中国烟草 CHINA TOBACCO | 四川中烟工业有限责任公司 CHINA TOBACCO SICHUAN INDUSTRIAL CO.,LTD.

Characterization and functional prediction of microbial community in agricultural processing of cigar leaves from Shifang, Sichuan

Author　　ZHANG Q.Y., CAI W., LIU Y., et al.

Reporter　　ZHANG Q.Y.

Cigar Fermentation Technology Key Laboratory of China Tobacco

(China Tobacco Sichuan Industrial Co., Ltd.)

CONTENTS

01 **Introduction**

02 **Results**

03 **Conclusion**

1 Introduction

Freshly harvested

Air-curing

Agricultural fermentation

Cigar leaves sampling

☐ **Shifang, located in Sichuan Province, is known as the Hometown of Cigar in China. In Shifang, cigar leaf has been cultivated for nearly 400 years.**

☐ **Microbes play important roles in cigar production.** However, microbial community diversity is poorly understood in agricultural processing of cigar leaves from Shifang.

☐ Based on <u>16S rRNA and ITS gene illumina Miseq high-through sequencing</u>, characterization and functional prediction of microbial community in freshly harvested, air-cured and agricultural fermented cigar leaves were revealed.
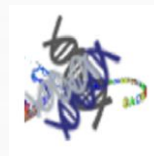
DNA extraction and sequencing
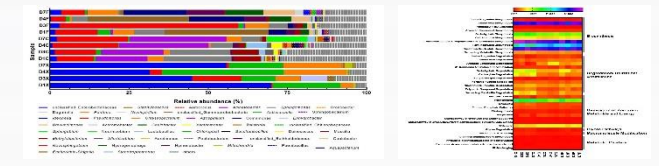
Microbial community characterization and functional prediction

2 Results

Table 1. Alpha diversity of microbes in cigar leaves

Sample	Bacterial diversity			Fungal diversity		
	Chao1 index	Shannon index	Coverage	Chao1 index	Shannon index	Coverage
D1X	236.83	3.41	0.997	219.25	3.10	0.999
D3X	479.34	4.62	0.993	171.91	2.86	0.998
D4X	322.07	3.74	0.996	186.68	1.84	0.998
D7X	362.49	3.80	0.999	262.66	3.11	0.998
D1C	872.47	6.95	0.991	122.42	3.19	0.999
D3C	1091.63	6.72	0.996	162.87	3.57	0.999
D4C	620.55	5.89	0.993	125.62	2.91	0.999
D7C	397.59	4.95	0.996	122.31	3.34	0.999
D1F	546.13	4.93	0.994	25.17	0.16	0.999
D3F	514.86	5.09	0.995	117.51	1.30	0.999
D4F	421.45	4.55	0.996	50.76	0.92	0.999
D7F	358.32	5.02	0.997	107.64	1.97	0.999

Freshly harvested
Dexue No.1 to No.7 cigar leaf

Air-cured
Dexue No.1 to No.7 cigar leaf

Agricultural Fermented
Dexue No.1 to No.7 cigar leaf

The coverages were more than 0.99, indicating that Illumina MiSeq sequencing was deep enough to represent all microbial communities detected.

2 Results

Table 1. Alpha diversity of microbes in cigar leaves

Sample	Bacterial diversity			Fungal diversity		
	Chao1 index	Shannon index	Coverage	Chao1 index	Shannon index	Coverage
D1X	236.83	3.41	0.997	219.25	3.10	0.999
D3X	479.34	4.62	0.993	171.91	2.86	0.998
D4X	322.07	3.74	0.996	186.68	1.84	0.998
D7X	362.49	3.80	0.999	262.66	3.11	0.998
D1C	872.47	6.95	0.991	122.42	3.19	0.999
D3C	1091.63	6.72	0.996	162.87	3.57	0.999
D4C	620.55	5.89	0.993	125.62	2.91	0.999
D7C	397.59	4.95	0.996	122.31	3.34	0.999
D1F	546.13	4.93	0.994	25.17	0.16	0.999
D3F	514.86	5.09	0.995	117.51	1.30	0.999
D4F	421.45	4.55	0.996	50.76	0.92	0.999
D7F	358.32	5.02	0.997	107.64	1.97	0.999

