Yin *et al. Chem. Biol. Technol. Agric.* (2023) 10:19
https://doi.org/10.1186/s40538-023-00394-8

Chemical and Biological Technologies in Agriculture

RESEARCH

Open Access

Effect of growth stage on Italian ryegrass (*Lolium multiflorum* Lam.) silage fermentation from microbiological perspective

Xuejing Yin, Jiangyu Long, Jie Zhao, Siran Wang, Zhihao Dong, Junfeng Li and Tao Shao*

Abstract

Background Italian ryegrass is a temperate climate crop, which is widely cultivated in the winter fallow paddy fields of subtropical China. The utilization efficiency of Italian ryegrass depends greatly on its growth stage at harvest. Previous studies have reported the optimum stage for harvesting various forage to balance their quality and quantity. However, when considering the practice condition, such as rainy or unavailability of harvest equipment, the harvest stage of forage cannot always be implemented according to the production schedules. Thus, to characterize the effect of growth stage on the silage fermentation profile, bacterial community construction and metabolisms of carbohydrates and amino acids, Italian ryegrass were naturally ensiled at the filling stage (FSN) and the dough stage (DSN), respectively. After ensiling for 1, 3, 7, 15, 30, and 60 days, triplicate silos were opened for sampling.

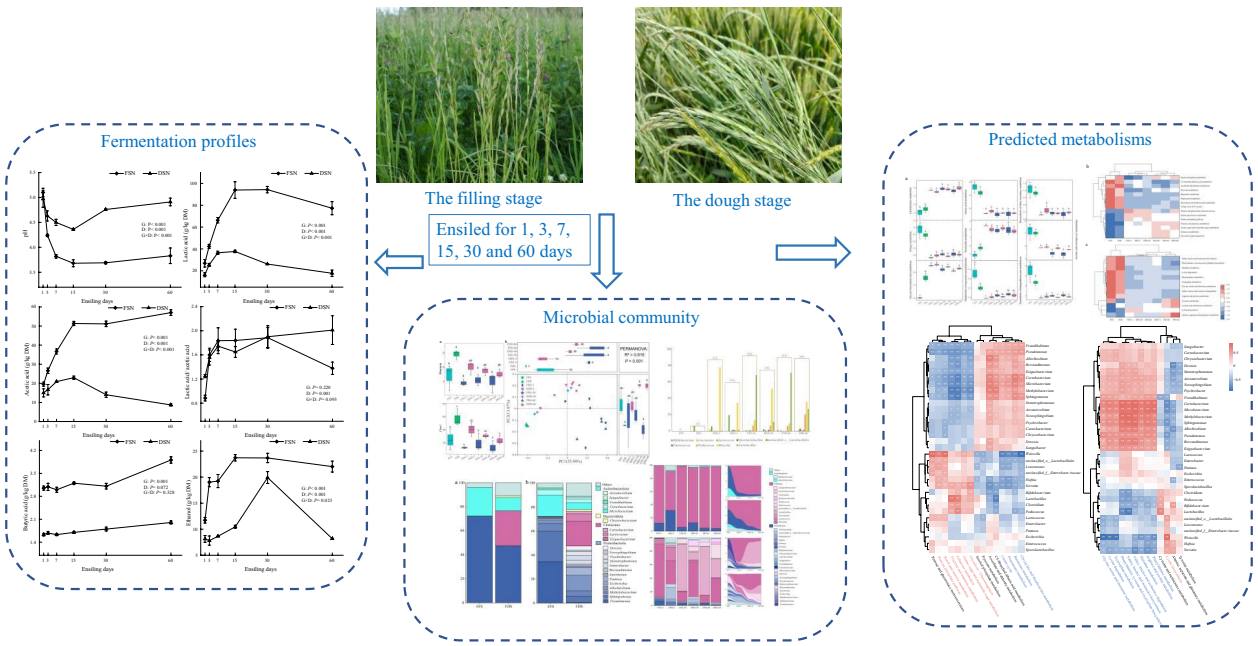
Results The growth of Italian ryegrass increased the pH, dry matter, neutral, and acid detergent fiber contents, but decreased buffering capacity, crude protein and water-soluble carbohydrates contents, and the epiphytic microbiota of Italian ryegrass harvested at the filling stage was simpler than that harvested at the dough stage. During ensiling, FSN had lower pH and higher organic acid content than DSN. The bacterial succession rate in FSN was also faster than DSN, which showed that *Lactobacillus* becomes the dominant genus in the early stage of ensiling. The predicted metabolisms revealed that carbohydrate and amino acid metabolisms were the two main metabolisms in silage fermentation. When compared with epiphytic microbiota, ensiling enhanced carbohydrate metabolism and diminished amino acid metabolism. The difference of these two metabolisms between FSN and DSN was obvious at the early stage of ensiling.

Conclusions Growth stage affected the chemical and microbial composition of Italian ryegrass, so as to the fermentation profile, bacterial community and its metabolisms intensity. Italian ryegrass harvested at the filling stage was prone to ferment. The complexity of epiphytic microbiota made Italian ryegrass harvested at the dough stage more difficult to ferment.

Keywords Italian ryegrass, Growth stage, Silage fermentation, Microbial community

*Correspondence:
Tao Shao
taoshaolan@163.com
Full list of author information is available at the end of the article

Graphical Abstract



Background

The development of animal husbandry is usually limited by the shortage of forage supply [1]. Italian ryegrass is a temperate climate crop, which is widely cultivated in the winter fallow paddy fields of subtropical China and can be utilized to meet the forage shortage of livestock consumption [2]. However, the utilization efficiency of Italian ryegrass depends greatly on its growth stage at harvest, as growth stage is the main factor influencing the nutrition and digestibility of forage [3]. The previous studies have reported the optimum stage for harvesting various forage to balance their quality and quantity. However, when considering the practice condition, such as rainy or unavailability of harvest equipment, the harvest stage of forage cannot always be implemented according to the production schedules [4]. Ensiling is a widespread practice to maintain the nutritional quality of forage and ensure the year-round supply of feed to meet the production requirement of animals [5]. The growth stage of plant harvest also affects the fermentation quality of the silage. Because factors affecting ensilability, such as buffering capacity [6], dry matter [7], and water-soluble carbohydrates [8] change with the growth of plants.

As known, ensiling is a microbial-driven fermentation process, which relies on the lactic acid bacteria (LAB) to produce acid to limit the growth of other microorganisms and nutrient loss. The role of LAB in the fermentation quality of silage has been extensively studied. Although the development of culture-independent technologies, such as next-generation sequencing enables researchers to understand the complex microbial community involved in silage fermentation, and understand the role of the microbial community (not only LAB) from an ecological perspective. However, most studies still remain at the level of description, and fail to establish a relationship between microbial community and function in silage fermentation. Through understanding the relationship between biodiversity, function, and fermentation products, exploring the role of microorganisms in silage may fully understand the function of growth stage on silage fermentation quality.

Therefore, the aim of this study was to evaluate chemical component, microbial structure, and functional differences in the two growth stages of Italian ryegrass and the effect of growth stage on silage fermentation.

Materials and methods

Forage material

Italian ryegrass cultivated on the Baima experimental field of Nanjing Agricultural University (31.61°N, 119.18°E, elevation 24.8 m, annual average precipitation

1106 mm, and temperature 15.4℃, Jiangsu, China) were harvested at the filling stage (FFS) and the dough stage (FDS) on 8th May and 12th June 2021, respectively. After harvest, Italian ryegrass was immediately chopped into approximately 2-cm lengths using a fodder chopper (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China). The chopped forages of each growth stage were mixed thoroughly to prepare silage and analyze raw material.

A total of 36 bags (2 growth stages × 6 store times × 3 replicates) were prepared. Briefly, 100 g of chopped Italian ryegrass was packed into a presterilized polyethylene plastic bag (size: 30 cm × 40 cm), and vacuum sealed by a vacuum sealing machine (DZD-400, Nanjing Aomitai Technology Co., Ltd., Nanjing, China). Then, the bag-silos were stored at ambient temperature (27 ± 2.5 ℃). Triplicate bag-silos of each growth stage of Italian ryegrass were sampled on days 1, 3, 7, 15, 30, and 60 of ensiling.

Analytical methods

Details relating to the sample preparation and analyses for the chemical and microbial composition of fresh Italian ryegrass and its silage were the same as used by Yin et al. [9].

