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西南民族大学
青藏高原研究院
饲草加工团队
SMU QINGHAI-TIBET PLATEAU
FORAGE UTILIZATION TEAM

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Microbiome and response surface methodology analyses reveal *Acetobacter pasteurianus* as the core bacteria responsible for aerobic spoilage of corn silage (*Zea mays*) in hot and humid areas

Dr. Hao Guan

Southwest Minzu University

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Outline

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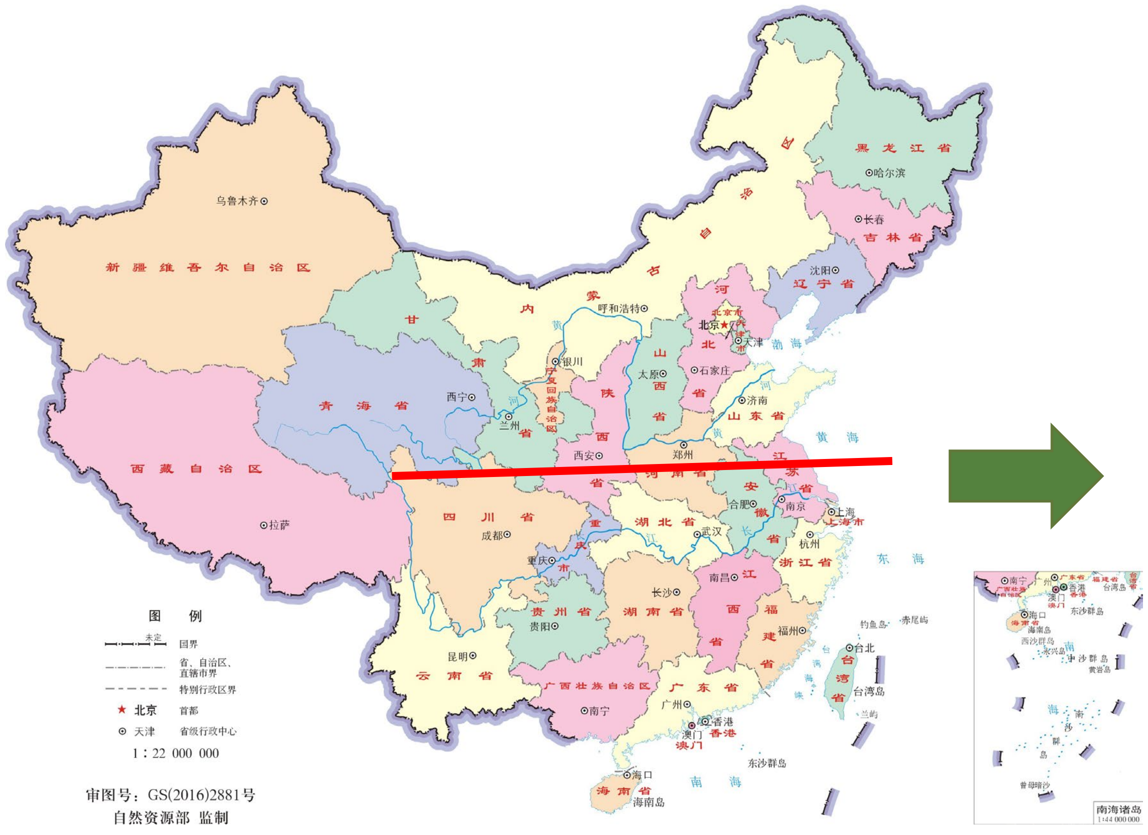
Background



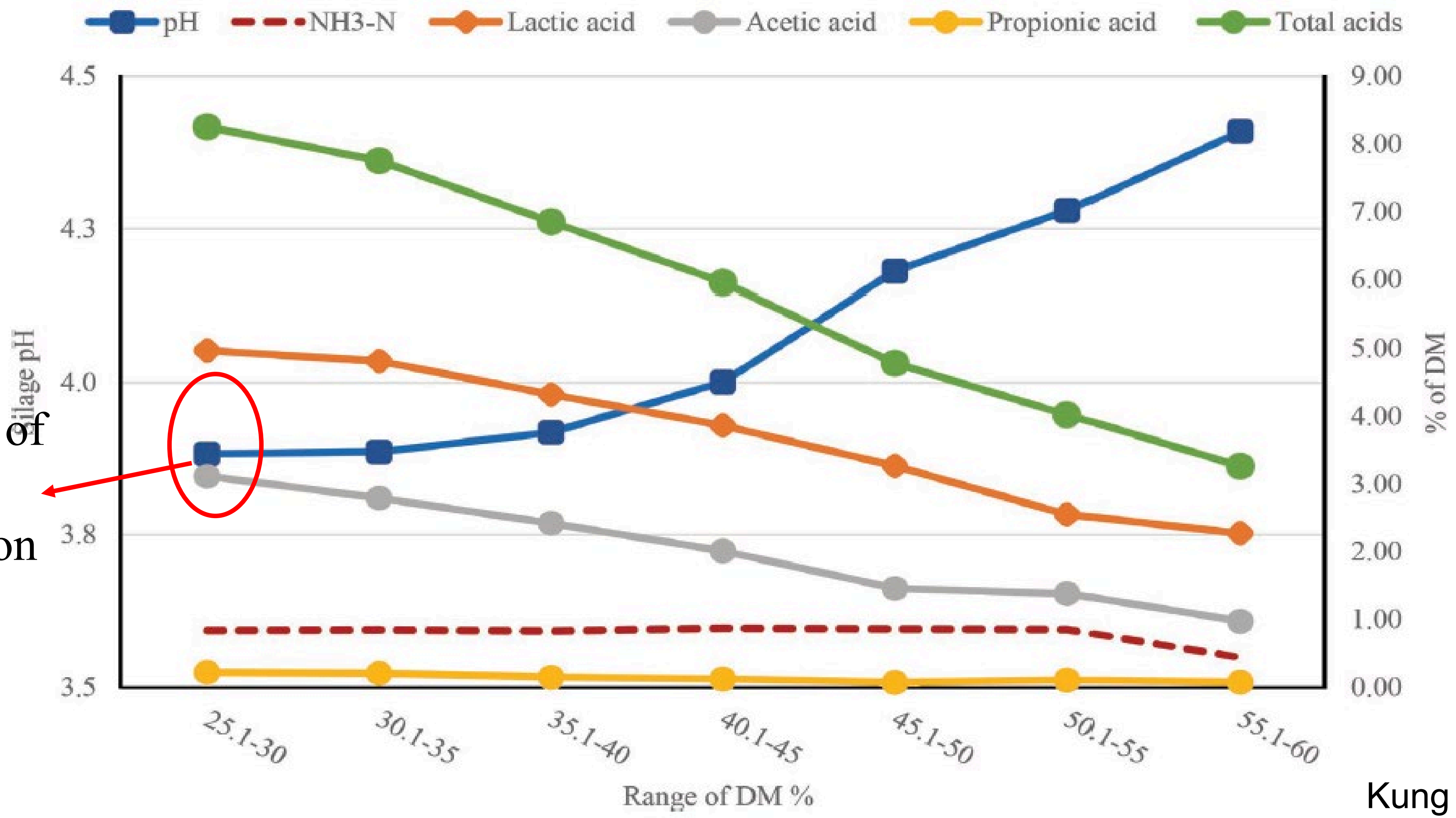
Southern China region

- ◆Rainy
- ◆Hot
- ◆Humid

- ➢ China's total demand for high-quality forage will exceed 120 million tons, leaving a gap of nearly 50 million tons;
- ➢ Difficult to produce hay in this area due to climate restrictions;
- ➢ The quality of silage is low due to the characteristics of forage and uncontrollable fermentation.

