Metagenomic sequencing analysis of bacterial biodiversity in the treatment of surplus sludge and chicken manure mixtures by black soldier fly larvae (Hermetia illucens)

Lei Zhang
Qi Zhao
Shuang He
Luyan Zhang
Liang Qiao
Cheng Ding
Ye Yuan
Feihong Wang
Tianming Chen (ycchentm@163.com)

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Abstract

The disposal of combined organic waste by black soldier fly larvae (*Hermetia illucens*) has drawn broad attention. However, changes of microflora during the treatment are lacking. In this study, livestock manure and surplus sludge were chosen as the target organic waste due to their wide produced in breeding industry and water treatment industry. Black solider fly (BSF) larvae were used to treat the mixture of chicken manure and surplus sludge for a week. Bacterial biodiversity of BSF larvae production residues were investigated with metagenomic sequencing technology to identify specific flora. The change trend of BSF larvae production residue derived from the mixture of chicken manure and surplus were closer to that resulting from chicken manure. *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* are the main bacteria in phylum level with the content of 75.39%,14.63%,5.69% and 2.42% in abundance. *Bacilli* were the major bacteria with 71.61% in class level. *Pseudogracilibacillus*(11.08%), *Virgibacillus*(9.86%) and *Oceanobacillus*(6.39%) were the key component in *Bacilli*. *Erysipelotrichales* as the probiotics of insect, also as pathogenic bacteria to poultry and human were found massive proliferation with the abundance of 2.71%. Overall, BSF has proved to be a sustainable management for organic waste by reducing its amount and whether the BSF larvae production residue can be used as organic fertilizer directly was controversial result from the bacterial biodiversity analysis.

Introduction

Sewage sludge, a kind of organic waste from wastewater treatment plants, will continue global growth because of the rising number of households connected to the central treatment plant. Untreated sewage sludge has a tendency to deteriorate the surrounding environment since they are general composited with degradable organic compounds, non-essential trace metals, microorganisms, and pathogens (Joo et al., 2019). Thermal treatments like catalytic pyrolysis (Xie et al., 2014), gasification (Freda et al., 2018) and combustion (Wang et al., 2019), can reduce the volume of sludge, diminish pathogenic bacteria and achieve energy recovery in a short time. However, undesirable aromatics would inevitably be formed during the thermal process (Dai et al., 2014).

The black solider fly (BSF), *Hermetia illucens* receive substantial attention as an ecologically sustainable method in the field of organic waste treatment. It can promote resource conversion efficiency and value-added product as pullulating new economic growth points for kitchen waste disposal industry and livestock and poultry manure treatment industry in developing countries (Anshika et al., 2019). By the biodegradation of organic matters, BSF larvae can grow on different substrates, varying from agricultural by-products to sewage sludge (Dennis et al., 2020; Dennis et al., 2021; Jonathan et al., 2021;Sarpong et al.,2019). BSF meal is expected to be a substitute of soybean and fish meal to reduce the cost of protein source in feed and food production (Onsongo et al., 2018; Wang Yu-Shiang et al., 2017; Thomas et al., 2018; Marianna et al., 2021; Guido et al., 2014).

Current research has shown that high-quantify substrate can provide efficient nutrient substance for the rapid growth of BSF larvae development, like chicken manure, pig manure and so on. On the contrary, growth of BSF larvae is very slow on low-quantify as sewage sludge from anaerobic digesters which is rich in proteins and other constituents of nutritional value (Lalander et al., 2019). Laganaro elucidated the feasibility of combined treatment in degassed sludge and chicken feed. When degassed sludge was included in substrate mixtures, the BSF larvae utilized more feed for respiration purposes at high rates of maintenance metabolism (Growth and metabolic performance of black soldier fly larvae grown on low and high-quality substrates). Although BSF larvae can convert different substrates into new biomass, specific flora in the bacterial biodiversity of BSF larvae production residues is needed to be identified for the further use.

Metagenomics is a direct genetic analysis of environment sample without cultivating clonal cultures (Anastasis et al., 2015). Metagenomics analysis can facilitate revealing the variation trend of bacterial communities to reply to the dynamic environment (Jithin et al., 2020). The changes of microbial community in chicken manure treated with black solider fly have showed that *Firmicutes*(97.72% in bacteria), *Penicillium*(46.82% in fungi) and *Aspergillus*(45.22% in fungi) dominated in manure after treatment (Zhang Xiaoxiao et al., 2020). *Protebacteria*(9.52–13.50%), *Bacteroidetes*(7.18%-10.65%) and *Firmicutes*(7.53%-9.46%) were the dominant bacterium group in anaerobic digestion sludge (Jeroen et al., 2020). Hence, metagenomics analysis is an efficiency measure for investigating changes of bacterial biodiversity during the disposal of combined organic waste.

