. 5, L) were also significantly reduced, and the signal transduction mechanism (shown in brown part of bar chart in Fig. 5, T) was significantly increased.

Figure 5 Genus correlation network and COG function classification. In network diagram (a, b, c), the main genuses include Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Fusobacteria, the size of the node represents the genus abundance, and different colors represent different genus; red is positive, green is negative; the more the lines, the closer the species is to other species.

Discussion

In this study, extracted deciduous teeth with large carious cavity from school-age children were collected and topically intervened in vitro with 38% SDF solution. Dental plaque from the carious cavity pre- and post-intervention were collected for microbial sequencing and the results were compared. The amount of most of the bacteria, the microbial diversity and the microbial community composition in the dental plaque after SDF intervention were obviously decreased or changed, and the relative contents of Pseudomonas, Fusobacterium and Pseudoramibacter were higher than before, although no statistically significant difference was found ($P > 0.05$). After SDF intervention, microbial association in the dental plaque became more complex with positive connections overwhelm the negative ones, and carbohydrate transportation and metabolic functions in the dental plaque were significantly reduced ($P < 0.05$).

The results supported that 38% SDF had broad spectrum of bactericidal effect, which suppressed the dental plaque as a whole. As a result, microbial diversity and the microbial community composition was reduced and shifted. The statistical indifference in this study may be due to the small sample size. A recent study on 38% SDF, which compared pre- and one-month-post-intervention dental plaque sample from the mouth of adult patients [29], found no significant difference in the plaque microorganisms composition either. However, difference in dentition, sampling condition and the follow-up interval should also be noticed.

Most of the bacteria presented before the intervention (Olsenella, Streptococcus, Bifidobacterium, Prevotella_7, Lactobacillus, Actinomyces, Leptotrichia, Selenomonas_3, Veillonella) were sensitive to SDF, and the amount decreased obviously. Streptococcus, Lactobacillus and Actinomyces has long

been recognized as classical cariogenic bacteria. Prevotella_7, Selenomonas_3, and Bifidobacterium were often found in deep dentin caries [30]. Olsenella, which could produce lactic acid, was seldom mentioned in the past, have been found being associated with dentin caries and root caries recently [31, 32]. Leptotrichia has a strong glycolysis effect [33], and its detection rate increased in the process of caries [34, 35]. Veillonella has a unique intracellular pH control mechanism [36], which might promote the cariogenicity of Streptococcus mutans [35, 37]. By inhibition of these bacteria, cariogenicity of the dental plaque was suppressed, microbial new balance was needed and the chance became possible.

It was also found that Pseudomonas, Fusobacterium and Pseudoramibacter could withstand the effect of topically applied SDF. The possible relation of Pseudomonas in dental caries was indicated recently. NavNeet Kaur et al. [38] detected a high level of Pseudomonas in the dentin caries. In Liang's study [39], Pseudomonas was mentioned as a potential pathogen of caries, but considered the relationship be further studied. Pseudomonas was obligate aerobic and negative gram staining, with capsular bacillus and no bud, belonged to the non-fermentation bacteria. It had no special nutritional requirements, was common in soil, fresh water, seawater. A total of twenty-nine species have been identified, of which at least three are pathogenic to animals or humans, causing infections. Pseudomonas aeruginosa was in the oral common flora and was a conditioned pathogen [40], it was one of the main pathogens of nosocomial infection, usually secondary infection. Small doses of the bacteria could produce local abscesses, while large doses of it could lead to death from systemic infection, which was also the main cause of pulmonary cystic fibrosis [41]. Fluorescent pseudomonas could cause spoilage of frozen meat, eggs, milk and dairy products. Pseudomonas birensis was also known as bacillus birensis, could be infected through mouth, respiratory tract or wound. Pseudoramibacter was a nonmotile, nonsporeforming, strictly anaerobic, Gram-positive rod, which was saccharolytic. The end products of its fermentation were formate, acetate, butyrate, caproate, and hydrogen [42]. It was the pathogen that could cause periapical infection [43–45], often found in deep dentin caries [30, 46] and infected root canals [42]. Similarly, Fusobacterium was often detected in deep dentin caries [30, 46] and infected root canals [44, 45, 47], It was a strict obligate anaerobe,

with negative gram staining, normally living in oral cavity, upper digestive tract, intestinal tract, urogenital tract of human or animal and soil. It was most commonly seen in oral dental plaque. Most of the strains did not ferment any sugars, only a few strains showed weak fermentation reaction to glucose and fructose.