Background

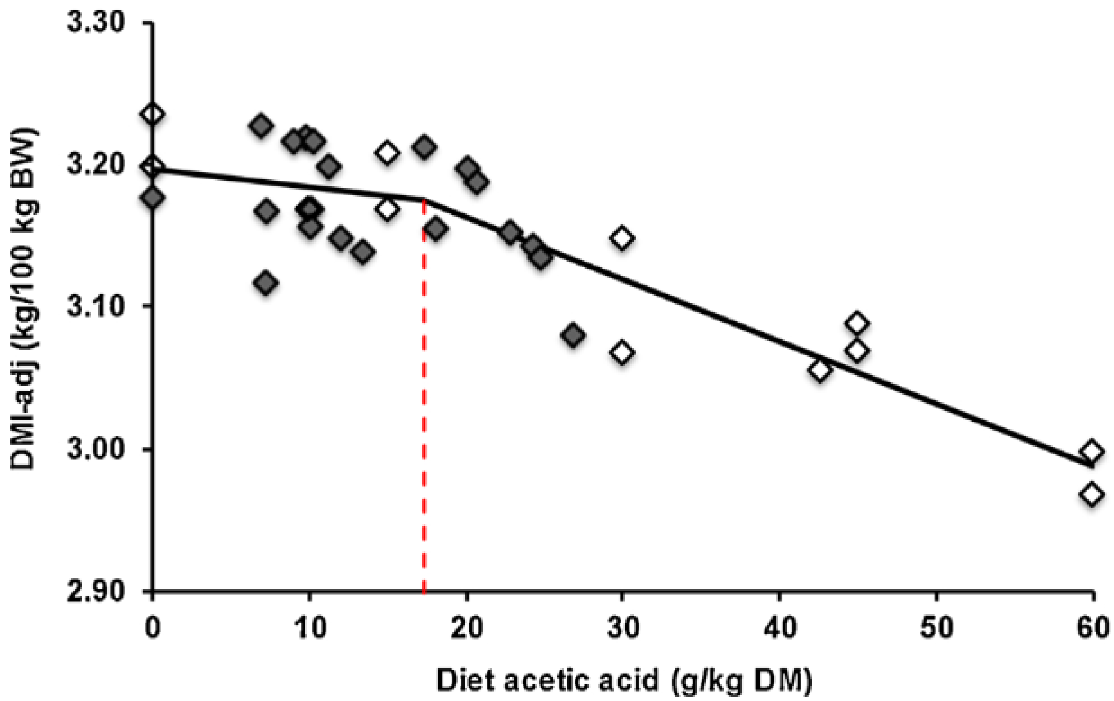
Effect of DM on Fermentation End Products: Corn Silage