In our study, livestock manure and surplus sludge were chosen as the target organic waste due to their wide produced in social development. The mixture of chicken manure and municipal sewage sludge have been proved to be treated by BSF lavae (Cai Minmin et al., 2017). Differences in the bacterium communities between chicken manure, sludge and their mixture were demonstrated through the metagenomic analysis of various substrates. We focused on the changes of bacterium community in combined treatment of chicken manure and surplus sludge by BSF larvae.

Experimental Section

2.1. Materials

The chicken manure was collected from the Taizhou Focusing Technology Co., Ltd in September 2021. The surplus sludge was collected from the sewage treatment platform of a local pig casing processing plant. The chicken manure and surplus sludge were mixed with 1:1 (weight ratio), then put in the experiment boxes. 5g BSF larvae growed in a transparent plastic box containing the rice bran of 40% moisture as hatchery for four days.

2.2. Experiment process

Isolating the larvae from rice bran, weighing and putting them on the mixture. The total weight of mixture was 100g. The composting device was put in a 30℃ incubator for two weeks without supplying water. BSF larvae production residue was collected and stored at -80 ℃ for metagenomics analysis. The chicken manure and surplus sludge were collected in the same way.

2.3. Metagenomics DNA isolation

The DNA Isolation kits were used to extract and purify the genomic DNA from samples (Omega, USA). Then they were tested by 1% agarose gel electrophoresis and placed at -80 °C until use. The primer-pairs 338F-806R were used to amplify bacterial 16S rRNA gene. The PCR products were sequenced by Majorbio (Shanghai, China) using the Illumina MiSeq PE300 platform. Then the sequencing data were analyzed by QIIME software.

2.4. Quantitative polymerase chain reaction (qPCR) analysis

Quantitative polymerase chain reaction (qPCR) on the bacterial 16S rRNA gene was analyzed by a company (Majorbio, Shanghai, China), which were diluted to a concentration of 10 μmol/L. The qPCR was employed on a Real-Time PCR Detection System in final 10 μL volume reaction mixtures consisting of the following components: 5 μL 2 × SYBR Green qPCR Master Mix (0.05 U/mL Taq DNA polymerase, 2 × GoTaq Flexi Buffer, 0.4 mM dNTPs, 4 mM $MgCl_2$ and SYBR Green dye), 1 μL template DNA (1–10 ng), 3.6 μL sterile water, and 0.4 μL forward and reverse primers (0.4 μM). Each qPCR amplification was employed in 40 cycles and followed by several analyses for melting curve. The plasmids containing specific total bacterial 16S rRNA were manufactured by Majorbio Co., Ltd.

Results And Discussion

3.1. Species composition

The BSF larvae production residue sample contained a large number of bacteria (Fig. 1). From the phylum point of view, *Firmicutes*(75.39%) was the richest, followed by *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*. The abundance of *Bacilli* reached 71.61% which dominated 94.97% of the bacterial in class level. *Actinobacteria*(14.74%), *Betaproteobacteria*(4.38%), *Erysipelotrichia*(2.8%), *Flavobacteriia*(2.12%), *Clostridia*(1.38%) and *Gammaproteobacteria*(4.38%) (as shown Fig. 2). *Pseudogracilibacillus*(11.08%), *Virgibacillus*(9.86%), *Bacillaceae*(7.75%), *Oceanobacillus*(6.39%) composed the first echelon in genus level while *Alcaligenaceae*, *Enteractinococcus*, *Atopostipes*, *Arysipelothrix*, *Cellulomonas*, *Gracillibacillus*, *Lentibacillus*, *Ornithinibacillus*, *Sporosarcina*, *Amphibacillus*, *Cerasibacillus* in the second echelon (Fig. 3).