Freshly harvested
Dexue No.1 to No.7 cigar leaf

Air-cured
Dexue No.1 to No.7 cigar leaf

Agricultural Fermented
Dexue No.1 to No.7 cigar leaf

**The diversity and richness of the fungal communities were generally lower than those of the bacterial communities.**

2 Results

Table 1. Alpha diversity of microbes in cigar leaves

Sample	Bacterial diversity			Fungal diversity		
	Chao1 index	Shannon index	Coverage	Chao1 index	Shannon index	Coverage
D1X	236.83	3.41	0.997	219.25	3.10	0.999
D3X	479.34	4.62	0.993	171.91	2.86	0.998
D4X	322.07	3.74	0.996	186.68	1.84	0.998
D7X	362.49	3.80	0.999	262.66	3.11	0.998
D1C	872.47	6.95	0.991	122.42	3.19	0.999
D3C	1091.63	6.72	0.996	162.87	3.57	0.999
D4C	620.55	5.89	0.993	125.62	2.91	0.999
D7C	397.59	4.95	0.996	122.31	3.34	0.999
D1F	546.13	4.93	0.994	25.17	0.16	0.999
D3F	514.86	5.09	0.995	117.51	1.30	0.999
D4F	421.45	4.55	0.996	50.76	0.92	0.999
D7F	358.32	5.02	0.997	107.64	1.97	0.999

Freshly harvested
Dexue No.1 to No.7 cigar leaf

Air-cured
Dexue No.1 to No.7 cigar leaf

Agricultural Fermented
Dexue No.1 to No.7 cigar leaf

The diversities of air-cured cigar leaf were the highest.

2 Results
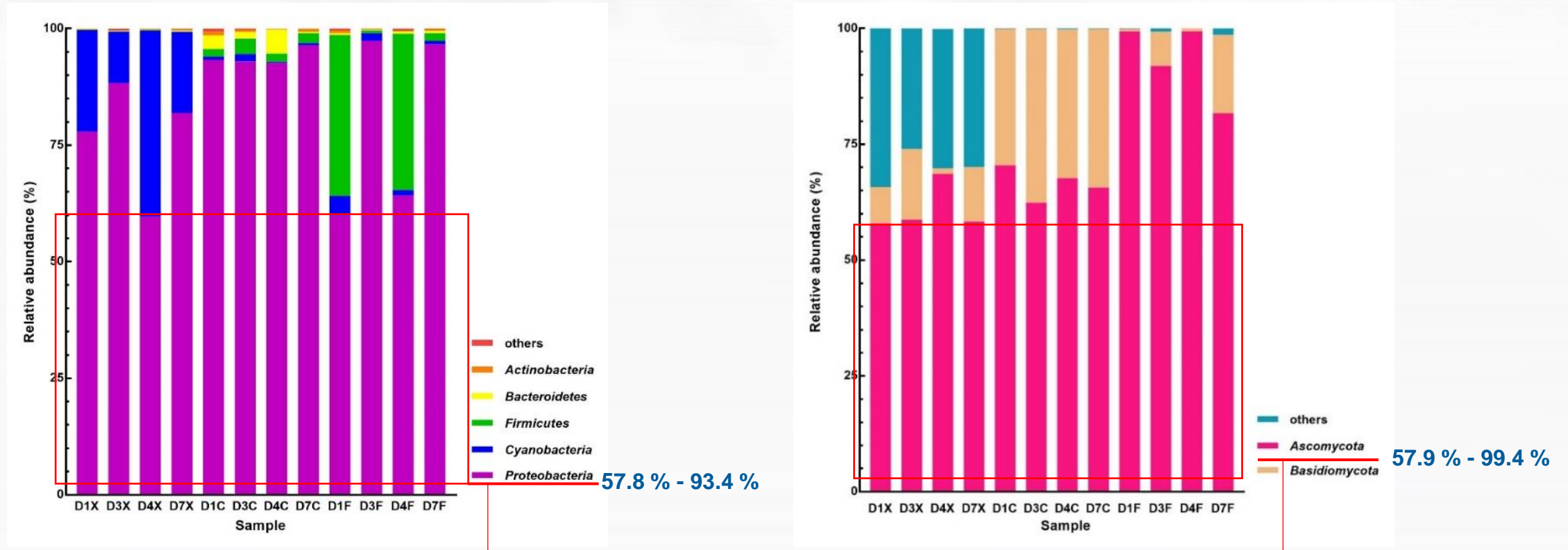
The dominant bacterial phylum was *Proteobacteria*,
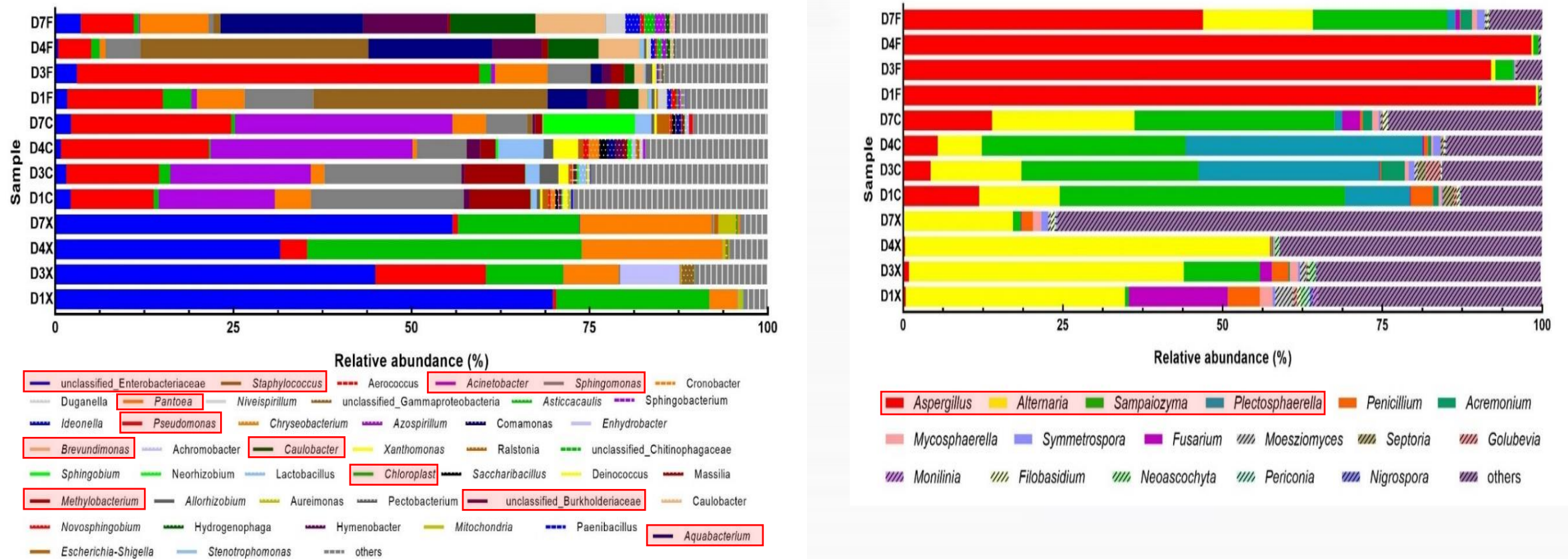and the dominant fungal phylum was *Ascomycota*.