Restricted by ethic issues, extracted deciduous teeth was used in this study. As a result, intervention and sampling after it were all carried out in vitro. In vitro study was different from real clinical situation. The effect of common oral flora, saliva, food and environmental microorganisms on the formation of new microbial balance could hardly be mimic and evaluated. New micro-ecological balance formation after SDF intervention in this study could only be based on the before-intervention status as well as the effect of SDF. The bacteria which was sensitive to SDF were controlled, while those with relatively high tolerance to SDF got better growth and development after the intervention. After SDF intervention, microbial association in the dental plaque became more complex with positive connections overwhelm the negative ones, indicating that the connections and collaborations among the remaining bacterial communities were enhanced.

The functional characteristics of plaque in caries were also compared and analyzed based on the COG database for homologous classification of gene products, carbohydrate transportation and metabolism function in plaque were significantly reduced 24 hours and 1 week after intervention (shown in yellow in Fig. 5 bar chart, G), and the replication, recombination and repair function in plaque (shown in pink in bar chart in Fig. 5, L) were also significantly reduced, revealed from another aspect the overall inhibitory effect of SDF on the bacterial community in dental plaque, which affecting the properties of the whole plaque, especially the cariogenicity within the plaque micro-ecology. The function of signal transduction mechanism was significantly increased, which could be correlated with the enhancement of synergistic effect between residual bacteria in plaque after intervention.

School-age children had mixed dentition, deciduous teeth with extensive carious cavity or obvious dental pulp symptoms may also affect the health of their permanent teeth. The results of the dental plaque from the extensive carious cavity could also be guiding information for the prevention and

control of deciduous caries associated with pulp infection or periapical disease.

Most parameters in this study showed that the greatest microbial difference was found at one week after SDF intervention, indicating a possible time effect of SDF. However, the observation period in this study was relatively short which may has its limitations, and there were differences between this in vitro study and the real mouth situation, time effect of SDF application on dental plaque may not be well explained, and should be studied further.

Conclusions

To sum up, by focusing on different perspective of plaque micro-ecology, this study fell in line with the concept of ‘ecological plaque hypothesis’ in understanding the development of dental caries. Topical application of 38% SDF had extensive inhibitory effect on dental plaque microorganisms, which led to a reduced carbohydrate metabolism and increased positive connections among survived bacteria within the dental plaque, provided the plaque a chance of micro-ecology balance re-distribution. This study may promote our understanding of the caries prevention and control mechanism of SDF on dental plaque from micro-ecology perspective.

Abbreviations

SDF, silver diammine fluoride.

Declarations

Ethics approval and consent to participate

Ethic approval was gain from the ethics committee of The First Affiliated Hospital of Zhengzhou University (NO. 2018-ky-35). Informed consent from parents or guardians were obtained prior to any clinical examination or sampling.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

We have no competing interests.

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Authors' contributions

LBY conceptualized and designed the study, guided the conduction of the experiment and was a major contributor in results explanation, drafting and revising the manuscript. LJ guided the conduction of the experiment, contributed in results explanation and drafting of the manuscript. ZD carried out the study and the experiment, conducted data analysis and involved in the drafting of this manuscript. YZL helped the conduction of the study and involved in the data analysis and results interpretation. FYP and WM assisted in the conduction of the study (clinical screening of experimental subjects and the sampling).