The dry matter (DM) content was determined by oven-dried for 72 h at 65 ℃. Water-soluble carbohydrates (WSC), total nitrogen (TN), neutral, and acid detergent fiber content (NDF and ADF) were determined on the forage powder ground after being used for dry matter analysis. The WSC content was analyzed by the Anthrone method [10]. The TN content was measured by the Kjeldahl method (Kjeltec 2300 Auto-Analyzer; FOSS Analytical AB, Hoganas, Sweden) [11], and crude protein (CP) content was calculated by the TN × 6.25. The NDF and ADF contents were determined according to the method of Van Soest et al. [12].

The filtrate extracted from fresh and ensiled Italian ryegrass was used to determine the pH, buffering capacity, ammonia nitrogen (NH_3-N), lactic, acetic, and butyric acid (LA, AA, and BA), and ethanol contents. The pH was measured by a pH meter (Hanna Instruments, Inc., Woonsocket, RI, USA). The buffering capacity was determined following the method described by Playne and McDonald [13]. The NH_3—N content was determined by the colorimetric method [14]. According to the method reported by Zhao et al. [15], the concentrations of organic acid and ethanol were measured by HPLC.

The culturable microorganisms from fresh and ensiled Italian ryegrass were enumerated by plate. Lactic acid bacteria and aerobic bacteria were enumerated, respectively, on MRS (de Man, Rogosa, and Sharp agar medium) and nutrient agar at 37 ℃ for 48 h; Enterobacteria were counted on Violet Red Bile agar at 37 ℃ for 24 h; Yeasts and moulds were counted on Rose Bengal agar, after incubating at 30 ℃ for 48 h. All microbial data were transformed to $\log_{10}$ on a fresh weight basis.

Bacterial DNA was extracted with the FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA, U.S.) according to the manufacturer's instructions. The DNA quantity and quality were tested by the agarose gel electrophoresis (1% agarose gel, 5 V/cm voltage, 20 min) and NanoDrop 2000 UV-vis Spectrophotometer (Thermo Scientific, Wilmington, DE). The PCR reactions were performed according to the procedure described in Dong et al. [16]. The PCR products were purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) and quantified using QuantiFluor™-ST according to the manufacturer's protocol.

Sequencing was done on an Illumina MiSeq PE300 platform using the paired-end method at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Obtained sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) software package version 1.9.1. Quality trimming of dataset removed sequences with a mean quality score less than 20. The remaining sequences were assigned to operational taxonomic units (OTUs) at a threshold of 97% pairwise sequence identity by UPARSE (version 7.0). OTU representative sequences were then classified taxonomically using the Silva database with a confidence threshold of 70%. Bacterial community structure was analyzed at the phylum and genus levels, and the relative abundance of bacteria shown in the figures is greater than 0.1%.

Statistical analysis

SPSS 22.0 software (IBM Corporation, New York, USA) was employed to perform the statistical analyses of chemical and microbial count data. Chemical and microbial data of the two growth stages of fresh Italian ryegrass were subjected to Student's *t* test. The data on the fermentation quality and microbial composition of silage were analyzed by two-way ANOVA. Alpha diversity (Shannon and Chao1) was calculated at QIIME (version 1.9.1). Beta diversity (Principal coordinates analysis, PCoA) was calculated and plotted based on the Unweight—Unifrac distance metric in R software (version 3.3.1). The permutational multivariate analysis of variance (PERMANOVA) was calculated to detect the difference in the microbiota structure. The correlation between microbes and fermentation products or metabolisms was calculated by Spearman's Correlation

Coefficient (R software, version 4.1.3). Functional profiles of bacterial community were predicted by the Tax-4fun tool based on the KEGG database [17]. The data were visualized using Origin and R software.

Results

Characteristics of fresh Italian ryegrass harvested at the filling and dough stages

The differences in chemical and microbial of two growth stages of Italian ryegrass are shown in Table 1. The contents of pH, DM, NDF, and ADF increased significantly ($P<0.05$) from the filling to dough stage of Italian ryegrass, while WSC content decreased significantly ($P<0.05$). The content of buffering capacity and CP did not change significantly, but decreased slightly with the growth of Italian ryegrass. Among the culturable microbes, only LAB counts increased with the growth of Italian ryegrass.

Fermentation characteristics of Italian ryegrass silage

Italian ryegrass harvested at the filling and dough stages were ensiled respectively, the corresponding changes of

Table 1 The chemical components and epiphytic microbiota of fresh Italian ryegrass at two growth stages

Items	The filling stage	The dough stage	SEM	*P* value
pH	6.12	6.75	0.143	< 0.001
Dry matter (g/kg FM)	226	347	7.089	< 0.001
Water-soluble carbohydrates (g/kg DM)	145	84.8	3.964	0.002
Buffering capacity (mEq/kg DM)	49.0	42.6	1.891	0.084
Neutral detergent fiber (g/kg DM)	527	570	2.182	0.014
Acid detergent fiber (g/kg DM)	322	347	5.905	0.007
Crude protein (g/kg DM)	62.7	59.1	1.200	0.142
Lactic acid bacteria (Log_{10} cfu/g FM)	4.33	5.42	0.198	0.032
Aerobic bacteria (Log_{10} cfu/g FM)	8.54	8.00	0.270	0.374
Yeasts (Log_{10} cfu/g FM)	5.29	3.07	0.587	< 0.001
Enterobacteria (Log_{10} cfu/g FM)	5.34	6.01	0.168	0.016

FM, fresh matter; TN, total nitrogen; cfu, colony-forming units; SEM, standard error of mean

Table 2 Effects of different growth stages on chemical components and microbial compositions during Italian ryegrass ensiling

Items	Treatments	Ensiling days						SEM	*P* value		
		1	3	7	15	30	60		G	D	G × D
Dry matter (g/kg FM)	FSN	220^{Ba}	217^{Bab}	213^{Babc}	210^{Bbc}	211^{Bbc}	205^{Bc}	12.97	< 0.001	0.001	0.228
	DSN	369^{A}	375^{A}	370^{A}	354^{A}	348^{A}	357^{A}				
Water-soluble carbohydrates (g/kg DM)	FSN	125^{Aa}	71.9^{Ab}	55.8^{Ac}	46.4^{Acd}	38.3^{Ade}	31.5^{Ae}	5.039	< 0.001	< 0.001	< 0.001
	DSN	48.7^{Ba}	40.5^{Bab}	36.2^{Bbc}	31.0^{Bbcd}	25.7^{Bd}	28.5^{cd}				
Ammonia nitrogen (g/kg TN)	FSN	39.0^{c}	55.1^{b}	67.7^{a}	75.2^{a}	75.4^{Aa}	74.6^{a}	2.374	0.039	< 0.001	0.810
	DSN	40.0^{c}	51.7^{b}	64.4^{a}	72.6^{a}	70.9^{Ba}	71.5^{a}				
Lactic acid bacteria (Log_{10} cfu/g FM)	FSN	8.96^{Bab}	9.90^{a}	9.49^{Aa}	8.11^{b}	6.03^{c}	6.91^{c}	0.214	0.272	< 0.001	0.005
	DSN	9.55^{Aa}	9.18^{ab}	8.96^{Bab}	8.22^{bc}	7.23^{c}	7.15^{c}				
Enterobacteria (Log_{10} cfu/g FM)	FSN	7.20^{Ba}	4.88^{Bb}	ND	ND	ND	ND	0.007	< 0.001	< 0.001	0.067
	DSN	7.54^{Aa}	6.20^{Ab}	ND	ND	ND	ND				
Aerobic bacteria (Log_{10} cfu/g FM)	FSN	8.95^{Ba}	7.90^{Bab}	6.06^{Bc}	6.74^{Bbc}	6.04^{Bc}	5.81^{c}	0.217	< 0.001	< 0.001	0.859
	DSN	9.57^{Aa}	8.97^{Aa}	7.20^{Ab}	7.34^{Ab}	6.79^{Ab}	6.66^{b}				
Yeasts (Log_{10} cfu/g FM)	FSN	7.07^{Ba}	4.99^{b}	4.75^{bc}	3.90^{bc}	4.03^{bc}	3.36^{c}	0.268	0.004	< 0.001	0.904
	DSN	7.56^{Aa}	6.11^{ab}	5.41^{bc}	4.54^{bc}	ND	4.02^{c}				

$^{A-B}$ Values with different capital letters within the same column indicate significant differences between treatments in the same ensiling day ($P<0.05$). $^{a-c}$Values with different lowercase letters indicate significant differences among ensiling days in the same treatment ($P<0.05$)

[1] FM, fresh matter; TN, total nitrogen; cfu, colony-forming units

[2] FSN, natural fermentation of Italian ryegrass harvested at the filling stage; DSN, natural fermentation of Italian ryegrass harvested at the dough stage; ND, not detected

[3] G, effect of growth stage; D, effect of ensiling day; G × D, interaction effect of growth stage and ensiling day