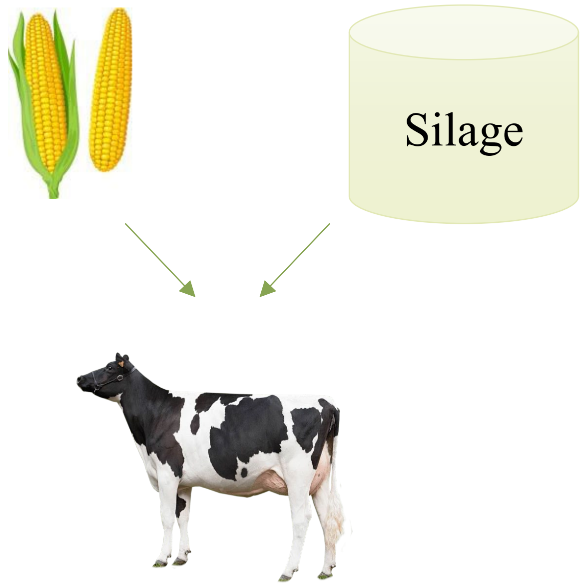


High level of acetic acid fermentation

Kung et al. 2018

Background



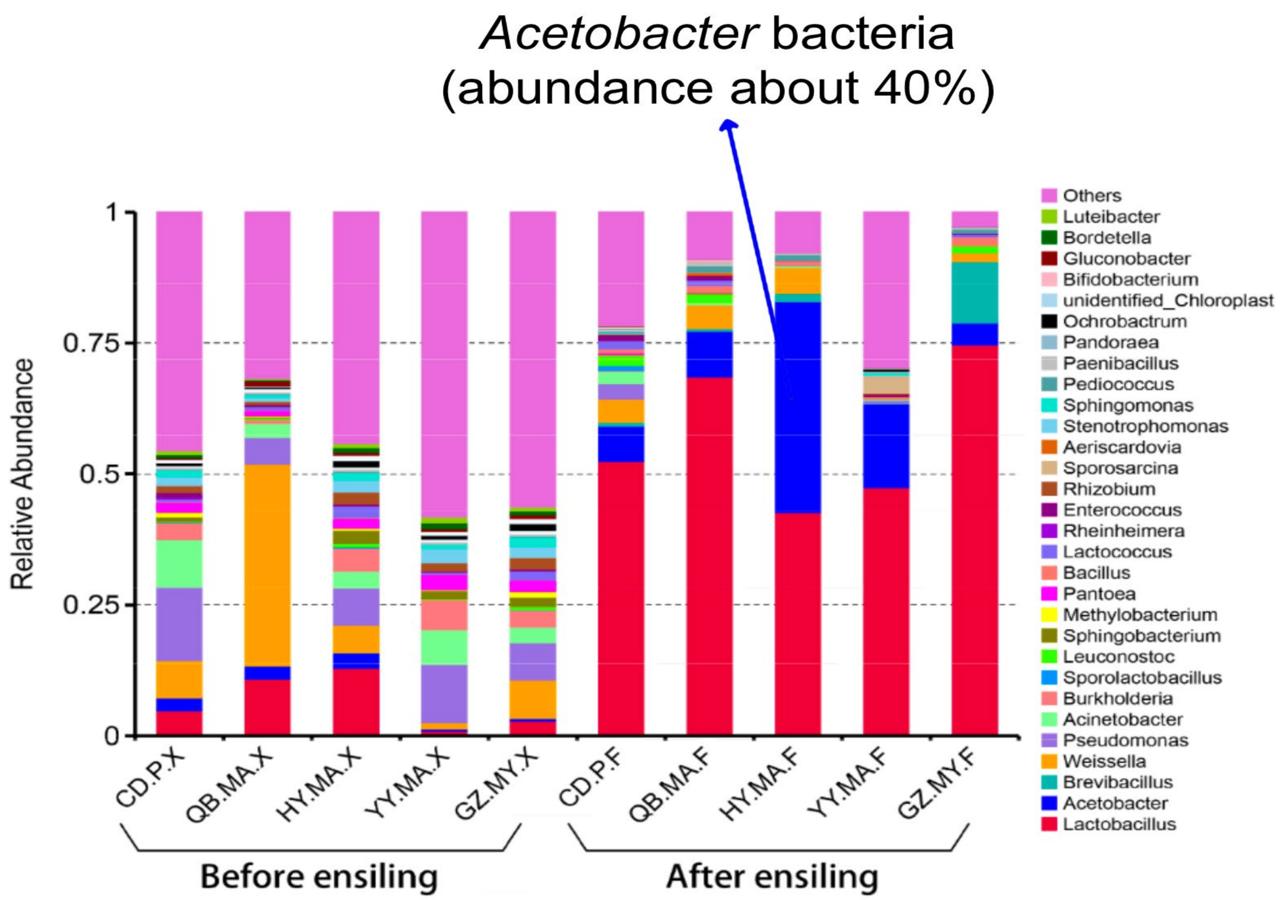


- Silage with a low DM content (less than 20%) can exhibit acetic acid levels as high as **3% to 6%** DM.
- Excessive acetic acid fermentation (greater than 3% DM) has been observed to results in significant **DM loss, accelerate aerobic spoilage, and markedly reduce animal intake** (Gerlach et al., 2021).

Background

Acetobacter Sp. may cause high acetic acid fermentation in corn silage in humid and hot regions

In particular, during the opening stage of silage in the hot and humid regions, a significant presence of *Acetobacter* bacteria has been observed (Guan et al., 2018).



Scientific Questions

1. What environmental factors lead to high acetic acid fermentation?

2. What is the silage fermentation process dominated by Acetobacter?

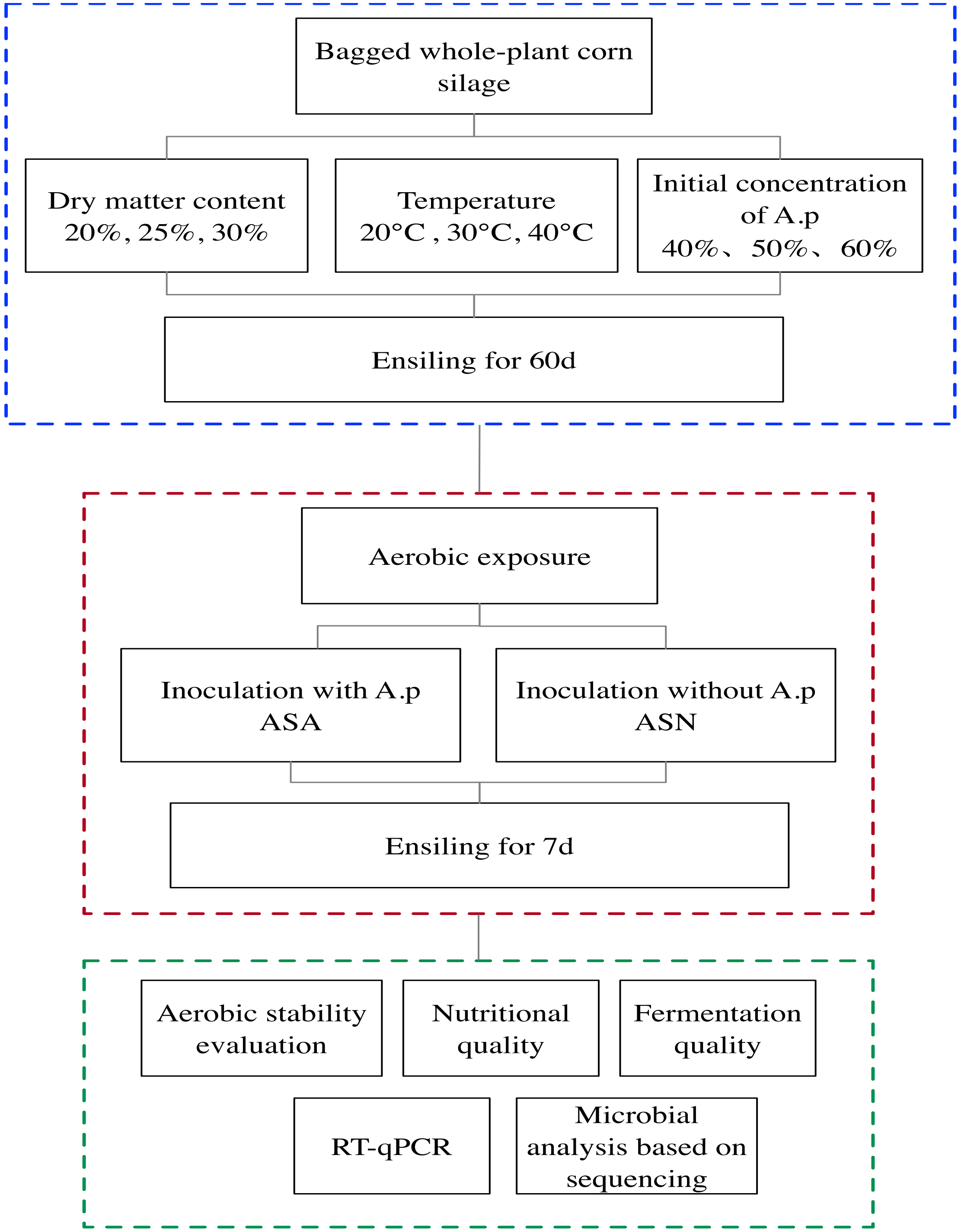
How do microorganisms interact?

Technology Roadmap

Table 1 Group information

Group	T (°C)	M (%)	A (%)
1 (T30M75A50)	30	25	50
2 (T20M80A50)	20	20	50
3 (T30M75A50)	30	25	50
4 (T30M70A60)	30	30	60
5 (T30M75A50)	30	25	50
6 (T20M75A60)	20	25	60
7 (T20M70A50)	20	30	50
8 (T30M80A40)	30	20	40
9 (T40M80A50)	40	20	50
10 (T40M70A50)	40	30	50
11 (T40M75A40)	40	25	40
12 (T40M75A60)	40	25	60
13 (T20M75A40)	20	25	40
14 (T30M75A50)	30	25	50
15 (T30M70A40)	30	30	40
16 (T30M80A60)	30	20	60
17 (T30M75A50)	30	25	50

Note: T, temperature; M, dry matter content; A, initial concentration of *A. pasteurianus*.