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Figures

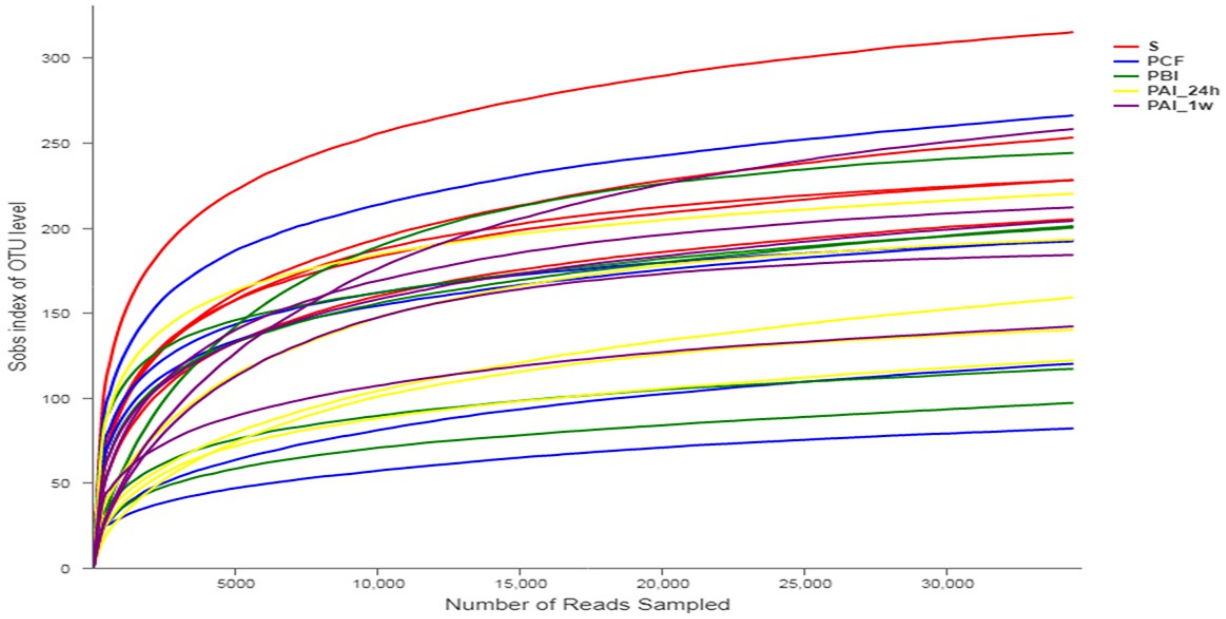


Figure 1

dilution curve of sample. The samples were grouped as following: saliva samples (S), supragingival plaque of sound teeth (PCF), plaque from carious cavity before SDF intervention (PBI), plaque from carious cavity 24 hours after SDF intervention (PAI_24h), and plaque from carious cavity 1 week after SDF intervention (PAI_1w).