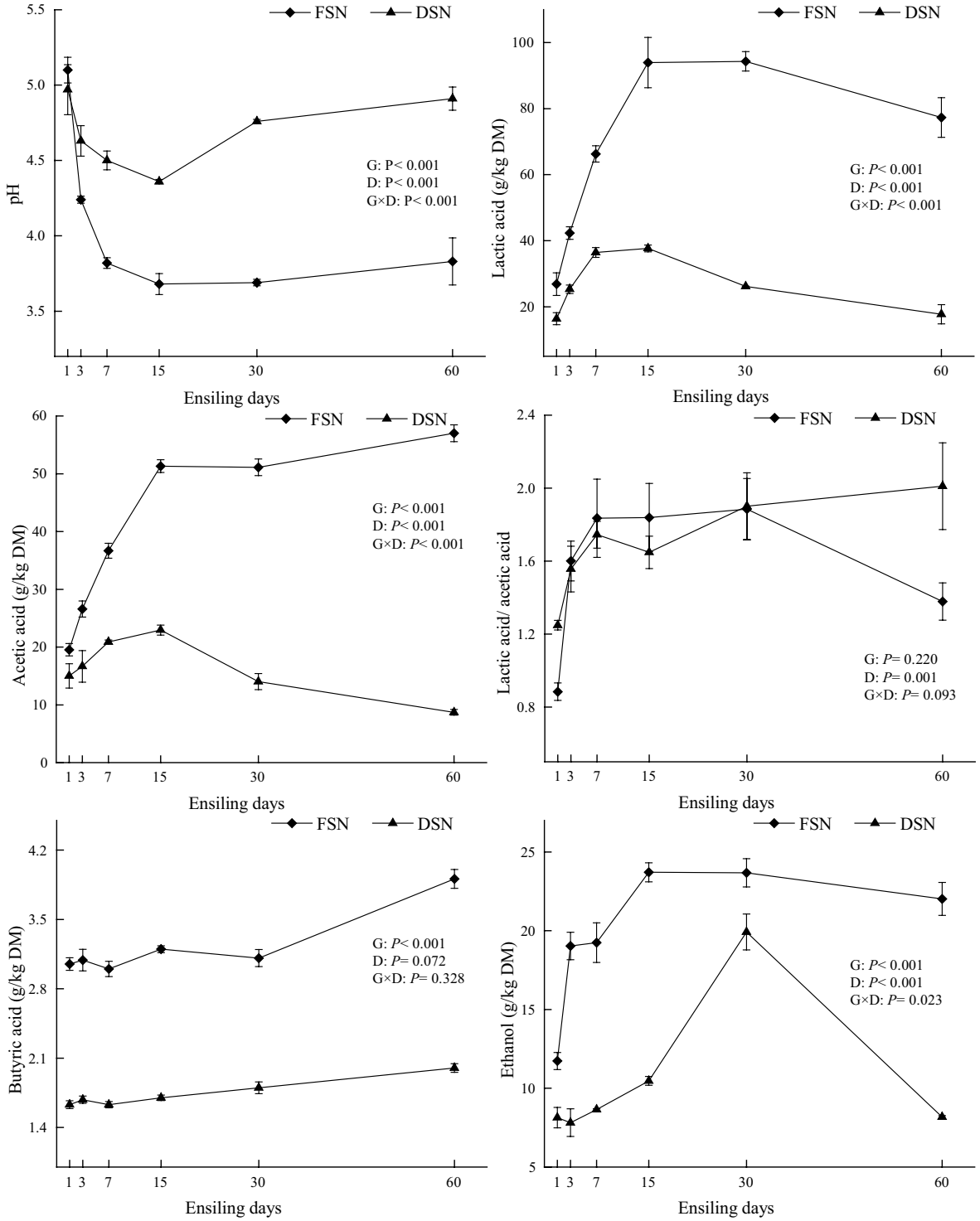


Fig. 1 Changes of pH, organic acid and ethanol contents during the ensiling of Italian ryegrass at different growth stages. FSN, natural fermentation of Italian ryegrass harvested at the filling stage; DSN, natural fermentation of Italian ryegrass harvested at the dough stage. G, effect of growth stage; D, effect of ensiling day; G × D, interaction effect of growth stage and ensiling day

chemical composition and fermentation products during ensiling are shown in Table 2 and Fig. 1. The interaction of growth stage and ensiling days significantly ($P<0.05$) affected the pH, WSC, LA, AA, and ethanol contents, but not the DM and NH_3−N contents ($P>0.05$). The ratio of lactic to acetic acid (LA/AA) was only affected ($P<0.05$) by ensiling day, and BA content was only affected ($P<0.05$) by the growth stage.

DM content in FSN decreased with the ensiling days, while there were no significant changes in DSN. The rapid decline of WSC in FSN occurred in the first 7 days of ensiling, while that in DSN occurred in the first day of ensiling. There was more WSC consumed in FSN during ensiling. Except for the first day of ensiling, the pH of FSN was lower than that of DSN (Fig. 1A). In the first 15 days of ensiling, the pH decreased to the lowest value of each group, thereafter, the pH presented different growth trend. In FSN, the pH increased slightly at the end of ensiling, while in DSN, it dramatically increased from 15 to 30 days of ensiling. The LA content of FSN was significantly higher than that of DSN during ensiling (Fig. 1B). However, the overall trend of LA content in FSN and DSN was similar. The LA content of FSN reached the plateau after 15 days of ensiling, and the decrease occurred after 60 days of ensiling. Although in DSN, the plateau appeared after 7 days of ensiling, and began to decrease after 30 days of ensiling. As shown in Fig. 1C, FSN had a higher AA content than DSN throughout the ensiling process. The AA content in FSN increased during ensiling, although after 30 days of ensiling the increase became slow. Although in DSN, the AA content increased gradually in the first 15 days of ensiling, then decreased until the end of ensiling. The ratio of LA/AA reflects the type of lactic acid fermentation. In the present study, the ratio of LA/AA in FSN and DSN was both lower than 2.0, indicating that hetero-fermentation was prevalent in both groups (Fig. 1D). The BA content of FSN and DSN was stabilized at a narrow range during ensiling, but the former had a higher content than the latter. The changing trend of ethanol content is shown in Fig. 1F. The ethanol content in FSN reached the peak in the first 15 days of ensiling, then decreased slightly. In DSN, the ethanol content increased significantly in the first 30 days of ensiling and decreased rapidly after 60 days of ensiling. While in the whole ensiling process, the ethanol content in FSN was always higher than that in DSN.

Characteristics of silage microorganisms

The counts of typical microorganisms in silage are shown in Table 2. Except for LAB, other microorganisms were significantly affected by the growth stage of Italian ryegrass ($P<0.05$). All microorganisms significantly decreased by the effect of the ensiling day ($P<0.05$). The interaction of growth stage and ensiling days only significantly affected the LAB counts ($P<0.05$). The LAB counts in FSN increased at the first 7 days of ensiling, then decreased. Although in DSN, its count decreased throughout the ensiling process. The enterobacteria count in DSN was significantly higher than that in FSN in the first 3 days of ensiling ($P<0.05$). However, after 7 days of ensiling, no enterobacteria was detected in FSN and DSN. Throughout the whole ensiling process, the number of aerobic bacteria and yeasts in DSN was always higher than that in FSN.

Bacterial community by Illumina analysis

In order to better understand the role of microorganisms in silage, bacterial community as a whole were detected by high-throughput sequencing.

Bacterial community diversities of fresh Italian ryegrass and their changes during ensiling are illustrated in Fig. 2. The epiphytic microbiota of FDS was more diverse than that of FFS. Ensiling process decreased the alpha diversity of the epiphytic microbiota of FDS, and narrowed the difference in alpha diversity between the two bacterial communities. The beta diversity was illustrated by the PCoA plot. The plot shows that on the PC1 axis, the bacterial community structure of FSN and DSN was clearly separated by growth stage, indicating that bacterial communities from the same growth stage were more similar during ensiling.

At the phylum and genus levels, the epiphytic microbiota and their changes during ensiling are shown in Figs. 3 and 4. The relative abundance of Proteobacteria and Actinobacteriota in FFS was higher than that in FDS. Although the relative abundance of Firmicutes in FDS was higher than that in FFS. *Pseudomonas*, *Sphingomonas,* and *Microbacterium* were the three most abundant bacteria in FFS, of which the first two belong to Proteobacteria, accounting for about 60% of the whole bacterial community, and the latter belongs to Actinobacteriota. In FDS, the number of genera was obviously higher than that in FFS, so the relative abundance of dominant genus was lower than that in FFS. *Exiguobacterium* belonging to Firmicutes, *Allorhizobium,* and *Pantoea* belonging to Proteobacteria were the three most abundant bacteria in FDS. *Lactococcus* was the epiphytic LAB presented in FDS, but no LAB in FFS was higher than 0.1%.