Methods

Activation and propagation of acetic acid bacteria

chop corn

Production of bagged whole plant corn silage

60d

Open bag sampling

Making aerobic exposure silage

- Nutritional quality
- Fermentation quality
- Microbial community structure
- RT-qPCR

Results-*Fermentation Quality*

Table 2 Changes in fermentation quality during silage fermentation to aerobic exposure stages

With AP **No AP**

Items	pH			NH_3-N (mg·dL^{-1})			LA (%DM)			AA (%DM)			PA (%DM)			BA (%DM)		
	D60	ASA	ASN	D60	ASA	ASN	D60	ASA	ASN	D60	ASA	ASN	D60	ASA	ASN	D60	ASA	ASN
1 (T30M75A50)	3.64^{cd}	4.21^{ij}	4.31^{b}	24.92^{abc}	292.92^{ghi}	226.51^{ef}	4.28^{bc}	3.09^{a}	3.11^{f}	1.26^{bc}	1.2^{b}	1.43^{bc}	ND	ND	ND	ND	ND	ND
2 (T20M80A50)	3.48^{hi}	5.62^{cd}	4.09^{d}	22.39^{defg}	311.16^{abc}	234.01^{bcdef}	4.28^{bc}	ND	2.05^{i}	1.08^{cde}	0.37^{hij}	0.91^{ef}	ND	0.3^{cd}	ND	ND	ND	ND
3 (T30M75A50)	3.71^{bc}	5.56^{d}	4.30^{b}	26.34^{a}	305.01^{bcdef}	243.08^{abcde}	3.97^{ef}	1.30^{d}	2.2^{hi}	1.07^{cde}	0.73^{ef}	1.21^{cde}	ND	ND	ND	ND	ND	ND
4 (T30M70A60)	3.56^{efgh}	5.66^{c}	3.90^{fg}	24.17^{abcd}	295.73^{fghi}	230.24^{cdef}	4.84^{a}	ND	6.52^{a}	1.42^{b}	1.39^{a}	1.54^{b}	ND	0.78^{ab}	ND	ND	ND	ND
5 (T30M75A50)	3.61^{de}	4.12^{j}	4.32^{b}	25.59^{ab}	307.2^{abcde}	226.85^{ef}	4.07^{cde}	1.75^{c}	2.37^{gh}	1.12^{cd}	0.94^{cd}	1.23^{bcd}	ND	ND	ND	ND	ND	ND
6 (T20M75A60)	3.50^{hi}	6.21^{a}	3.88^{fg}	20.38^{g}	316.18^{a}	217.98^{f}	3.14^{ghi}	ND	3.77^{e}	0.78^{gh}	0.93^{cd}	1.14^{cde}	ND	0.24^{cd}	ND	ND	ND	ND
7 (T20M70A50)	3.53^{efghi}	4.77^{f}	3.86^{g}	22.3^{defg}	316.31^{a}	219.56^{f}	4.35^{b}	1.13^{d}	3.11^{f}	1.13^{cd}	0.47^{ghi}	1.14^{cde}	ND	0.34^{c}	ND	ND	ND	ND
8 (T30M80A40)	3.51^{ghi}	6.15^{a}	4.91^{a}	20.48^{g}	309.85^{abcd}	244.2^{abcde}	2.92^{i}	ND	0.68^{j}	0.73^{h}	0.65^{fg}	0.65^{fg}	ND	ND	ND	ND	ND	ND
9 (T40M80A50)	3.56^{efgh}	6.14^{a}	3.86^{g}	20.15^{g}	299.57^{defgh}	250.84^{ab}	3.13^{ghi}	ND	4.91^{c}	0.74^{h}	0.28^{ij}	1.2^{cde}	ND	0.5^{bc}	ND	ND	ND	ND
10 (T40M70A50)	3.76^{ab}	4.52^{g}	4.18^{c}	23.86^{bcde}	286.83^{i}	247.39^{abc}	3.04^{hi}	1.57^{c}	3.15^{f}	1.84^{a}	0.87^{de}	2.07^{a}	ND	0.9^{a}	ND	ND	ND	ND
11 (T40M75A40)	3.60^{def}	4.29^{hi}	3.90^{fg}	22.96^{cdef}	304.75^{bcdef}	254.07^{a}	3.24^{gh}	1.75^{c}	3.96^{e}	0.89^{efgh}	0.65^{fg}	1.23^{bcd}	ND	ND	ND	ND	ND	ND
12 (T40M75A60)	3.64^{cd}	4.32^{h}	3.95^{ef}	21.8^{efg}	309.26^{abcde}	246.51^{abcd}	3.75^{f}	2.17^{b}	4.33^{d}	1.02^{def}	0.48^{gh}	1.24^{bcd}	ND	ND	ND	ND	ND	ND
13 (T20M75A40)	3.52^{fghi}	5.42^{e}	4.00^{e}	21.25^{fg}	291.14^{hi}	222.4^{f}	3.34^{g}	0.72^{e}	3.17^{f}	0.86^{fgh}	0.25^{j}	0.95^{de}	ND	0.22^{cd}	ND	ND	ND	ND
14 (T30M75A50)	3.59^{defg}	4.30^{hi}	4.28^{b}	25.38^{ab}	302.04^{cdefg}	227.56^{def}	4.04^{de}	2.14^{b}	2.54^{g}	1.13^{cd}	1.06^{bcd}	1.29^{bc}	ND	ND	ND	ND	ND	ND
15 (T30M70A40)	3.54^{efghi}	5.76^{b}	3.84^{g}	21.56^{efg}	313.45^{ab}	243.26^{abcde}	3.81^{f}	0.43^{f}	5.24^{b}	0.91^{efgh}	0.72^{ef}	1.45^{bc}	ND	ND	ND	ND	ND	ND
16 (T30M80A60)	3.46^{i}	5.33^{e}	4.14^{cd}	20.85^{fg}	298.39^{efgh}	242.41^{abcde}	3.32^{g}	0.84^{e}	2.64^{g}	0.96^{defg}	0.41^{hij}	0.54^{g}	ND	0.48^{bc}	ND	ND	ND	ND
17 (T30M75A50)	3.80^{a}	4.27^{hi}	4.32^{b}	25.79^{ab}	301.69^{cdefg}	247.45^{abc}	4.22^{bcd}	3.14^{a}	3.08^{f}	1.21^{c}	1.12^{bc}	1.41^{bc}	ND	ND	ND	ND	ND	ND
SEM	0.0355	0.0453	0.0351	1.02614	4.7048	8.2729	0.103	0.112	0.145	0.085	0.090	0.133		0.140				
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.001				

D60, whole-plant corn ensiled for 60 days; ASA, silages inoculated with *A. pasteurianus* during aerobic exposure; ASN, silages without *A. pasteurianus* during aerobic exposure. LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid. Values with different superscripts in the same column indicate significant differences (*P* < 0.05).