The abundance of *Firmicutes* and *Actinobacteria* added up to 90.02% which was similar with the worm feces treating by BSF larvae from chicken manure (Zhang Xingxiao et al., 2020). But in that study, *Firmicutes*(97.72%) were the most phylum and the proportion of abundance related to the gut microbes of BSF larvae. *Pseudogracilibacillus* were the most microbiology in our and his research, 33% and 11.08% respectively. But in different studies about gut microbes of BSF larvae, *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* were main phylum (Yuan Zhineng et al., 2020). It was hard to find the specific bacteria in class, order or other levels. Because gut microbes were related to food sources, regions, varieties and so on (Jeroen et al., 2020; Fathiya et al., 2020). Even suppling microbial agents like *Arthrobacter*, *Rhodoccoccus*, *Bifidobacterium* also changed the microbial diversity (Emilia et al., 2020).

3.2. Species Differences

From the abundance to see, the microbes changed a little in phylum and genus level. Even *Pseudogracilibacillus*, *Bacillaceae*, *Planococcaceae*(three belong to *Bacilli* in *Firmicutes*), *Lachnospiraceae*, *Peptostreptococcaceae* (two belong to *Clostridia* in *Firmicutes*) had altered after natural fermentation. But the dominant taxa changed significantly after BSF larvae treatment by the heatmap analysis (Zhang Xingxiao et al., 2020). Chicken manure and surplus sludge were two kinds of organic waste. We gathered some study about microbe community of these source after treated or not.

From the Table 1, we can see the *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the dominant bacteria in phylum in both sludge and chicken manure. Each substance had a special microbial community. *Spirochaetae* and *Tenericutes* can be found widely in manure and sludge(Zhang Xingxiao et al., 2020; Yang Ying et al., 2014). *Acidobacteria, Cyanobacteria, Deinococcus-Thermus, Euryarchaeota, Fibrobacteres, Gemmatimonadetes, Nitrospirae, Planctomycetes, Saccharibacteria, Synergistetes* were hard to form the advantagement but always present in organic waste(Zhang Xingxiao et al., 2020; Shailendra and Atya, 2019; Ai Chenbing etal., 2019; Yang Ying wt al., 2014; Lv Xiaomei wt al., 2015; Song Liuying wt al., 2019). During the treatment, the bacteria diversity had changed but keep the associated with the raw material. After natural fermentation, the dominant bacteria had no significant change in phylum but a little change in content. BSF larvae used the digestion in vivo and intestinal microbiome to change the manure to be worm feces which had different microbial diversity. *Firmicutes* became the most advantageous bacteria in worm feces in our study and other research (Zhang Xingxiao et al., 2020).

Table 1
Bacterial diversity in sludge and manure in available literature

Materials	Dominant Bacteria(Phylum)	Dominant Bacteria (Order)	
Activated Sludge	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Actinomycetales(3%), Bacteroidales(2%),Burkholderiales(> 8%), Desulfuromonadales(> 3%), Flavobacteriales(> 5%), Methylophilales(> 2%), Myxococcales(> 3%), Pseudomonadales(> 3%), Rhizobiales(> 9%), Rhodobacterales(> 3%), Rhodocyclales(> 9%), Xanthomonadales(> 3%)	
	Actinobacteria, Bacteroidetes, Proteobacteria	Actinomycetales(> 2%), Burkholderiales(> 15%), Caulobacterales(> 1%), Chromatiales(> 1%), Enterobacterales(0.9%), Flavobacteriales(> 3%), Nitrosomonadales(> 2%), Pasteurellales(> 1%), Pseudomonadales(> 2%), Rhizobiales(> 20%), Rhodobacterales(> 12%), Rhodocyclales(> 6%), Rhodospirillales(> 3%), Sphingomonadales(> 6%), Xanthomonadales(> 2%)	
Fresh Chicken Manure	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetae, Tenericutes	Bacteroidales(17%), Lactobacillales(20%)	
Chicken Manure After Natural Fermentation	Actinobacteria, Bacteroidetes, Firmicutes, Spirochaetae Tenericutes, Proteobacteria	Bacillales(> 24%), Ignatzschineria(27%)	
Chicken Manure Treated by BSFL	Actinobacteria, Firmicutes	Bacillales(> 33%), Lactobacillales(30%)	
Fresh Chicken Manure	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Actinomycetales(1.04%), Aeromonadales(1%), Bacillales(13.75%), Burkholderiales(4.24%), Corynebacteriales(24.43%), Enterobacyerales(4.94%), Flavobacteriales(1.06%), Lactobacillales(19.30%), Micrococcales(16.93%), Pseudomonadales(1.87%), Sphingobacteriales(3.02%), Thiotrichales(1.82%)	
Surplus Sludge	Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Saccharibacteria	Bacteroidales(4.53%), Burkholderiales(7.92%), Clostridiales(3.01%), Corynebacteriales(1.26%), Flavobacteriales(1.9%), Micrococcales(10.13%), Nitrosomonadales(1.24%), Propionibacteriales(4.39%) Rhodobacteria(5.56%), Rhodocyclales(3.58%), Xanthomonadales(1.54%)	
Mixture of Chicken Manure and Surplus Sludge Treated by BSF larvae	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Bacillales(60.71%),Burkholderiales(4.31%),Corynebacteriales(1.02%),Clostridiales(1.37%),Erysipelotrichales(2.71%), Micrococcales(10.86%), Flavobacteriales(2.05%), Lactobacillales(5.32%)	