When compared with epiphytic microbiota, bacteria community structure has changed greatly after 3 days of ensiling. Both in FSN and DSN, Firmicutes became the dominant phylum (Fig. 4a), while the shift from Proteobacteria to Firmicutes in the former was faster than that in the latter (Fig. 4b, c). After 3 days of ensiling, the

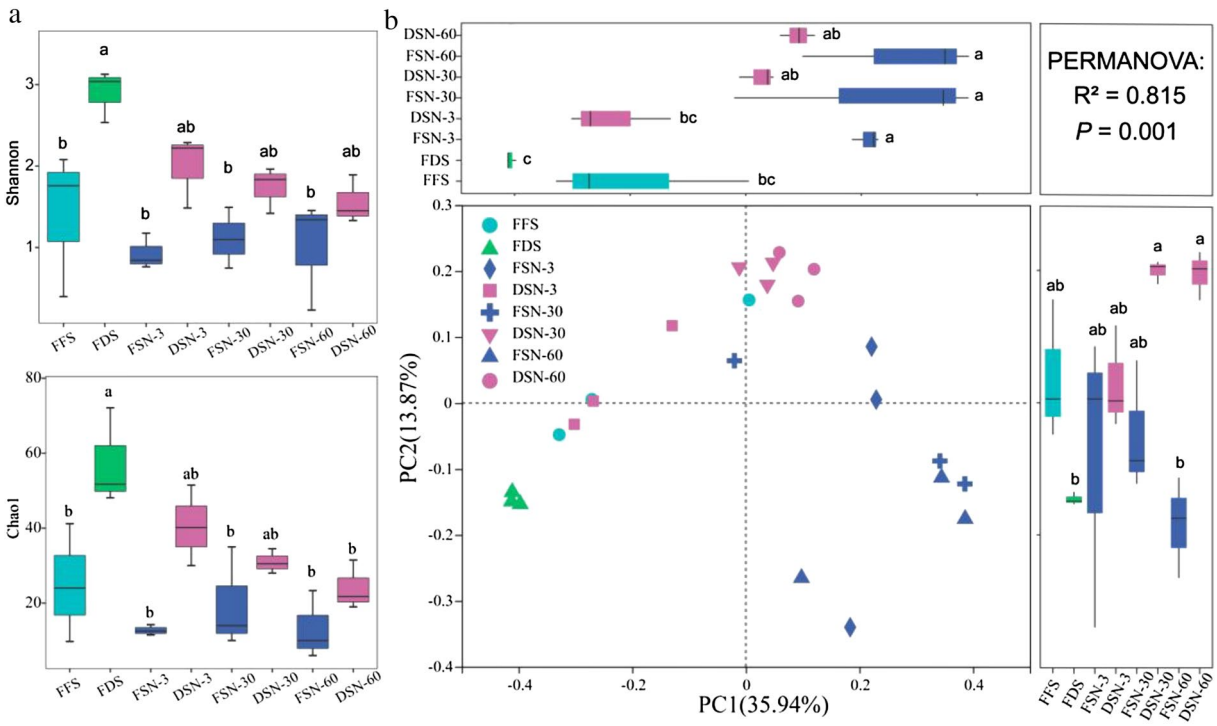


Fig. 2 The alpha diversity **a** and beta diversity **b** of fresh and ensiled Italian ryegrass. FFS, Italian ryegrass harvested at the filling stage; FDS, Italian ryegrass harvested at the dough stage; FSN, natural fermentation of Italian ryegrass harvested at the filling stage; DSN, natural fermentation of Italian ryegrass harvested at the dough stage. $^{a-c}$Means with different lower case were significant at $P < 0.05$

relative abundance of most bacteria belonging to Proteobacteria in FSN decreased, while there were some bacteria belonging to Enterobacteriaceae increased slightly, such as *Serratia*, *Pantoea*, and *Hafnia*. However, their relative abundance decreased to a very small amount in the later ensiling process. In DSN, bacteria belonging to Proteobacteria decreased after 3 days of ensiling, except for *Pantoea* and *Enterobacter*, which increased at the early stage of ensiling, and decreased to a small amount in the later stage of ensiling. When compared with FSN, the slow shift of bacteria community in DSN was also reflected in the change of LAB genera (cocci-type LAB to rod-type LAB). In FSN, *Weissella* became the dominant bacteria after 3 days of ensiling, and after nearly 30 days of ensiling, *Lactobacillus* replaced *Weissella* as the dominant genus, accompanied by the increase of *Pediococcus*. At the end of ensiling, the relative abundance of *Lactobacillus* and *Pediococcus* did not obviously change, but the relative abundance of *Weissella* further decreased, and the relative abundance of *Clostridium* and *Bifidobacterium* increased instead. In DSN, *Weissella* and *Lactococcus* were the two dominant LABs after 3 days of ensiling. After 30 days of ensiling, the relative abundance of *Weissella* further increased, while the relative abundance of *Lactococcus* decreased greatly. *Lactobacillus* and *Pediococcus* were the two LABs that increased at the later of ensiling. At the end of ensiling, the relative abundance of *Weissella* began to decrease, and *Lactobacillus* and *Pediococcus* further increased. However, the relative abundance of *Weissella* was still higher than that of *Lactobacillus* and *Pediococcus*. These three LAB dominated the bacterial community of DSN after 60 days of ensiling. When compared with FSN, *Lactobacillus* failed to become the dominant LAB. Although the relative abundance of epiphytic LAB in FFS was lower than that in FDS, the total relative abundance of LAB in FSN was higher than that in DSN in the first 30 days of ensiling (Fig. 5).

Metabolic prediction of bacterial community

Metabolisms changed by ensiling are illustrated in Fig. 6a. When compared with silage, epiphytic microbiota of fresh Italian ryegrass had more amino acid, energy, cofactors, vitamins, xenobiotics, terpenoids, and polyketides metabolisms, and the biosynthesis of other secondary metabolites. Among them, metabolisms of terpenoids, polyketides and xenobiotics in FFS were significantly higher than that in FDS ($P < 0.05$). The metabolisms of carbohydrate, glucan, and nucleotide were upregulated in silage. Among them, the glycan metabolism in FFS was

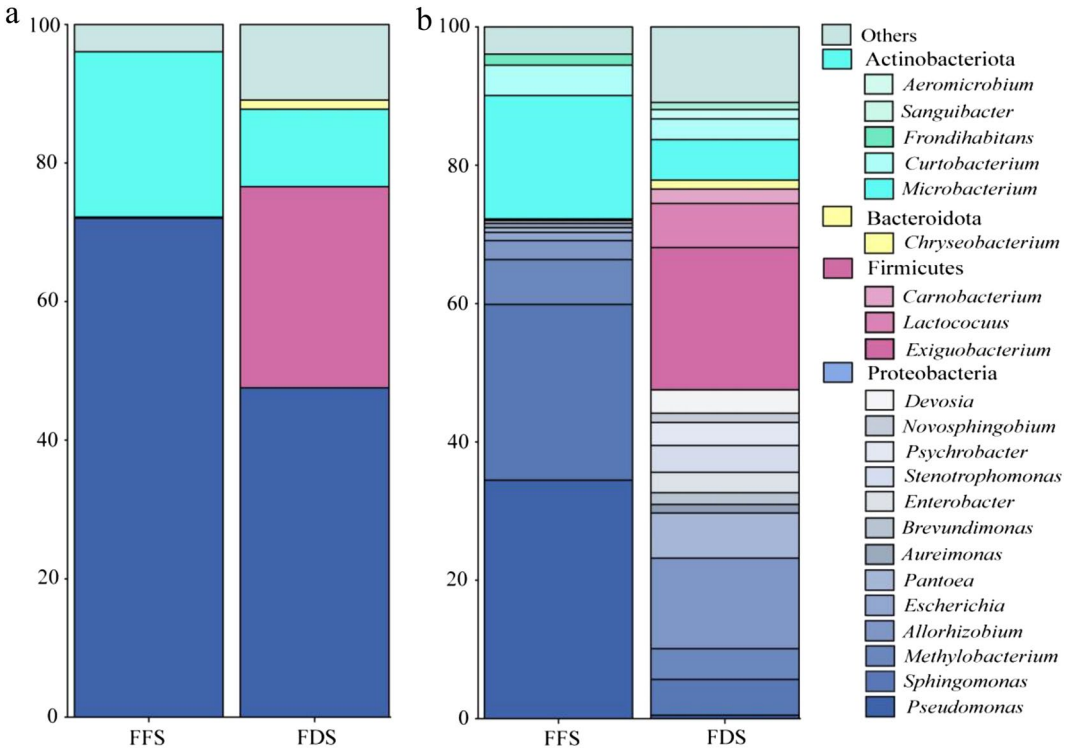


Fig. 3 Epiphytic microbiota of Italian ryegrass. **a** at phylum level. **b** at the genus level. FFS, Italian ryegrass harvested at the filling stage; FDS, Italian ryegrass harvested at the dough stage