Figure 1. Plot of phylum level relative abundances of bacterial and fungal communities in cigar leaves

2 Results

There were **16 dominant microbial genera**, including unclassified Enterobacteriaceae, *Pseudomonas*, *Chloroplast*, *Acinetobacter*, *Pantoea*, *Sphingomonas*, *Staphylococcus*, *Aquabacterium*, unclassified Burkholderiaceae, *Methylobacterium*, *Caulobacter*, *Brevundimonas*, *Aspergillus*, *Alternari*, *Sampaiozyma*, and *Plectosphaerella*.

Figure 2. Plot of genus level relative abundances of bacterial and fungal communities in cigar leaves

2 Results

The dominant microbial community succession was from unclassified_Enterobacteriaceae, *Chloroplast*, *Pantoea*, and *Alternaria*, followed by *Acinetobacter*, *Sphingomona*, *Methylobacterium*, *Sampaiozyma*, and *Plectosphaerella*, and finally to *Pseudomonas*, *Staphylococcus*, *Aquabacterium*, unclassified_Burkholderiaceae, *Brevundimonas*, and *Aspergillus*.
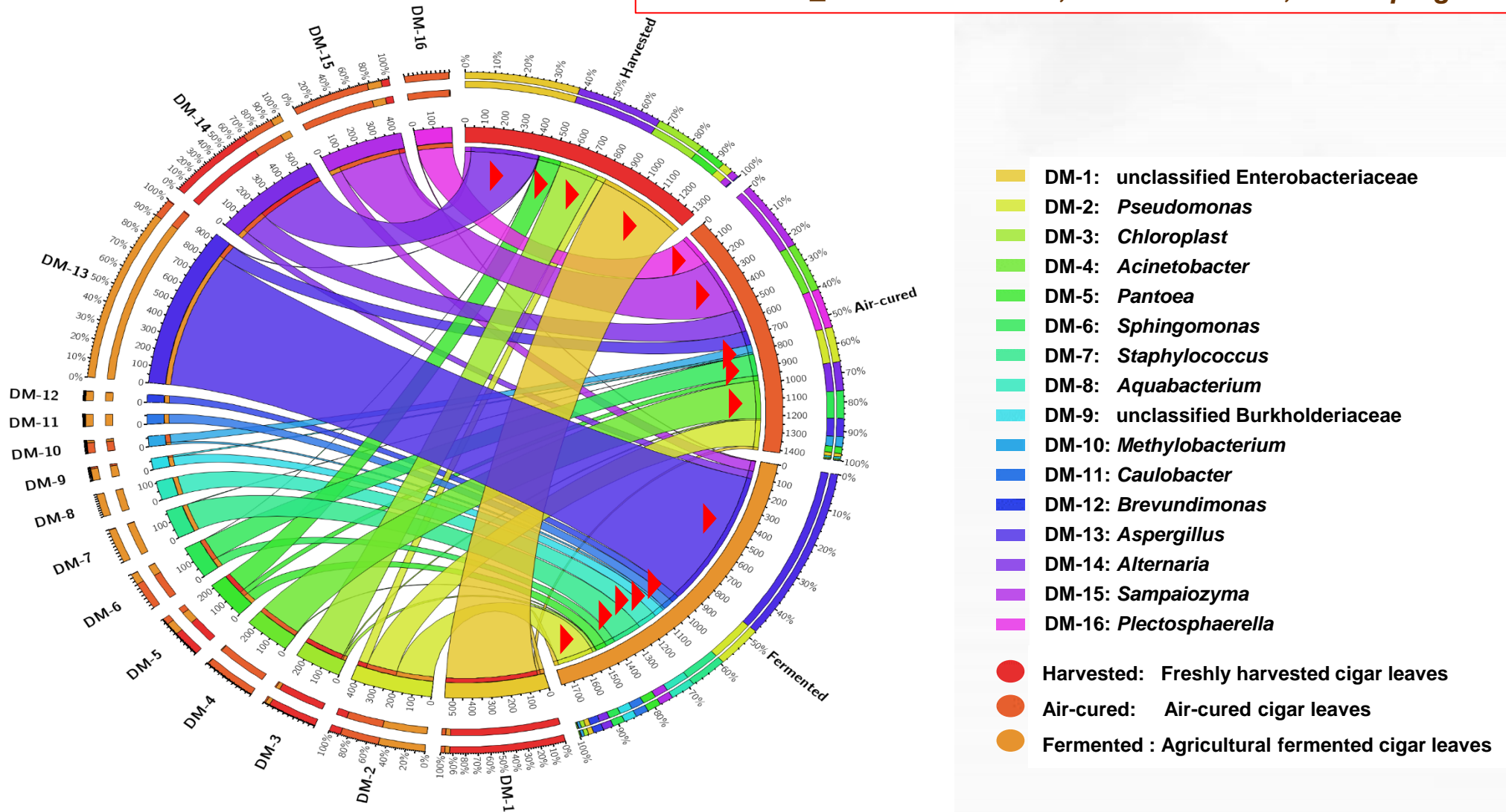
Figure 3. Circos diagram of the relationship between dominant microbes and sampling points

Legend:
- DM-1: unclassified Enterobacteriaceae
- DM-2: *Pseudomonas*
- DM-3: *Chloroplast*
- DM-4: *Acinetobacter*
- DM-5: *Pantoea*
- DM-6: *Sphingomonas*
- DM-7: *Staphylococcus*
- DM-8: *Aquabacterium*
- DM-9: unclassified Burkholderiaceae
- DM-10: *Methylobacterium*
- DM-11: *Caulobacter*
- DM-12: *Brevundimonas*
- DM-13: *Aspergillus*
- DM-14: *Alternaria*
- DM-15: *Sampaiozyma*
- DM-16: *Plectosphaerella*

- Harvested: Freshly harvested cigar leaves
- Air-cured: Air-cured cigar leaves
- Fermented: Agricultural fermented cigar leaves

2 Results

The R2X, R2Y and Q2 were 0.831, 0.932 and 0.919.