Results

The duration of aerobic stability

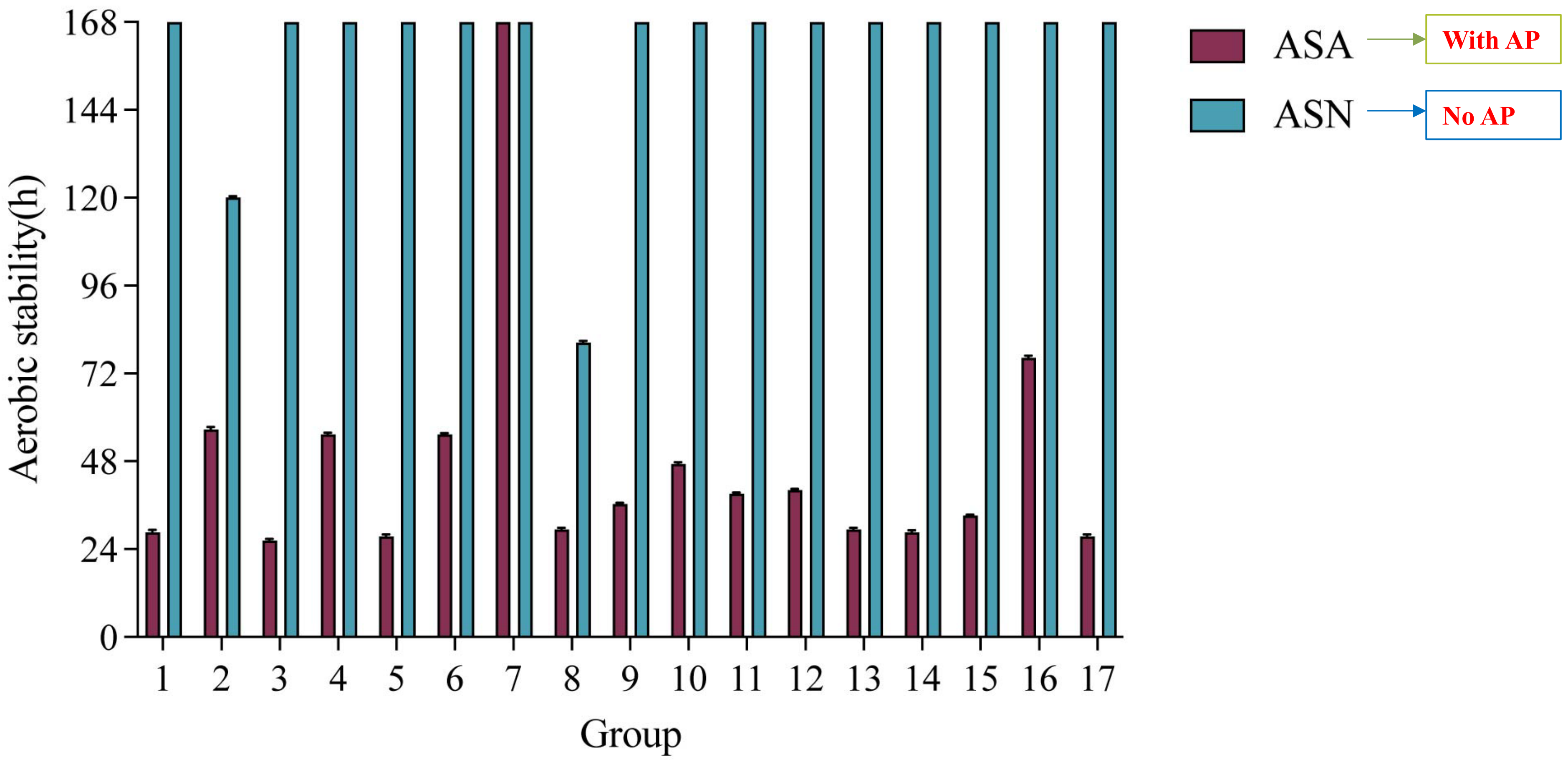


Figure 1 Effect of *A. pasteurianus* on the aerobic stability of whole-plant corn silage after 60 days of fermentation

Note: ASA, silages inoculated with *A. pasteurianus* during aerobic exposure; ASN, silages without *A. pasteurianus* during aerobic exposure.

Results

The microbial communities

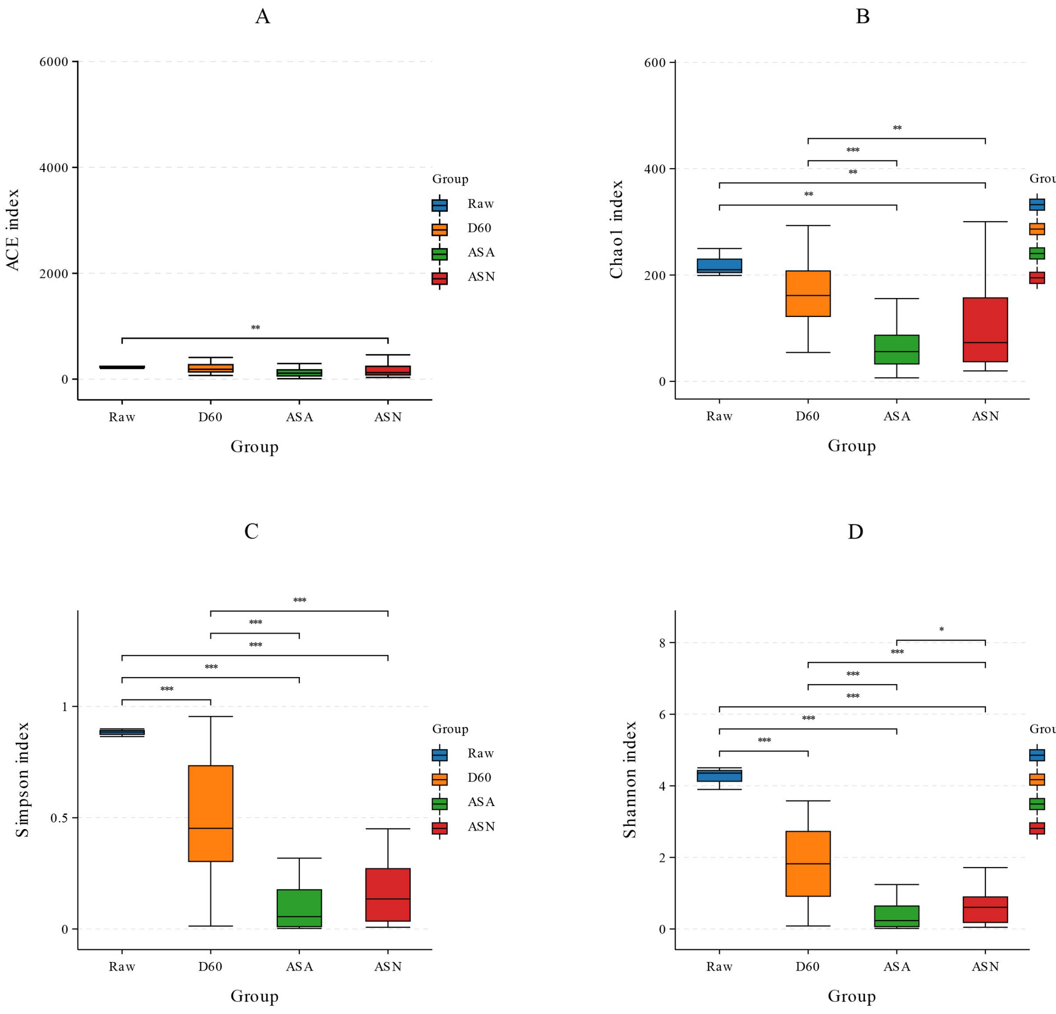


Figure 3 Bacterial alpha diversity of silage (A, ACE index; B, Chao1 index; C, Shannon index; D, Simpson index)