significantly lower than that in FDS ($P < 0.05$). Carbohydrate and amino acid were the two metabolisms with the highest proportion in fresh and ensiled Italian ryegrass, so we specifically analyzed the carbohydrate and amino acid metabolisms (Fig. 6b, c). In terms of carbohydrate metabolism, the citrate cycle, butanoate, propanoate, glyoxylate and dicarboxylate metabolisms in fresh Italian ryegrass were higher than that in their corresponding silage. Ensiling upregulated the metabolisms of starch, sucrose, amino and nucleotide sugar, galactose, glycolysis/gluconeogenesis, fructose and mannose. Most amino acid metabolisms were downregulated in silage, such as glycine, serine, threonine, arginine and proline metabolisms, Lysine biosynthesis was the only obviously upregulated amino acid metabolism in silage. Carbohydrate and amino acid metabolisms in FFS and FDS were similar, while amino acid metabolisms downregulated by ensiling were higher in FFS than those in FDS. Carbohydrate metabolism in the first 3 days of ensiling of FSN was similar to that in the first 30 days of ensiling of DSN, while amino acid metabolism of FSN in the late stage of ensiling was quite different from DSN. Thus, for the metabolic level, it also showed that the fermentation degree of DSN was lower than that of FSN.

In order to find out the contribution of bacteria to the carbohydrate and amino acid metabolisms, the correlation between bacteria and these metabolisms was analyzed (Fig. 7). Bacteria, which was abundant in epiphytic microbiota were positively correlated to the downregulated carbohydrate and amino acid metabolisms during ensiling, and negatively correlated to the upregulated carbohydrate and amino acid metabolisms. *Lactobacillus*, *Weissella*, *Pediococcus*, *Bifidobacterium*, *Serratia,* and *Hafnia* were positively correlated to the upregulate carbohydrate and amino acid metabolic pathway during ensiling, indicating that these bacteria exerted the main function in Italian ryegrass silage.

Discussion

Chemical and microbial changes of Italian ryegrass during growth

In the present study, with the growth of Italian ryegrass, the increase of DM, NDF, and ADF contents and the decrease of CP and WSC are typical, and consistent with the previous studies. These were mainly due to the deposition of cell wall composition, such as hemicellulose and cellulose [7] and the increase of stem to leaf ratio—because the protein fraction of plant was dominated

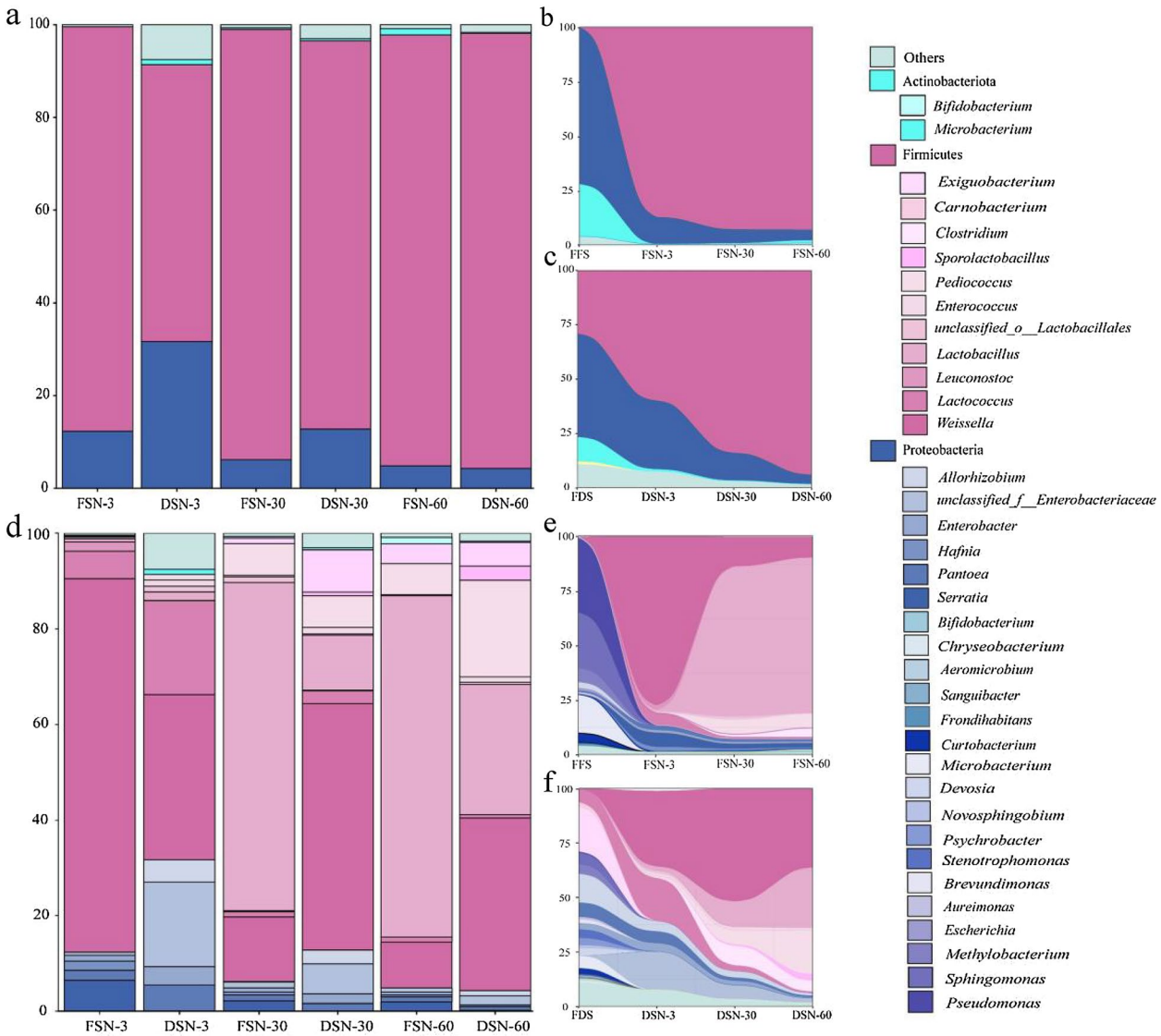


Fig. 4 Bacterial community composition and succession of Italian ryegrass silage (relative abundance > 0.1%). Relative abundance of bacteria in Italian ryegrass silage at the phylum level **a** and at the genus level **d**. Bacterial community succession of Italian ryegrass during ensiling are aggregated and colored on a stream-graph at the phylum level **b** and **c** and genus level **e** and **f**

by leaf protein [18, 19]. Organic acid is the main factor affecting the buffering capacity of forage, in Italian ryegrass, the main buffers are malate and citrate [13]. The decrease of buffering capacity in FDS, may be due to the decrease of these organic acid content.

Phyllosphere constitutes a very large microbial habitat, which is sufficiently numerous to contribute in many processes of importance to global process. However, it has long been considered as a hostile environment for bacterial colonists, and bacteria need a high degree of adaptation to colonize this environment [20]. In the present study, the dominant bacteria in the two growth stages of Italian ryegrass belonged to Proteobacteria, indicating that the metabolic type or physiological characteristics of this kind of bacteria can adapt to the complex environment of the phyllosphere. However, with the growth of Italian ryegrass, the relative abundance of Proteobacteria decreased and the diversity of epiphytic microbiota increased. *Pseudomonas* was the dominant genus in FFS, O'May and Tufenkji [21] pointed out that the swimming ability relying on the biofilm of *Pseudomonas* is crucial to its