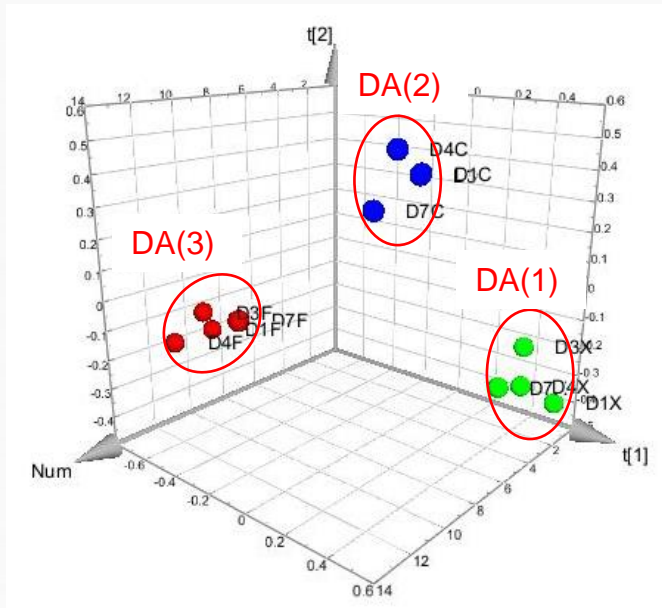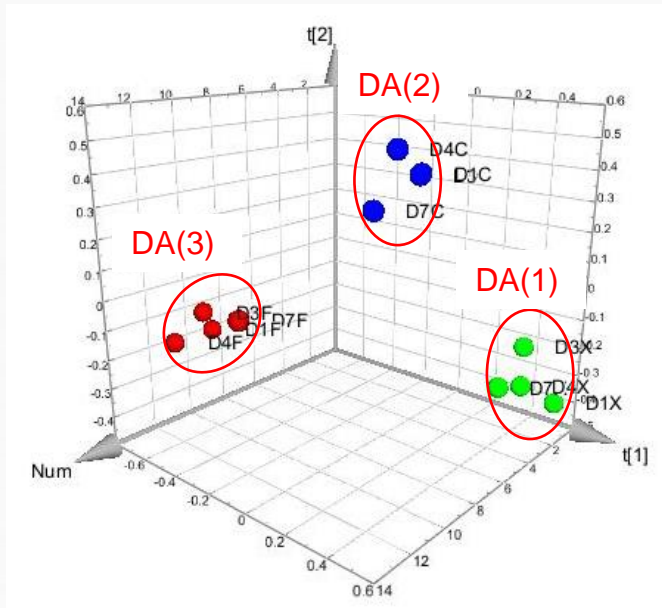**The PLS-DA model was suitable for this research.**

Figure 4. Score scatter three-dimensional plot of PLS-DA

According to different periods of agricultural processing, samples were divided into three groups.

Table 2. Coefficient values between variables and groups

Variable	CoeffCS[1]			CoeffCS[2]		
	DA(1)	DA(2)	DA(3)	DA(1)	DA(2)	DA(3)
unclassified_Enterobacteriaceae	0.5778*	0.1442	-0.7220*	1.6971*	-1.4545*	-0.2426
unclassified_Burkholderiaceae	-0.0826*	-0.0206	0.1032	-0.0530*	-0.0629	0.1159
Novosphingobium	-0.0052*	-0.0013	0.0065*	-0.0146*	0.0122*	0.0024
Pectobacterium	-0.0032*	-0.0008	0.0041*	-0.0018*	-0.0029*	0.0047*
Aspergillus	-1.2266*	-0.3060	1.5326*	-0.6147*	-1.1800*	1.7947*
Alternaria	0.4261*	0.1063	-0.5324*	0.8697*	-0.5273	-0.3424*
Penicillium	0.0318*	0.0079	-0.0397*	0.0481	-0.0153	-0.0328*
Mycosphaerella	0.0132*	0.0033	-0.0164*	0.0238*	-0.0119	-0.0119
Moesziomyces	0.0124	0.0031	-0.0155*	0.0315	-0.0241	-0.0074
Filobasidium	0.0039*	0.0010	-0.0049*	0.0013	0.0047	-0.0060
Nigrospora	0.0024	0.0006	-0.0030*	0.0048	-0.0028	-0.0020

Note: CoeffCS[1] and CoeffCS[2] represent significant principal component 1 and 2, respectively.
 * means significant at the level of 0.05.

2 Results

The R2X, R2Y and Q2 were 0.831, 0.932 and 0.919.

The PLS-DA model was suitable for this research.

Figure 4. Score scatter three-dimensional plot of PLS-DA

According to different periods of agricultural processing, samples were divided into **three groups**.