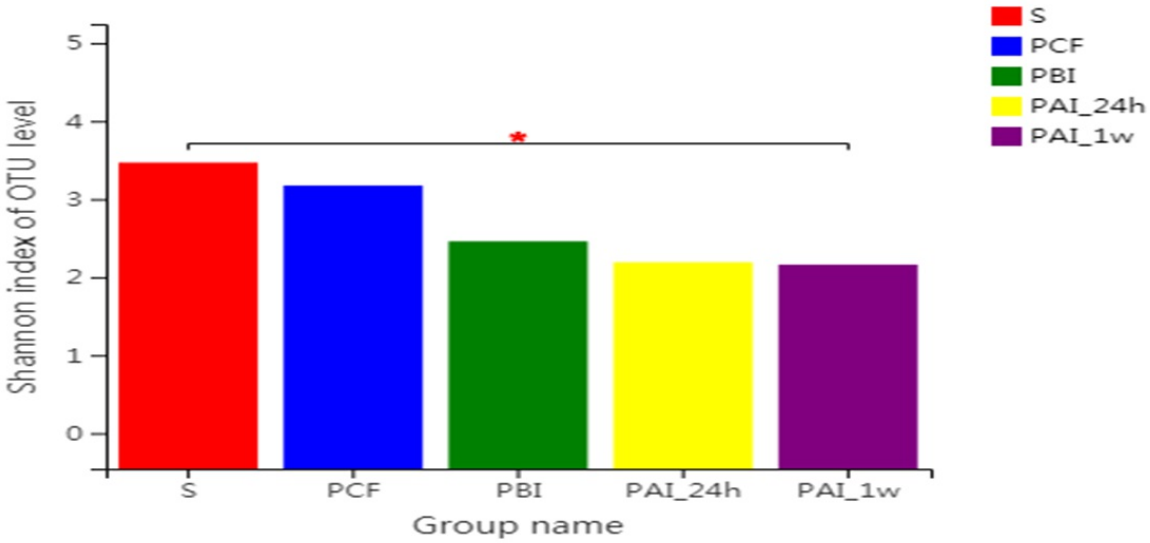
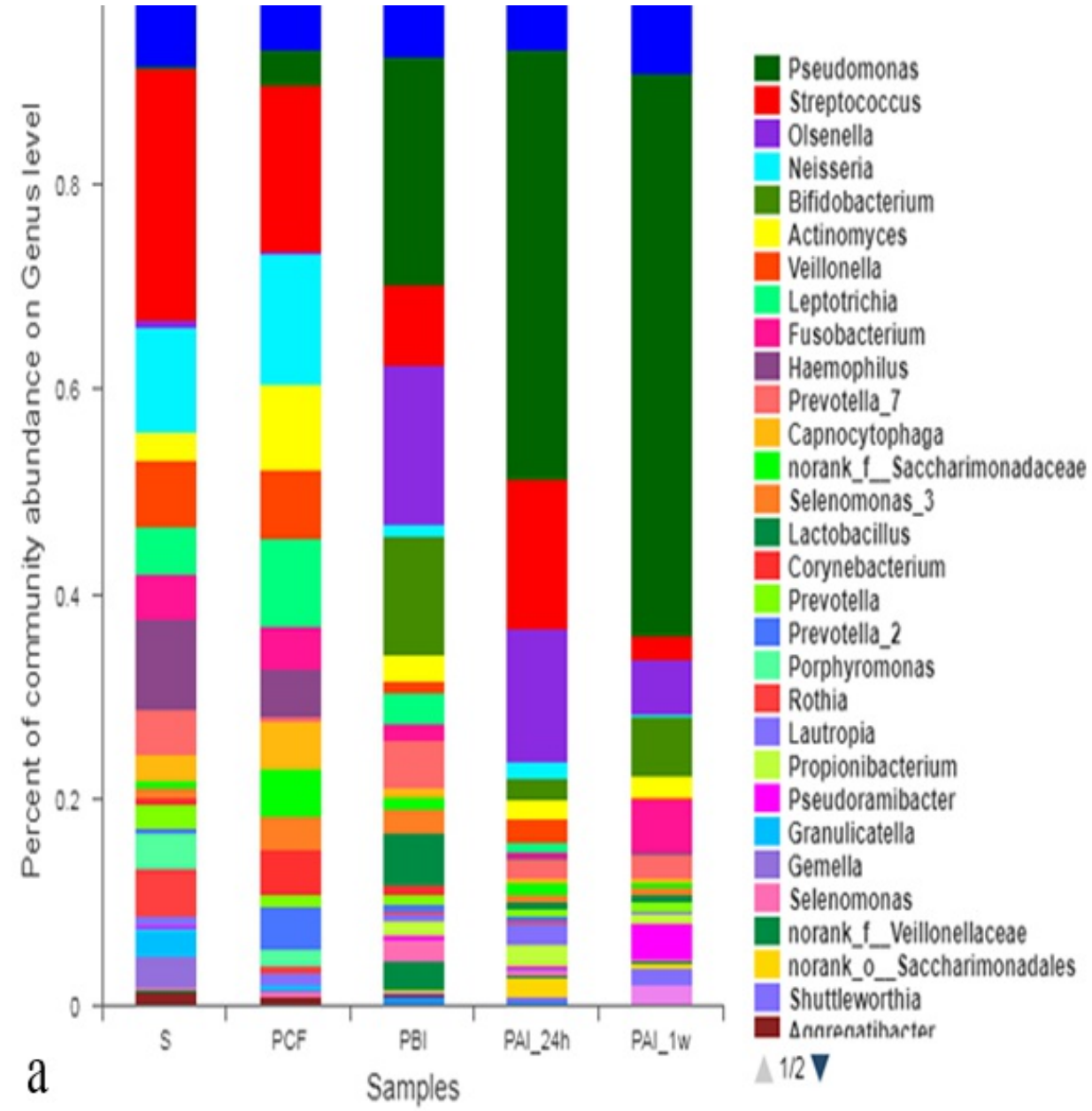