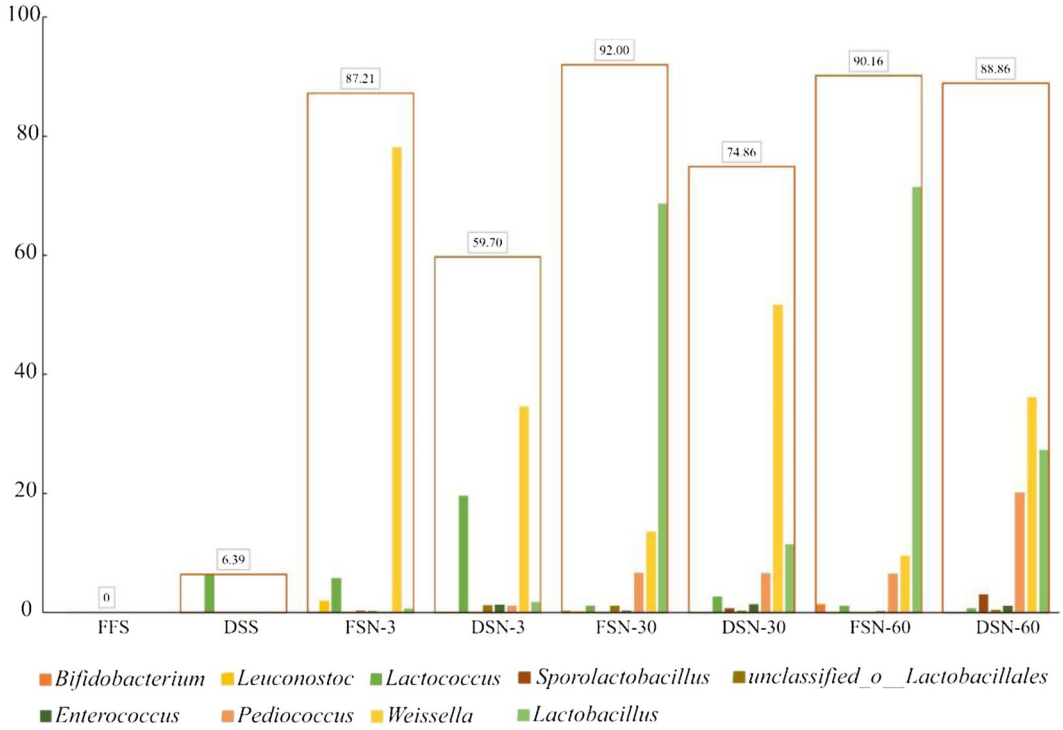


Fig. 5 Lactic acid bacterial succession in Italian ryegrass silage

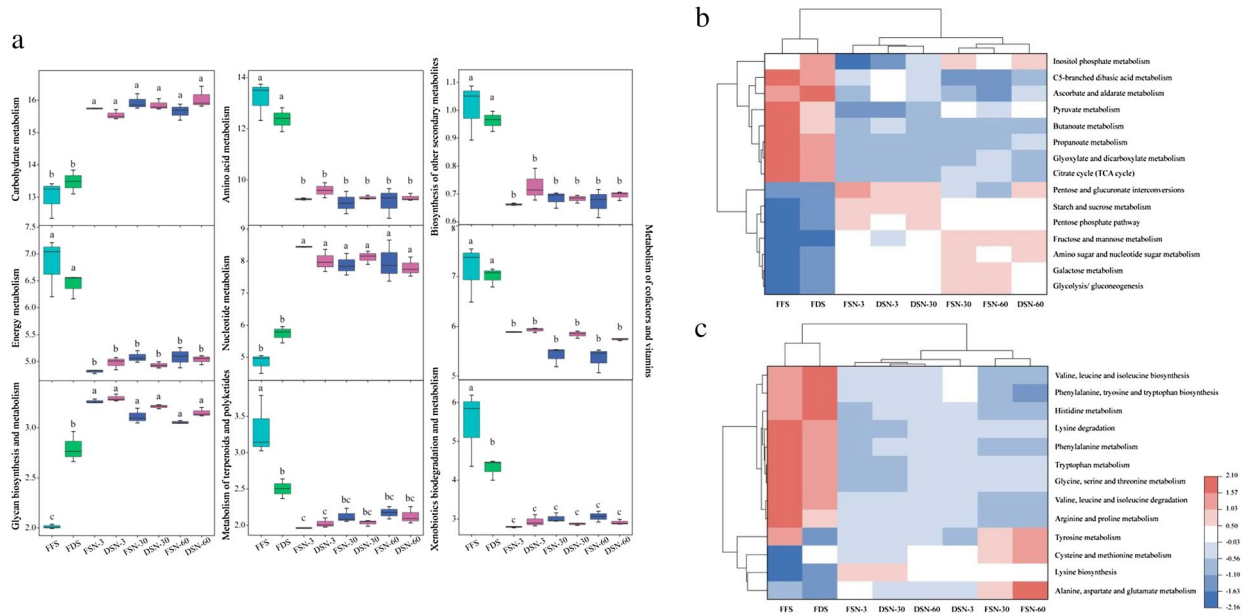


Fig. 6 Microbial metabolism of Italian ryegrass harvested at the two growth stages during ensiling. **a** the level 2 metabolisms predicted by KEGG. Differential analysis of specific carbohydrate **b** and amino acid **c** metabolisms

colonization, and the formation of biofilm depends on certain humidity conditions. Therefore, the decreased moisture content during the growth of Italian ryegrass may limit the colonization of *Pseudomonas*, and the vacated niche expanded the living space of other bacteria. Although the epiphytic microbiota of FFS and FDS was quite different, the metabolisms in the two epiphytic microbiotas were relatively consistent. This

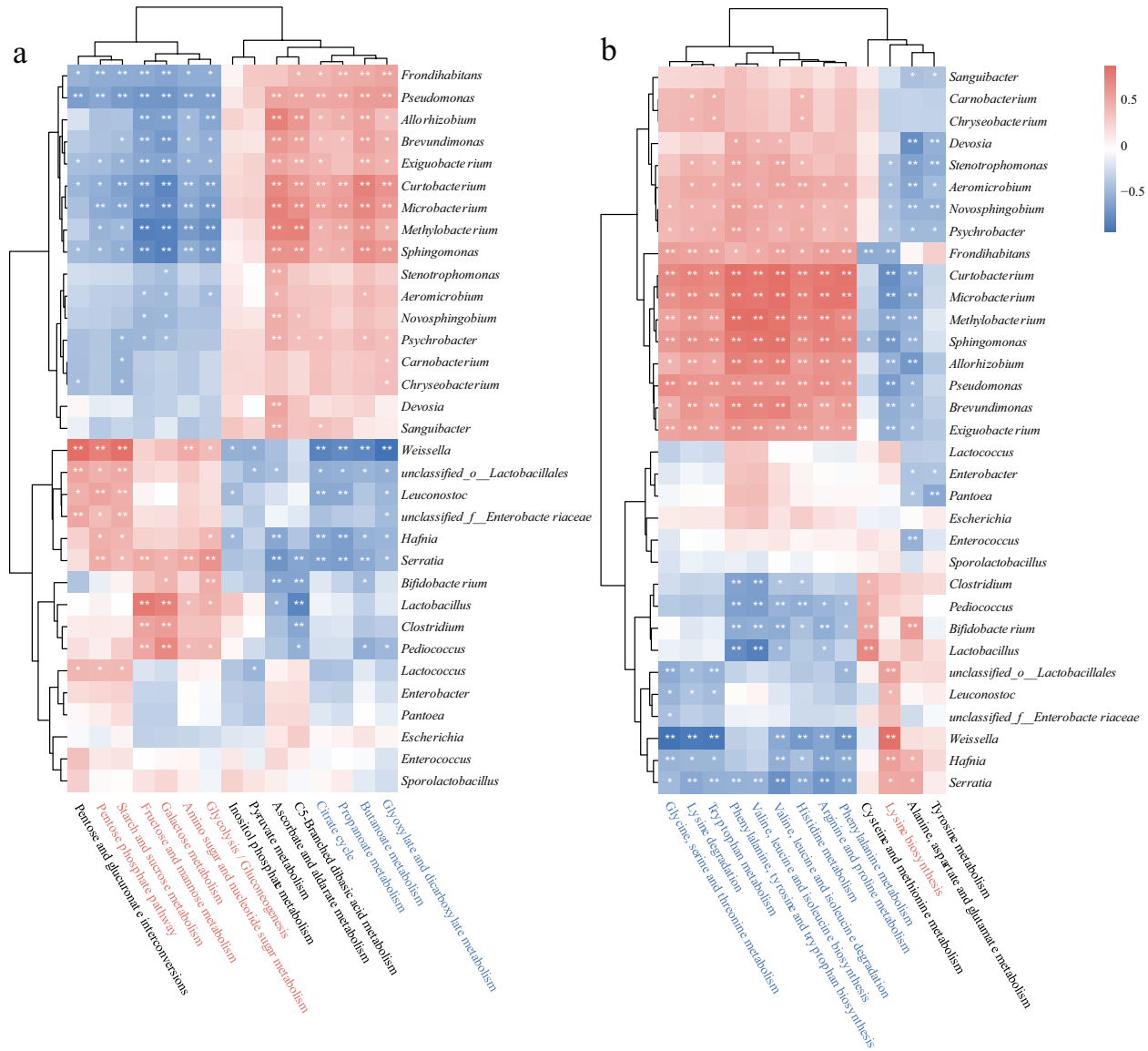


Fig. 7 Correlation analysis of bacterial community and specific carbohydrate **a** and amino acid **b** metabolisms of Italian ryegrass silage. The metabolism names in red highlight the upregulated metabolism after ensiling. The metabolism names in blue highlight the downregulated metabolism after ensiling. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

may be due to the similar selection directions in similar environments, resulting in similar functions of bacteria in the same environment.