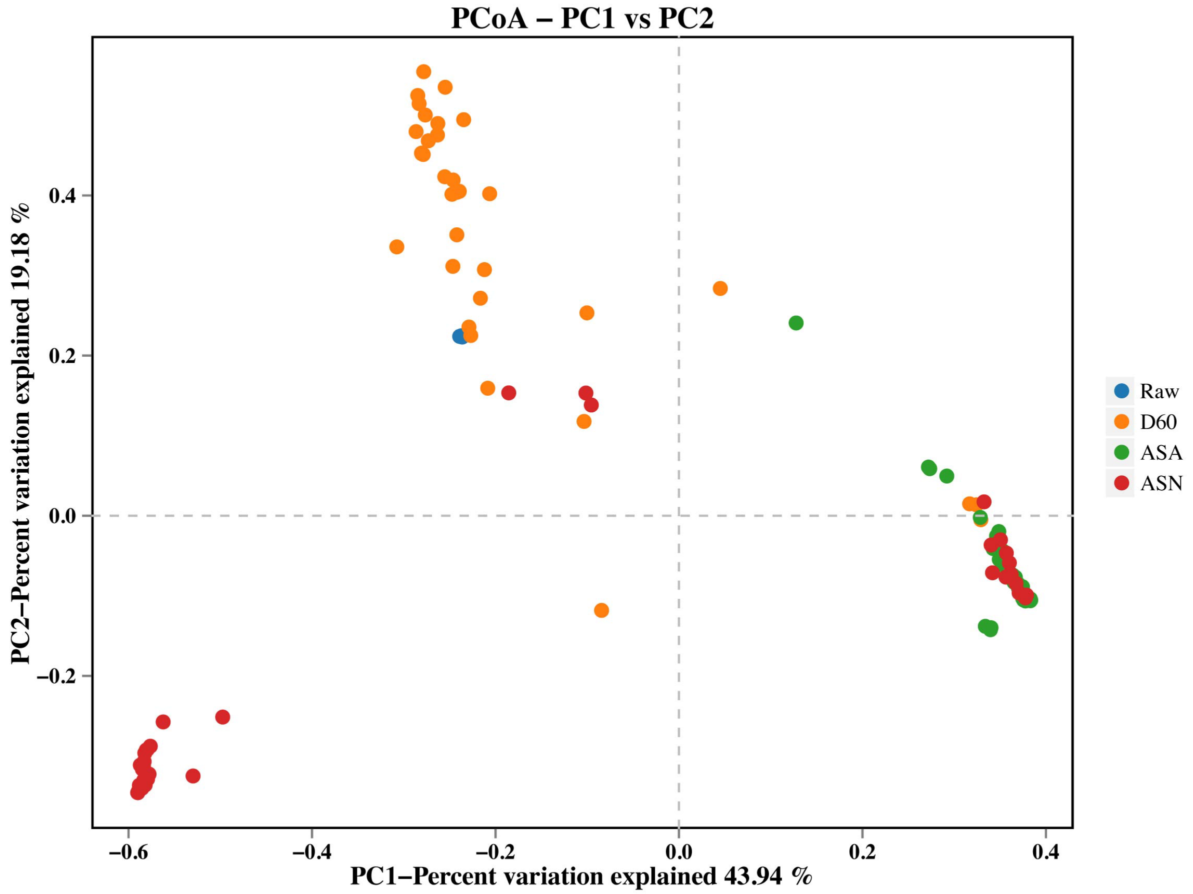


Figure 4 Bacterial Beta Diversity of Silage

Results

The relative abundance

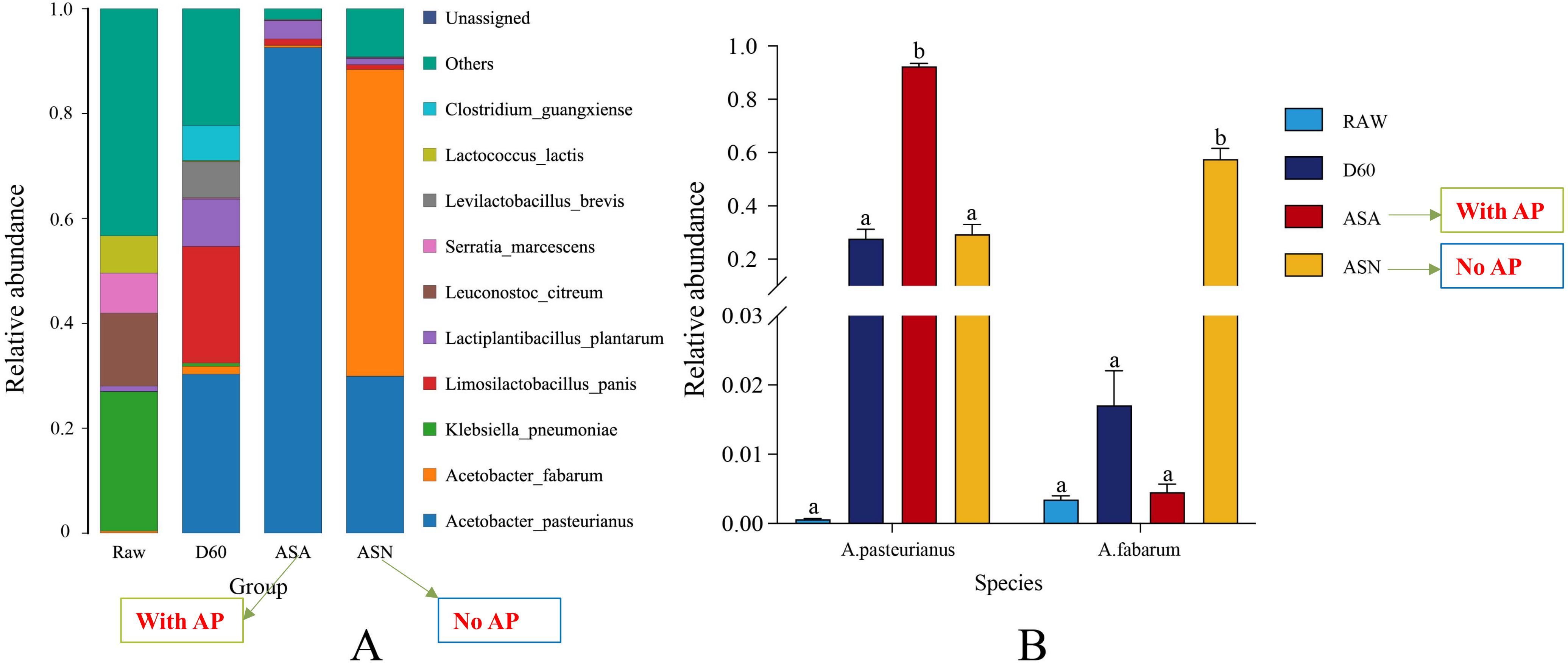
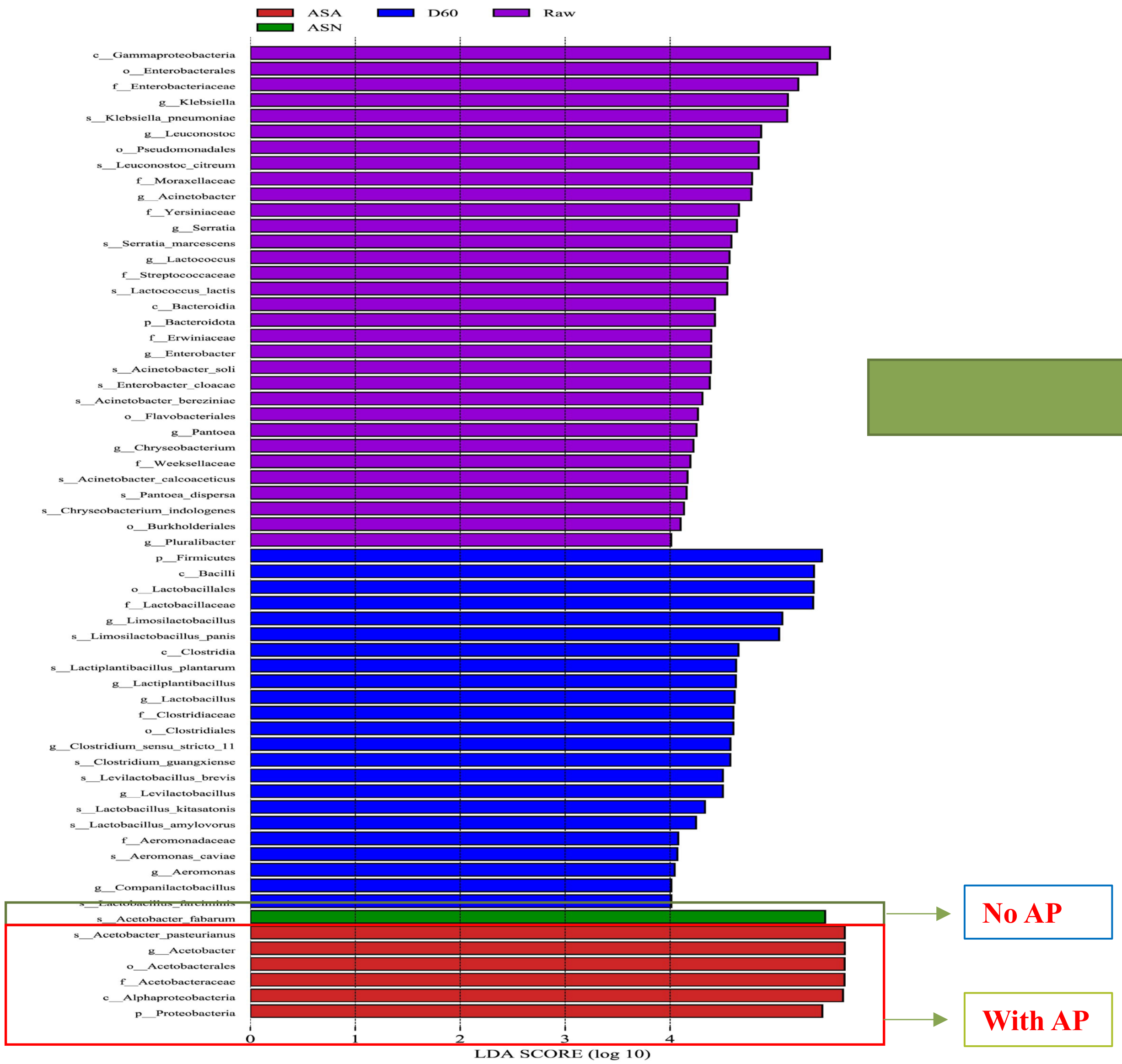