Bacillales grew rapidly into the core bacteria which contained the 80.53% in *Firmicutes* and 60.71% in abundance of all bacteria. To further observe the changes in microbial populations, we used the heatmap analysis to show the top 30 of total abundance of classification levels in Fig. 4. *Rubrivivax* as a kind of photosynthetic bacteria were hard to find in worm feces but common in sludge. The bacterial population structure in worm feces and chicken manure were highly similar. A lot of bacteria formed advantagements in BSF larvae, like *Pseudogracilibacillus*, common degradable microorganisms in digestion and composting, were found to decomposing monensin in a combined bioaugmentation-vermicomposting approach with housefly larvae (Li Hao et al., 2020). *Virgibacillus*, putative beneficial microbes which widely present in composting system, significantly correlated with disease suppression(Shen Zongzhuan et al., 2019). *Bacillus, Oceanobacillus, Paenibacillus* were the dominant genera in manure compost (Mukesh et al., 2018). *Cellulomonas* which usually decomposed the fibre of manure and soil in nature, as used to accelerate fibrous substances like straw (Brijesh and Lata, 2012). *Gracilibacillus*, with the production of xylanase and alginate in nature, could be effective in composting (Tang Jingchun et al., 20; Giridhar and Chandra, 2021). *Erysipelothrix* were found in manure and animal with pathogenicity in some varieties, and they could become the dominant in chicken manure thermophilic digestion (Helena, 2013; Ao Tianjie et al., 2021). bacteria in worm feces in our study and other research (Zhang Xingxiao et al., 2020).

Discussion

In the BSF larvae composting is prevailing in organic waste treatment in China, especially applied to kitchen waste, livestock and poultry manure. According to incomplete statistics, the amount of daily throughput used by BSF larvae composting exceeded 3,000 ton. Mixed processing of kitchen waste with other organic waste like sludge, manure and stalks had proved to work by biogas production and composting. Thinking about the huge population and farmed animals of China, BSF larvae composting was in line with the Chinese policy of economic, agriculture development and environment protection. Using sludge

as the only food source to feed BSF larvae had reported no effect(Cai Minmin et al., 2017; Norgren et al., 2019). Mixture of sludge and manure could provide enough nutrition to BSF larvae in available literature (Cai Minmin et al., 2017). But from the data, we found the sludge in mixture were unused.

It is first report about the bacterial community of mixture of sludge and chicken manure treatment of BSF larvae. Whether the worm feces need to be treated is controversial. Obviously, worm feces were rich in nutrients which can used as fertilizer (Dennis et al., 2020). But there are several questions need to be attentive. a):If it is harmful; b):Where do the harmful factors come from; c):Whether there is untransformed waste.

In our paper, we want to clear the biosecurity of BSF larvae production residue which were excreted by BSF larvae using the mixture of chicken manure and sludge as food source. *Erysipelothrix* as probiotics were found to promote insect intestinal digestion and disease (Farah et al., 2019). We observed that *Erysipelotrichales* had proliferated to become dominant bacterium with 2.71% of gene richness. As pathogenic bacteria, it is hard to ensure the biosecurity when they release in environment with worm feces. Kiyonori found *Erysipelotrichaceae*(2.92%) in BSF larvae production residue by treating experimental household organic waste (Kiyonori et al., 2020). Erysipelas was associated to *Erysipelotrichales* which widely resist in poultry (Chirico et al., 2003).