Table 2. Coefficient values between variables and groups

Variable	CoeffCS[1]			CoeffCS[2]		
	DA(1)	DA(2)	DA(3)	DA(1)	DA(2)	DA(3)
unclassified_Enterobacteriaceae	0.5778*	0.1442	-0.7220*	1.6971*	-1.4545*	-0.2426
unclassified_Burkholderiaceae	-0.0826*	-0.0206	0.1032	-0.0530*	-0.0629	0.1159
Novosphingobium	-0.0052*	-0.0013	0.0065*	-0.0146*	0.0122*	0.0024
Pectobacterium	-0.0032*	-0.0008	0.0041*	-0.0018*	-0.0029*	0.0047*
Aspergillus	-1.2266*	-0.3060	1.5326*	-0.6147*	-1.1800*	1.7947*
Alternaria	0.4261*	0.1063	-0.5324*	0.8697*	-0.5273	-0.3424*
Penicillium	0.0318*	0.0079	-0.0397*	0.0481	-0.0153	-0.0328*
Mycosphaerella	0.0132*	0.0033	-0.0164*	0.0238*	-0.0119	-0.0119*
Moesziomyces	0.0124	0.0031	-0.0155*	0.0315	-0.0241	-0.0074*
Filobasidium	0.0039*	0.0010	-0.0049*	0.0013	0.0047	-0.0060*
Nigrospora	0.0024	0.0006	-0.0030*	0.0048	-0.0028	-0.0020*

Note: CoeffCS[1] and CoeffCS[2] represent significant principal component 1 and 2, respectively.
 * means significant at the level of 0.05.

2 Results

The number of functional genes of bacteria was higher than that of fungi.
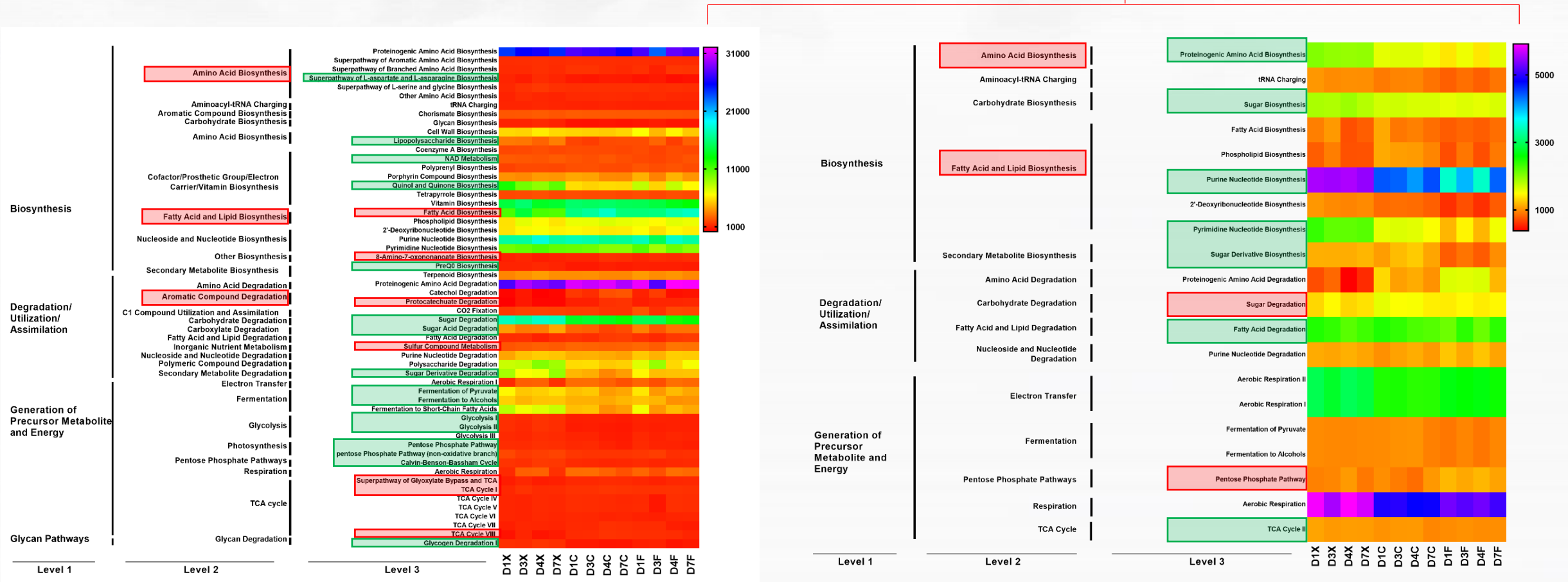

Figure 5. Function prediction of bacteria and fungi of cigar leaves

The functions of bacteria and fungi were associated with fatty acid, amino acid, and aromatic compound biosynthesis.