Effect of growth stages on fermentation quality of Italian ryegrass silage

In the present study, the changing trend of pH in both growth stages of Italian ryegrass silage was opposite to that of lactic acid content. As known, lactic acid (pKa, 3.86) is the most effective organic acid to reduce pH in silage circumstances [22], and its acid strength is 10–12 times that of other organic acids, such as acetic acid (pKa, 4.75) and propionic acid (pKa, 4.87). The different upward trends of pH in FSN and DSN at the end of ensiling were related to the different production rates of lactic acid during ensiling. The lactic acid production rate of FSN was significantly higher than that of DSN, resulting in more lactic acid and lower pH in FSN during the same ensiling days. The dry matter content can affect the process, rate, and type of silage fermentation by affecting the activity of microorganisms [23]. As the fermentation substrate, WSC content can determine the fermentation intensity. In the present study, the moisture and WSC

contents in FSN were higher than that in DSN, jointly resulting in more organic acids and lower pH in FSN.

In general, the acetic acid content increased with ensiling days is related to the decrease of WSC content in the later stage of ensiling, and the hetero-fermentation of LAB [24]. In the present study, the increase of acetic acid content in FSN at the later stage of ensiling may be due to the above reasons. Although the linear increase of acetic acid content in the first 15 days of ensiling in FSN was probably related to other acetic acid-producing bacteria. The result of Bjorkroth et al. [25] showed that bacteria in the genus *Weissella* can carry out heterofermentative, such as *Weissella cibaria*. Therefore, the rapid increase of acetic acid content in FSN was caused by the abundant *Weissella* at the early stage of ensiling. The acetic acid content in DSN also increased obviously in the first 15 days of ensiling, which may be related to abundant Enterobacteriaceae besides *Weissella*. The decrease of acetic acid content in the later stage of ensiling may be partly due to esterification [26]. Overall, the acetic acid content in FSN group was higher than that in DSN, which was related not only to the DM and WSC contents, but also to the abundance of bacteria. The abundance of bacteria that can produce acetic acid in FSN was higher than that in DSN.

The NH_3−N content of FSN and DSN was lower than the upper limit (100 g/kg TN) required for quality silage, indicating that the protein of Italian ryegrass was not degraded significantly during ensiling. The result of Zhao et al. [27] showed that Naiper grass harvested in early vegetable stage had a lower NH_3−N than that of harvest at the late vegetative stage (95.7 vs. 155). Although in this study, there is no significant difference in NH_3−N content of Italian ryegrass harvested at the two growth stages. Previous study showed that the degradable protein of Italian ryegrass decreased with the growth stage [28]. Therefore, FSN may have more degradable proteins than DSN. However, the activity of plant protease will become higher in the mature stage of forage [29], which may lead to no significant difference in NH_3−N content between the two growth stages.

Effect of growth stages on microbial community of Italian ryegrass silage

When compared with the epiphytic microbiota, ensiling significantly changed bacterial community structure, showing an increase in Firmicutes and a decrease in Proteobacteria. For DSN, ensiling also decreased the alpha diversity of bacterial community, but not for FSN. Therefore, the difference in alpha diversity between the two epiphytic microbiotas disappeared after ensiling. On day 3 of ensiling, the relative abundance of *Weissella* in FSN reached 78.16%. The greater the abundance of dominant bacteria in bacterial community, the lower its diversity. Therefore, when compared with epiphytic microbiota, the bacterial diversity of FSN decreased obviously. However, the bacterial community of FSD on day 3 of ensiling was not stable, because its dominant bacteria were *Weissella*, which is intolerant to low pH, as the continuous accumulation of organic acid content and reduction of pH, *Weissella* was gradually replaced by *Lactobacillus* [30, 31]. On day 30 of ensiling, *Lactobacillus* became the dominant bacteria, and since then, the bacterial community became relatively stable. On day 3 of ensiling, the bacterial community structure of DSN also change greatly as compared to its epiphytic microbiota. However, the relative abundance of its dominant bacteria was relatively low, accounting for 34.62%, and many genera were retained. In the subsequent ensiling, *Lactobacillus* did not occupy a large advantage, so the bacterial community of DSN maintained a high diversity in the ensiling process.

However, the bacterial community structures of FSN and DSN were still different. In other words, the growth stage still has an effect on the bacterial community of silage, which may be related to the epiphytic microbiota of Italian ryegrass and the contents of WSC and DM. The diversity of epiphytic microbiota of FDS was higher than that of FFS. The higher the microbial diversity, the more stable the community structure, and the stronger resistance to external environmental stresses [32]. In addition, the WSC and moisture contents of FDS were obviously lower than that in FFS, and the lower moisture content will limit the activity of most microorganisms, resulting in a lower succession rate of the bacterial community in DSN than that in FSN.

Weissella, *Lactococcus*, and *Pediococcus* are common LAB in the early stage of ensiling [33], but these three types of bacteria showed different changes in the process of Italian ryegrass silage. In the present study, *Weissella* is the LAB with the highest relative abundance in the early stage of ensiling of Italian ryegrass, followed by *Lactococcus*, and the relative abundance of *Pediococcus* appeared to increase in the later stage of ensiling. The relative abundance of *Weissella* in FSN decreased with ensiling days, while that decrease in DSN presented at the later stage of ensiling, and at the end stage of ensiling, DSN had a higher abundance of *Weissella*, which may be related to the slow fermentation process. At the early stage of ensiling, both FSN and DSN did not show abundant *Pediococcus*, but it increased since day 30 of ensiling. Cai et al. [34] showed that some strains of *Pediococcus* can grow normally at pH 3.5, which may be the reason why they can exist in FSN. The relative abundance of *Pediococcus* in DSN was higher than that in FSN, which may be related to the relatively high pH value.

Effect of growth stage on bacterial metabolism of Italian ryegrass silage

By predicting the metabolisms of bacterial community, the dynamics of bacterial community and its role in silage fermentation were further clarified. Consistent with Bai et al. [35] that most metabolisms were downregulated by ensiling, such as amino acid metabolism. Amino acid metabolism provides essential component (like nitrogen nutrients) for microbial growth [36, 37]. The decreased amino acid metabolism was positively correlated to the relative abundance of undesirable bacteria, thus it can be speculated the simplified bacterial community during ensiling caused the downregulated of most metabolisms. Compared with epiphytic microbiota, carbohydrate and nucleotide metabolism were upregulated in silage microbiota. Anaerobic environment promoted fermentation metabolism, bacteria (most were enterobacteria and LAB) adapted to this environment and fermented carbohydrate to produce ATP and intermediate metabolites to maintain their activities. The upregulated nucleotide metabolism may be related to bacterial death and nucleotide degradation. Although the specific prediction of carbohydrate and amino acid metabolism showed that not all carbohydrate and amino acid metabolisms were upregulated and downregulated after ensiling, respectively, indicating that ensiling was intensified by those upregulated carbohydrate and amino acid metabolism, such as fructose, mannose, cysteine, and methionine metabolism. Although the ensiling process simplified the bacterial community structure and downregulated most of the metabolic pathways, there was no significant difference in the changes of the metabolic pathways of Italian ryegrass harvested at the two growth stages during ensiling, and no obvious difference between the two growth stages, indicating that although the bacterial community was different, the metabolic pathway was similar, it might be caused by functional redundancy in similar environments [38].

At present, researches on silage metabolism mainly focus on phenomena. It has not been reported to regulate the fermentation of silage microorganisms by regulating the metabolic pathway, which may be the research direction of silage microbiology in the future.

Conclusion

When compared with the dough stage, Italian ryegrass harvested at the filling stage had higher WSC and moisture content, and its silage showed lower pH, higher lactic acid content, and rapid bacterial succession. The diversity of epiphytic microbiota made Italian ryegrass harvested at the dough stage more difficult to ferment, showing a delayed succession process. Metabolic analysis revealed that carbohydrate and amino acid metabolism played an important role in silage fermentation. Silage quality may be regulated through these metabolic pathways in the future.