The growth of BSF larvae, waste conversion, heavy metal transfer and drug-resistant gene are our ongoing studies. We will continue to focus on biosecurity of BSFL production residue and how to identify the conversion efficiency of waste. Pretreatment, to kill disease-causing bacteria or sludge modification for further utilized by BSF larvae, will become our research directions.

Conclusions

The present study demonstrates the bacterial biodiversity in the BSF larvae production residue from treating the mixture of surplus sludge and chicken manure (mix ratio 1:1). *Firmicutes* (75.39%)were the most dominant bacteria which distinguished from sludge(5.34%) and chicken manure(35.81%). *Pseudogracilibacillus*(11.08%), *Virgibacillus*(9.86%) and *Oceanobacillus*(6.39%) were the key component in Firmicutes. *Erysipelotrichaceae* as pathogenic bacteria were be found in BSF larvae production residue more than 2% when the wastes contain chicken manure.

Declarations

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Figures

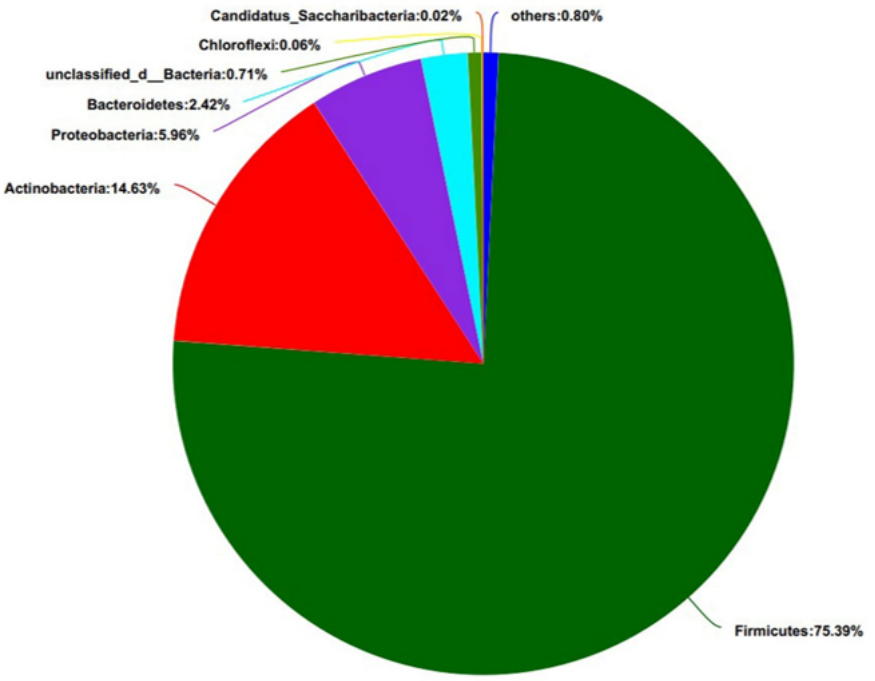

Figure 1

Relative abundance of the most abundant bacterial in phylum level in BSF larvae production residue.

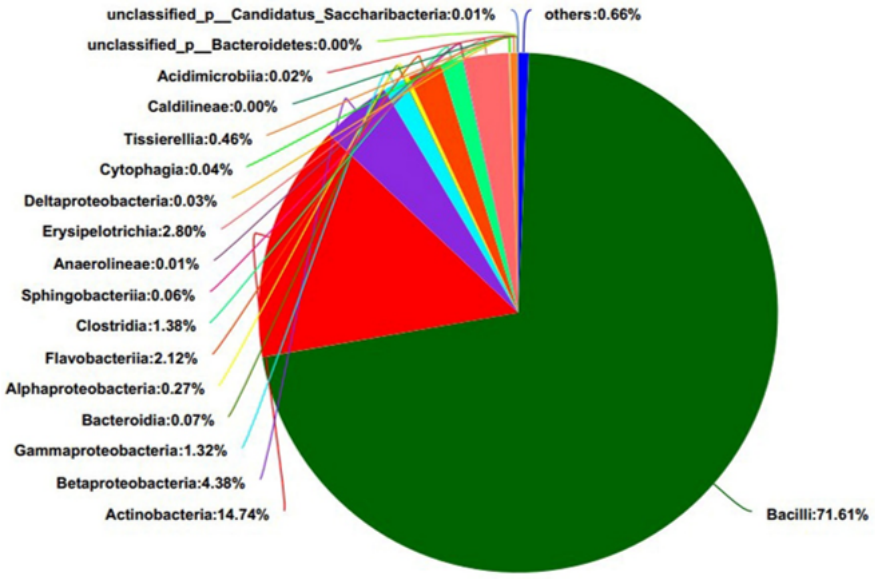

Figure 2

Relative abundance of the most abundant bacterial in class level in BSF larvae production residue.