2 Results

Functions were significantly positively correlated with unclassified Enterobacteriaceae, *Chloroplast*, *Pantoea*, *Mycosphaerella*, *Sampaiozyma*, *Symmetrospora*, *Penicillium*, *Filobasidium*, and *Alternaria*.
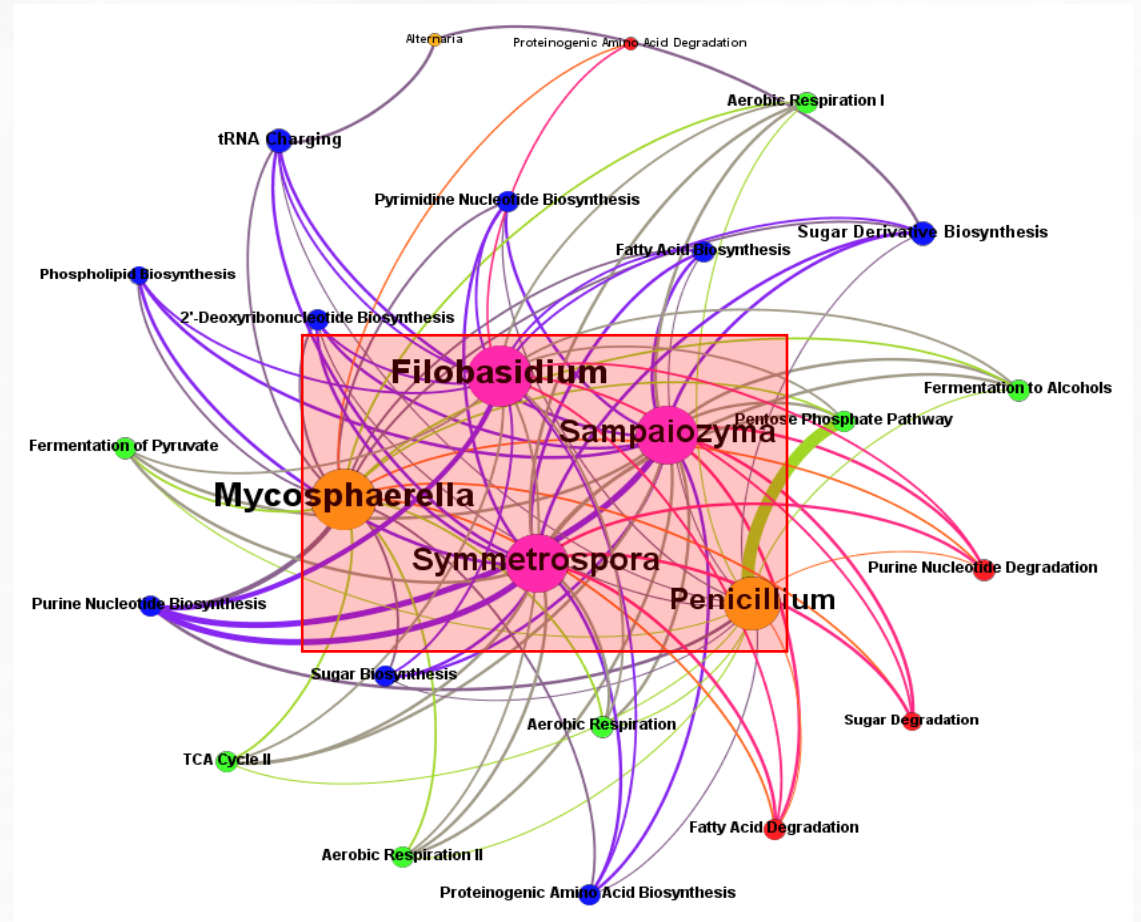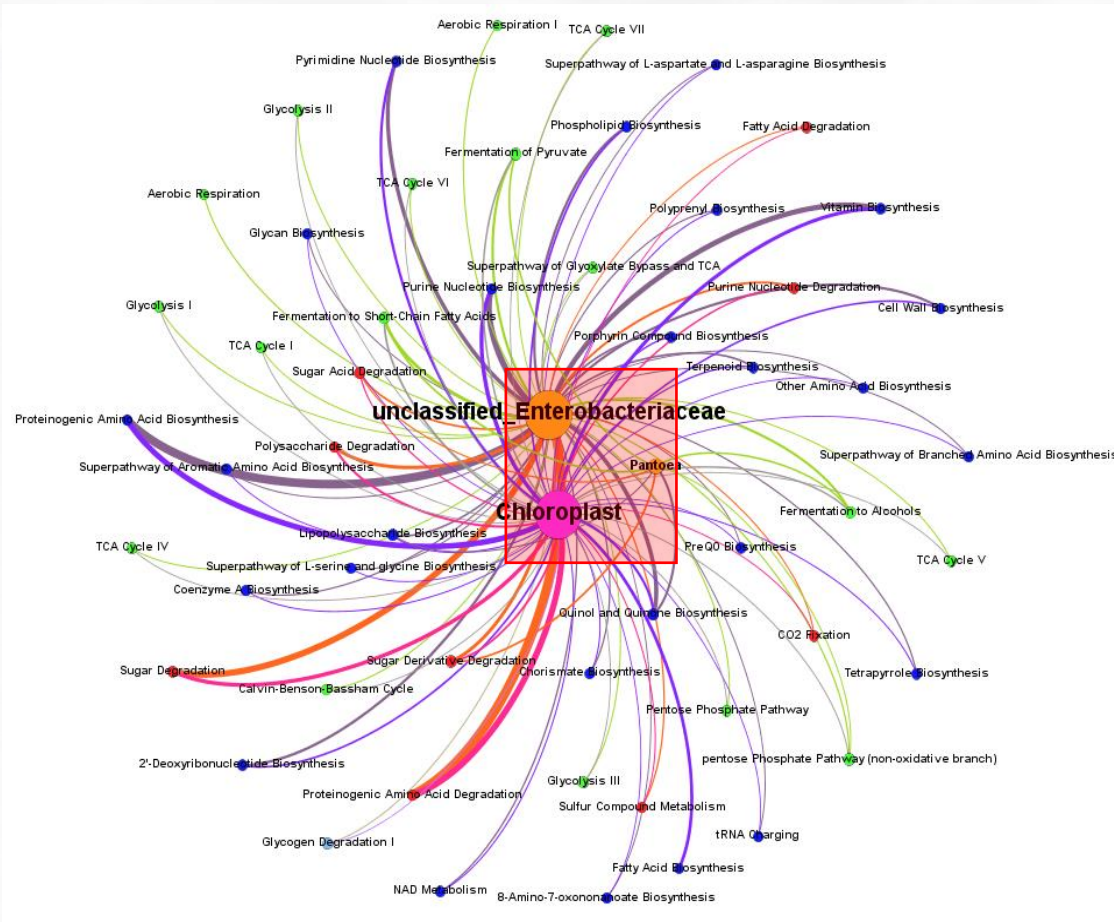