Figure 5 Bacterial community structure at the species level (A) and relative abundance of *A. pasteurianus* and *A. fabarum* the species level (B) in pre-ensiled, ensiled, and aerobically exposed silage

Results

LEfSe analysis



➪ *Klebsiella* was enriched in RAW group.

➪ Limosilactobacillus, *Lactiplantibacillus* and *Clostridium* were enriched in D60A group.

➪ *Lactobacillus* such as *Levilactobacillus, Lactobacillus* and *Companilactobacillus* were enriched in the D60N group.

➪ The ASA group was enriched with *Acetobacter pasteurianus*, and the ASN group was enriched with *Acetobacter fabarum*.

Results

The response surface statistical process and the ANOVA analysis of the model

Table 3 The Box-Behnken design with independent factors and observed values for the absolute quantitative results of *A. pasteurianus*

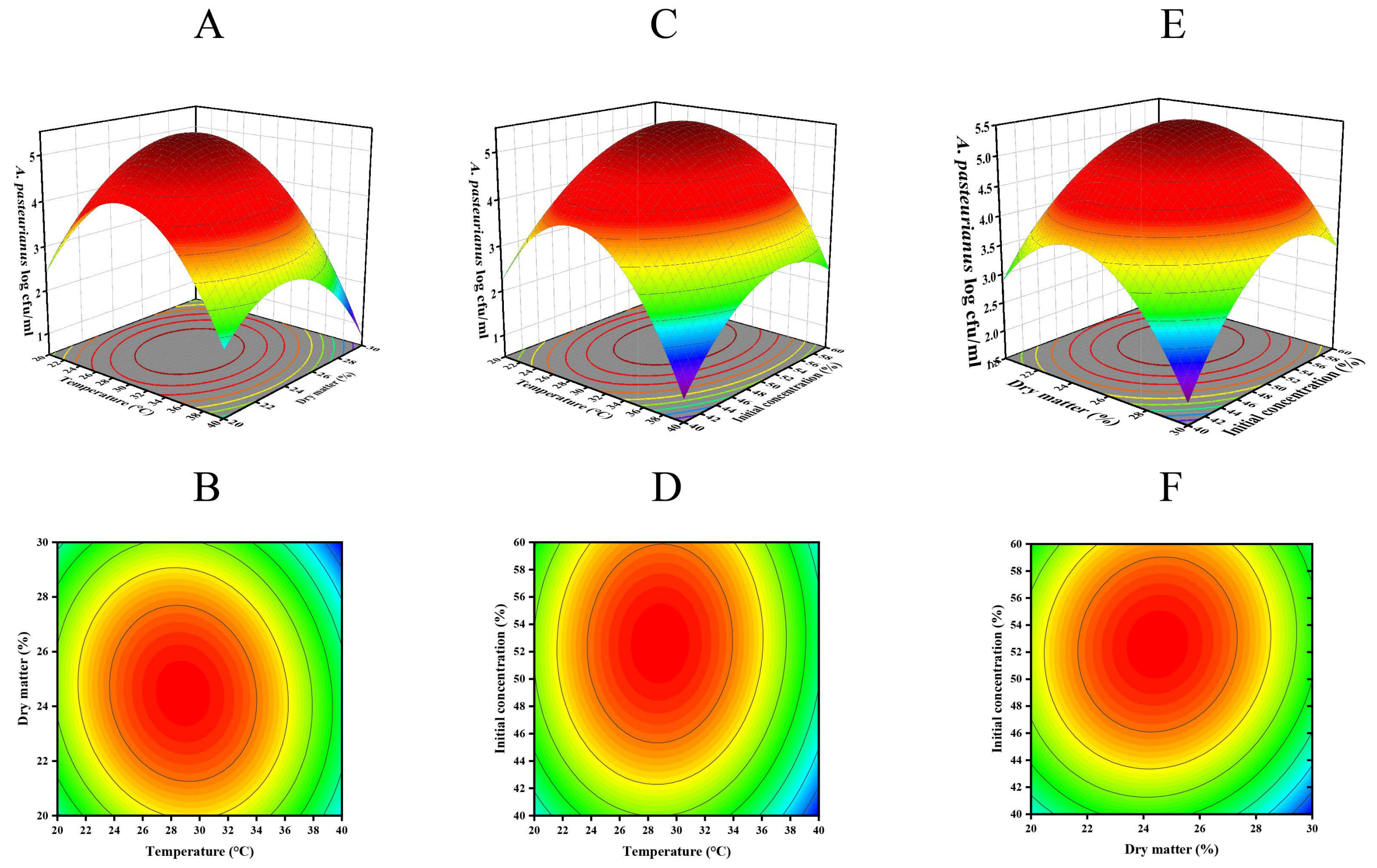
Runs	Level of Independent Factors			Concentration of *A. pasteurianus* (log cfu·ml^{-1})
	X_1(°C)	X_2(%)	X_3(%)	
1	30	25	50	5.59
2	20	30	50	2.31
3	40	30	50	0.67
4	30	25	50	5.36
5	30	25	50	5.36
6	30	25	50	5.3
7	30	20	60	3.64
8	20	25	40	2.26
9	20	25	60	3.1
10	30	20	40	2.81
11	30	30	60	3.41
12	20	20	50	2.45
13	30	25	50	5.26
14	30	20	40	1.84
15	40	25	40	1.02
16	40	25	60	2.28
17	40	20	50	1.93