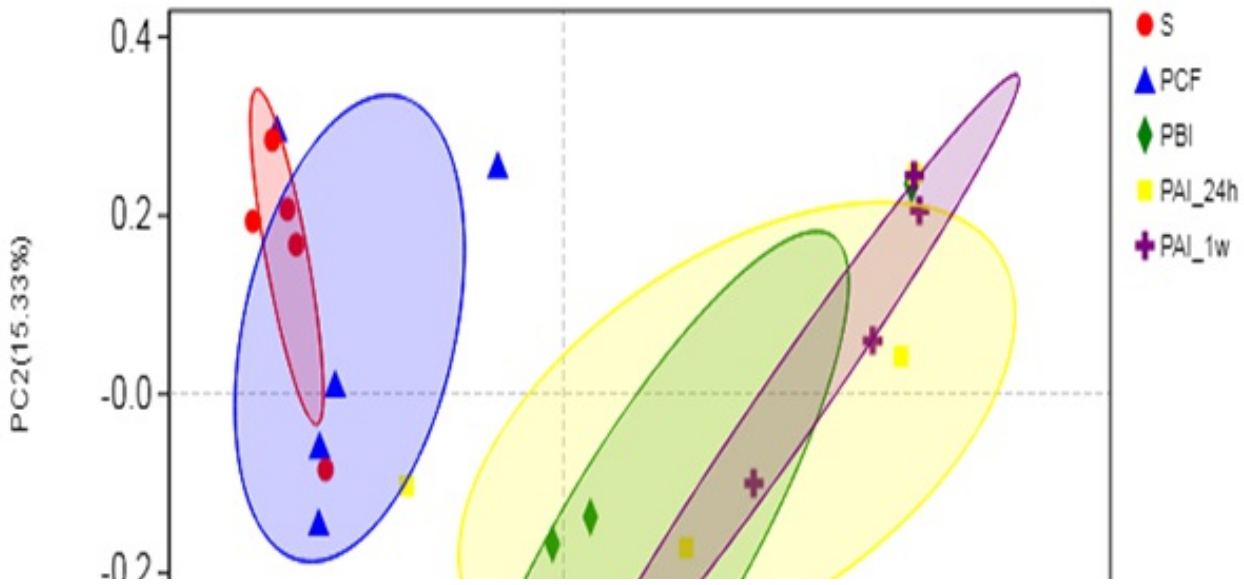