Figure 6. Correlation between microbial genera and functional genes

3 Conclusion

**Microbes played important roles in improving the flavor and aroma of cigar leaves.**

The differences of microbes in different periods during the agricultural processing were bigger than those among different varieties.

Species and functions of microbes of cigar leaves varied with different periods in the agricultural processing.

The numbers of bacterial population and function in all samples were higher than those of fungal.
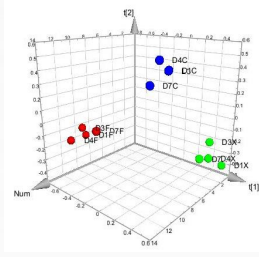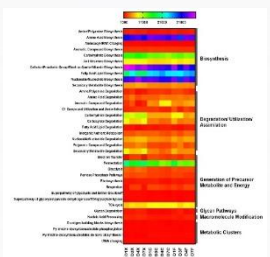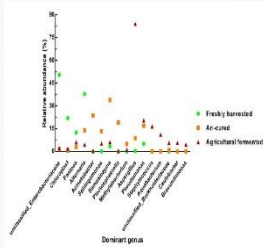
□ Microbial community succession was from **unclassified Enterobacteriaceae**, *Chloroplast*, *Pantoea*, and *Alternaria*, followed by *Acinetobacter*, *Sphingomona*, *Methylobacterium*, *Sampaiozyma*, and *Plectosphaerella*, and finally to *Pseudomonas*, *Staphylococcus*, *Aquabacterium*, *unclassified_Burkholderiaceae*, *Brevundimonas*, and *Aspergillus*.

□ Unclassified Enterobacteriaceae, *Chloroplast*, *Pantoea*, *Mycosphaerella*, *Sampaiozyma*, *Symmetrospora*, *Penicillium*, *Filobasidium*, and *Alternaria* might be important functional microbes in agricultural processing of cigar leaves.