Table 4 Analysis of variance for absolute quantitative response surface modelling of *A. pasteurianus*.

Source	Sum of squares	df	Mean square	F-value	p-value	Significance
Model	41.7254	9	4.6362	391.28	<0.0001	**
A- Temperature	2.2261	1	2.2261	187.87	<0.0001	**
B- dry matter	0.8450	1	0.8450	71.32	<0.0001	**
C- Initial concentration	2.5313	1	2.5313	213.63	<0.0001	*
AB	0.3136	1	0.3136	26.47	0.0013	**
AC	0.0441	1	0.0441	3.72	0.0950	NS
BC	0.1369	1	0.1369	11.55	0.0115	*
A^2	19.4145	1	19.4145	1638.53	<0.0001	**
B^2	8.1037	1	8.1037	683.93	<0.0001	**
C^2	4.7516	1	4.7516	401.02	<0.0001	**
Residual	0.0829	7	0.0118			
Lack of Fit	0.0175	3	0.0058	0.3566	0.7884	NS
Pure Error	0.0654	4	0.0164			
Cor Total	41.8083	16				
R^2	0.9980					
Adjusted R^2	0.9955					
Predicted R^2	0.9909					
Adeq Precision	55.9033					

Results

Response surface plots



- The three-dimensional response surface map and two-dimensional contour map of the content of Acetobacter pasteurianus in the ASN stage are shown in Figure 2.
- The interaction between storage temperature and moisture content (AB), moisture content and initial acetate concentration (AC) was significant.
- Figs.a-f showed that with the increase of temperature and water content, temperature and concentration of *A.pasteurianus*, DM content and concentration of *A.pasteurianus*, the content of *A.pasteurianus* in ASN increased first and then decreased.

Figure 2 Three-dimensional response surface plots (A, C, E) and two-dimensional contour plots (B, D, F) of ASN *A. pasteurianus* content. a-f respectively represent the interactions between temperature, dry matter content, and initial *A. pasteurianus* concentration.

Results

The predicted conditions

Number	Temperature	Dry matter content	Initial *A. pasteurianus* concentration	Absolute quantity	Desirability
1	28.889	24.557	52.517	5.489(Max)	0.979
2	39.853	29.909	56.690	0.667(Min)	1

Predicted value of *A. pasteurianus* content

- The conditions with the **highest quantity** of *A.pasteurianus* : temperature at 28.889 °C, DM content at 24.557 %, and initial concentration of *A.pasteurianus* at log 5.7 cfu·ml^{-1}.
- The conditions with the **lowest quantity** of *A.pasteurianus:* temperature at 39.853 °C, DM content at 29.909 %, and the initial concentration of *A.pasteurianus* at log 5.9 cfu·ml^{-1}.

Conclusion

- *A. pasteurianus* did not negatively impact the quality of corn silage when properly sealed, however, it can cause rapid aerobic spoilage during the feed out stage.

- Not all Acetobacter have the negative impact on the aerobic stability of silage, such as *A. fabarum*.

- RSM analysis demonstrated that at temperature of 28°C, a DM content of 25% of forage, and an initial *A. pasteurianus* concentration of log 5.7 cfu·ml^{-1}, the highest concentration of *A. pasteurianus* was observed during the aerobic exposure stage.

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Prospect

Future efforts should be focused on the prevention and control of *A. pasteurianus* in whole-plant corn silage to improve aerobic stability in hot and humid regions.

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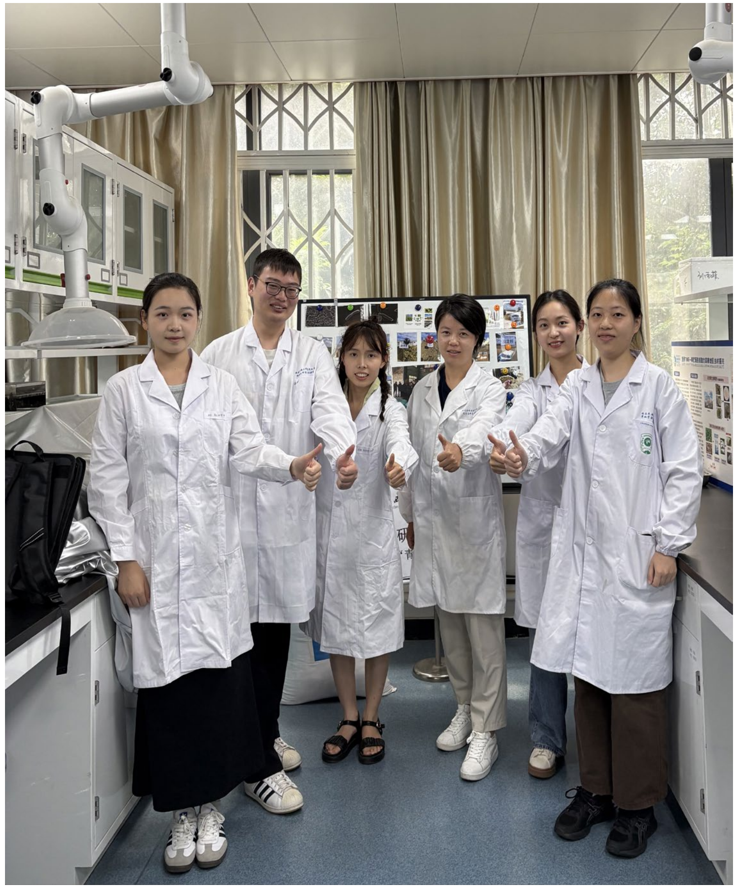

Thank you !

guanh0427@outlook.com

Welcome to the National Field Scientific Observation and Research Station of the Ruoergai Alpine Wetland Ecosystem!