Figure 2

Results of Alpha diversity index (Shannon index). * 0.01 < P ≤0.05 .


Percent of community abundance on Genus level
0
0.2
0.4
0.6
0.8
S
PCF
PBI
PAI_24h
PAI_1w
Samples
a
Pseudomonas
Streptococcus
Olsenella
Neisseria
Bifidobacterium
Actinomyces
Veillonella
Leptotrichia
Fusobacterium
Haemophilus
Prevotella_7
Capnocytophaga
norank_f__Saccharimonadaceae
Selenomonas_3
Lactobacillus
Corynebacterium
Prevotella
Prevotella_2
Porphyromonas
Rothia
Lautropia
Propionibacterium
Pseudoramibacter
Granulicatella
Gemella
Selenomonas
norank_f__Veillonellaceae
norank_o__Saccharimonadales
Shuttleworthia
1/2


PC2(15.33%)
0.4
0.2
-0.0
-0.2
S
PCF
PBI
PAI_24h
PAI_1w

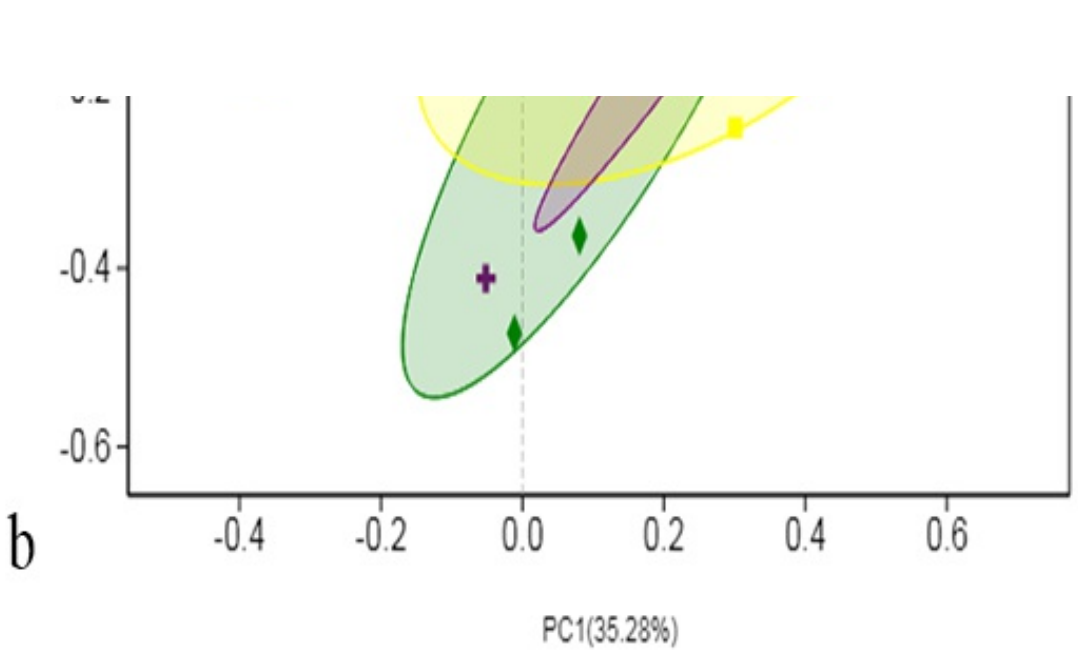


Figure 3

Community bar plot analysis and PCoA diagram. a is the Community barplot, the abscissa is the sample name, and the ordinate is the proportion of species in the sample. b is the PCoA diagram on OUT level. The X-axis and Y-axis represent the two selected primary axes, and the percentage represents the explanatory value of the primary axis to the difference in sample composition, the scale of the X-axis and Y-axis is the relative distance, which is meaningless; Dots of different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition of the two samples is.