Acknowledgements
No applicable

Author contributions
XY: data curation, formal analysis, writing—original draft. JL, SW, and JZ: visualization and investigation. JZ, ZD, and JL: review and editing. TS: supervision, project administration and review. All authors read and approved the final manuscript.

Funding
This work was partially supported by the Joint Fund for Innovative Development (U20A2003, Beijing, China) and the Postgraduate Research and Practice Innovation Program of Jiangsu Province (KYCX20_0609, Nanjing, China).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
No applicable.

Consent for publication
No applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Institute of Ensiling and Processing of Grass, College of Agro-Grassland Science, Nanjing Agricultural University, Nanjing 210095, China.

Received: 6 January 2023 Accepted: 18 February 2023
Published online: 01 March 2023

References

1. Liu S, Cai Y, Zhu H, Tan Z. Potential and constraints in the development of animal industries in China. J Sci Food Agric. 2012;92(5):1025–30.
2. Xu L, Tang G, Tian J, Wang X, Zhang J. Effects of no-tillage sowing on soil properties and forage wheat and Italian ryegrass yields in winter fallow paddy fields. Peer J. 2021. https://doi.org/10.7717/peerj.10573.
3. Valente ME, Borreani G, Peiretti PG, Tabacco E. Codified morphological stage for predicting digestibility of Italian ryegrass during the spring cycle. Agron J. 2000;92(5):967–73.
4. Comino L, Tabacco E, Righi F, Revello-Chion A, Quarantelli A, Borreani G. Effects of an inoculant containing a Lactobacillus buchneri that produces ferulate-esterase on fermentation products, aerobic stability, and fibre digestibility of maize silage harvested at different stages of maturity. Anim Feed Sci Technol. 2014;198:94–106.
5. Souza AMd, Neumann M, Rampim L, Almeida ERd, Matchula AF, Cristo FB, et al. Effect of storage time on the chemical composition of whole and grainless corn plant silage harvested at different maturity stages. Revista Brasileira de Zootecnia. 2022. https://doi.org/10.37496/rbz5120200180.
6. Zhao J, Yin X, Dong Z, Wang S, Li J, Dong D, et al. Using gamma-ray irradiation and epiphytic microbiota inoculation to separate the effects of chemical and microbial factors on fermentation quality and bacterial community of ensiled Pennisetum giganteum. J Appl Microbiol. 2022;132(3):1675–86.
7. Xue Y, Bai C, Sun J, Sun L, Chang S, Sun Q, et al. Effects of locations and growth stages on nutritive value and silage fermentation quality

ofLeymus chinensisin Eurasian steppe of northern China. Grassland Sci. 2018;64(1):40–50.
8. Longland AC. Nutritional assessment of forage quality. In: Saastamoinen M, Fradinho MJ, Santos AS, Miraglia N, editors. Forages and grazing in horse nutrition. Wageningen: Wageningen Academic Publishers; 2012. p. 65–82.
9. Yin X, Zhao J, Wang S, Dong Z, Li J, Shao T. The effects of epiphytic microbiota and chemical composition of Italian ryegrass harvested at different growth stages on silage fermentation. J Sci Food Agric. 2022. https://doi.org/10.1002/jsfa.12232.
10. Arthur TT. An automated procedure for the determination of soluble carbohydrates in herbage. J Sci Food Agric. 1977;28(7):639–42.
11. AOAC. AOAC Official Methods of Analysis (15th ed.), Association of Official Analytical Chemists, VA. 1990.
12. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 1991;74(10):3583–97.
13. Playne MJ, McDonald P. The buffering constituents of herbage and of silage. J Sci Food Agric. 1966;17(6):264–8.
14. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J Dairy Sci. 1980;63(1):64–75.
15. Zhao J, Dong Z, Li J, Chen L, Bai Y, Jia Y, et al. Ensiling as pretreatment of rice straw: the effect of hemicellulase and Lactobacillus plantarum on hemicellulose degradation and cellulose conversion. Bioresour Technol. 2018;266:158–65.
16. Dong Z, Shao T, Li J, Yang L, Yuan X. Effect of alfalfa microbiota on fermentation quality and bacterial community succession in fresh or sterile Napier grass silages. J Dairy Sci. 2020;103(5):4288–301.
17. Asshauer KP, Wemheuer B, Daniel R, Meinicke P. Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics. 2015;31(17):2882–4.
18. Fluck AC, Costa OAD, Junior JS, Rosa PPd, Cardozo L, Rizzo FA, et al. In vitro and in situ digestibility of annual ryegrass silage from distinct phenological growth stages and pre-dehydration times. Biosci J. 2018. https://doi.org/10.14393/BJ-v34n5a2018-39421.
19. Contreras-Govea FE, Albrecht KA. Forage production and nutritive value of oat in autumn and early summer. Crop Sci. 2006;46(6):2382–6.
20. Lindow SE, Brandl MT. Microbiology of the phyllosphere. Appl Environ Microbiol. 2003;69(4):1875–83.
21. O'May C, Tufenkji N. The swarming motility of Pseudomonas aeruginosa is blocked by cranberry proanthocyanidins and other tannin-containing materials. Appl Environ Microbiol. 2011;77(9):3061–7.
22. Mcdonald P, Henderson AR, Heron S. The biochemistry of silage. 1991.
23. Weinberg ZG, Khanal P, Yildiz C, Chen Y, Arieli A. Effects of stage of maturity at harvest, wilting and LAB inoculant on aerobic stability of wheat silages. Anim Feed Sci Technol. 2010;158(1–2):29–35.
24. Parvin S, Nishino N. Bacterial community associated with ensilage process of wilted guinea grass. J Appl Microbiol. 2009;107(6):2029–36.
25. Bjorkroth KJ, Schillinger U, Geisen R, Weiss N, Hoste B, Holzapfel WH, et al. Taxonomic study of Weissella confusa and description of Weissella cibaria sp. nov., detected in food and clinical samples. Int J Syst Evol Microbiol. 2002;52(1):141–8.
26. Weiss K, Kroschewski B, Auerbach H. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. J Dairy Sci. 2016;99(10):8053–69.
27. Zhao J, Yin XJ, Wang SR, Li JF, Shao T. Separating the effects of chemical and microbial factors on fermentation quality and bacterial community of Napier grass silage by using gamma-ray irradiation and epiphytic microbiota transplantation. Anim Feed Sci Technol. 2021. https://doi.org/10.1016/j.anifeedsci.2021.115082.
28. Borba AE, Ribeiro J. Nutritive value, and in situ dry matter and protein degradability of fresh and ensiled Italian ryegrass at three stage of growth[J]. Iolium multiflorum, 1996.
29. Faria Júnior WG, Gonçalves LC, Ribeiro Júnior GO, Carvalho WTV, Maurício RM, Rodrigues JAS, et al. Effect of grain maturity stage on the quality of sorghum BRS-610 silages. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2011;63(5):1215–23.
30. Dong Z, Li J, Chen L, Wang S, Shao T. Effects of freeze-thaw event on microbial community dynamics during red clover ensiling. Front Microbiol. 2019;10:1559.
31. Guo L, Lu Y, Li P, Chen L, Gou W, Zhang C. Effects of delayed harvest and additives on fermentation quality and bacterial community of corn stalk silage. Front Microbiol. 2021;12:687481.
32. Jizheng H, Jing L, Yuanming Z. Thoughts on the microbial diversity–stability relationship in soil ecosystems. Biodivers Sci. 2014;21(4):411–20.
33. Muck R. Recent advances in silage microbiology. Agric Food Sci. 2013;22(1):3–15.
34. Cai Y, Kumai S, Ogawa M, Benno Y, Nakase T. Characterization and identification of Pediococcus species isolated from forage crops and their application for silage preparation. Appl Environ Microbiol. 1999;65(7):2901–6.
35. Bai J, Ding Z, Ke W, Xu D, Wang M, Huang W, et al. Different lactic acid bacteria and their combinations regulated the fermentation process of ensiled alfalfa: ensiling characteristics, dynamics of bacterial community and their functional shifts. Microb Biotechnol. 2021;14(3):1171–82.
36. Wang H, Tao Y, Li Y, Wu S, Li D, Liu X, et al. Application of ultrasonication at different microbial growth stages during apple juice fermentation by Lactobacillus plantarum: investigation on the metabolic response. Ultrason Sonochem. 2021;73:105486.
37. Nsogning SD, Fischer S, Becker T. Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity. Food Microbiol. 2018;73:245–53.
38. Louca S, Parfrey LW, Doebeli M. Decoupling function and taxonomy in the global ocean microbiome. Science. 2016;353(6305):1272–7.

Publisher's Note