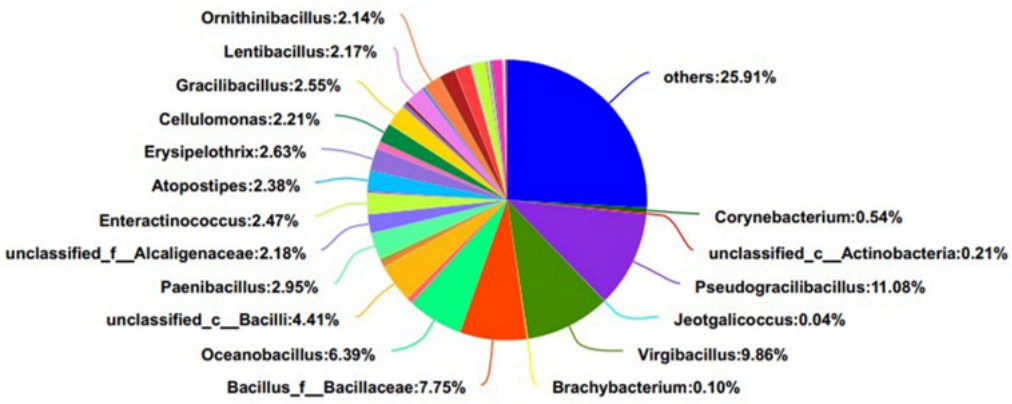

Figure 3

Relative abundance of the most abundant bacterial in genus level in BSFL production residue.

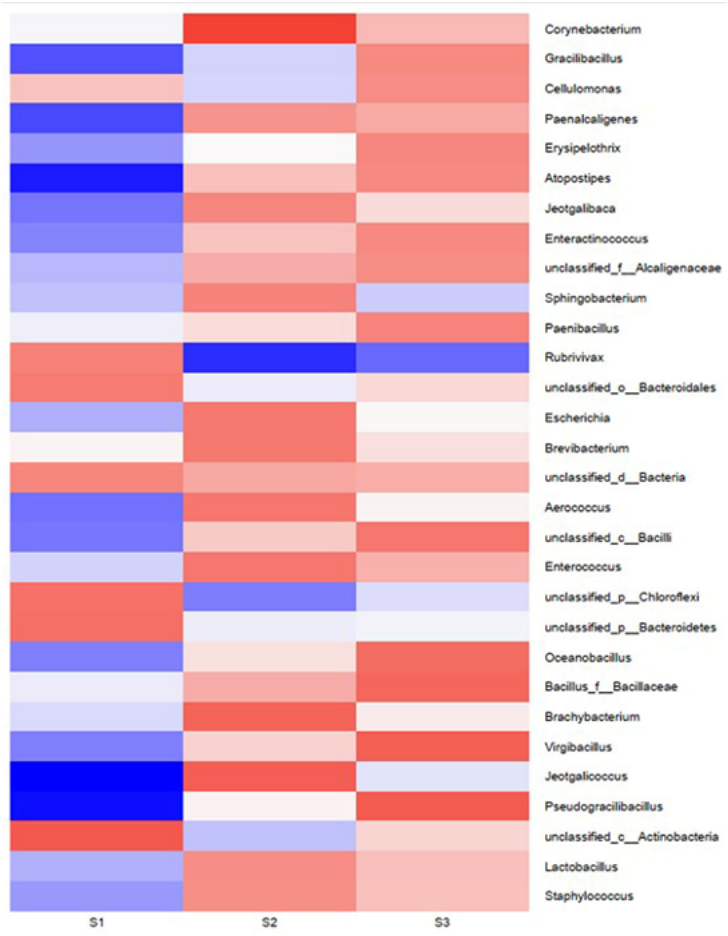

Figure 4

Heatmap analysis of top 30 common bacteria in chicken manure(S1), surplus sludge(S2) and BSFL production residue(S3).