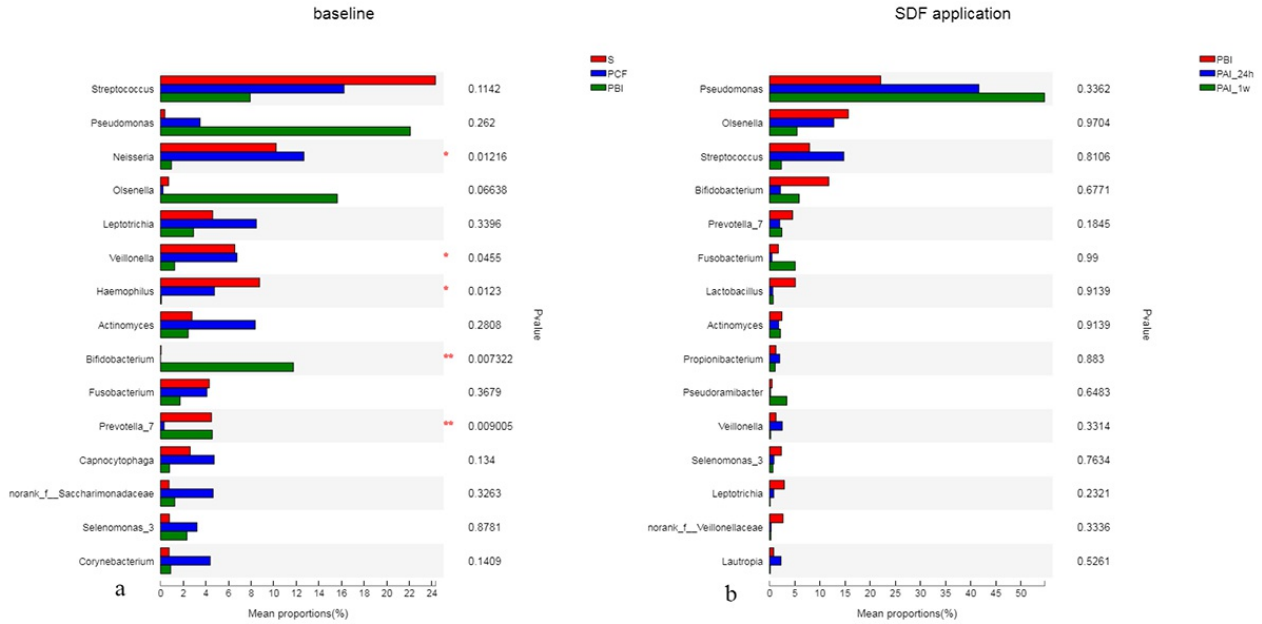


Figure 4

Community composition diversity test on the top 15 richest Genus among different groups of samples

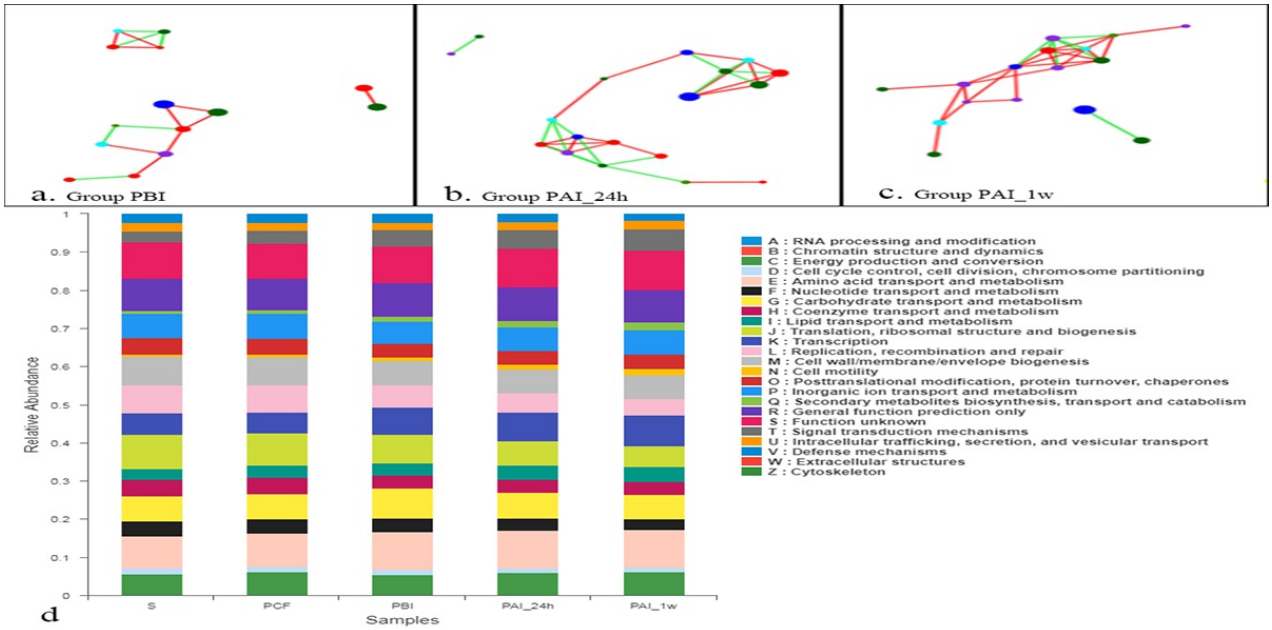


Figure 5

Genus correlation network and COG function classification. In network diagram (a, b, c), the main genuses include Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Fusobacteria, the size of the node represents the genus abundance, and different colors represent different genus; red is positive, green is negative; the more the lines, the closer the species is to other species.