applied sciences

MDPI

Article

Fermentation Characteristics and Microbiota during the Ensiling of *Myriophyllum aquaticum* Inoculated with Lactic Acid Bacteria

Qian Sun [1], Bo Sun [2], Shengjun Xu [2,*], Huanzhen Zhang [1], Jianlin Guo [3], Zhi Qian [4,*] and Xuliang Zhuang [2,3]

1 School of Water Sources and Environment, China University of Geosciences (Beijing), Beijing 100083, China; sqatcau@126.com (Q.S.); huanzhen@cugb.edu.cn (H.Z.)
2 Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China; bosun_st@rcees.ac.cn (B.S.); xlzhuang@rcees.ac.cn (X.Z.)
3 Ningxia Zhongke Jingke Testing Technology Company, Yinchuan 750000, China; 3005180051@cugb.edu.cn
4 College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: sjxu@rcees.ac.cn (S.X.); qianz@ucas.ac.cn (Z.Q.)

Abstract: *Myriophyllum aquaticum* (*M. aquaticum*) is a commonly used aquatic macrophyte for water purification and could be utilized as animal food. However, the high water content of *M. aquaticum* makes it difficult for long-term preservation, which leads to challenges as an ideal animal feed ingredient. The storage of Silage for long periods may be a proper method to solve the problem. In the present paper, we assess the effects of lactic acid bacteria *Lactobacillus buchneri* (LB), *Lactobacillus plantarum* (LP), or their combination on fermentation and microbial communities during the ensiling of *M. aquaticum* silage. The results show that the LP-treated silage displays a higher lactic acid concentration than that in the control silage. Both LB and LP increased the abundance of *Lactobacillus*, but decreased the abundance of *Serratia* and *Prevotella_9* in *M. aquaticum* silage after 60 days of ensiling. Both LB and LP increased the diversity and richness of fungi. Therefore, the inoculation of LP improved silage fermentation during ensiling. These results show that the inoculation of lactic acid bacteria improves the fermentation quality of *M. aquaticum* silage, which makes it possible for the application of *M. aquaticum* to animal forage in the future.

Keywords: *Myriophyllum aquaticum*; *Lactobacillus buchneri*; *Lactobacillus plantarum*; silage

Citation: Sun, Q.; Sun, B.; Xu, S.; Zhang, H.; Guo, J.; Qian, Z.; Zhuang, X. Fermentation Characteristics and Microbiota during the Ensiling of *Myriophyllum aquaticum* Inoculated with Lactic Acid Bacteria. *Appl. Sci.* **2022**, *12*, 5139. https://doi.org/10.3390/app12105139

Academic Editor: Ramona Iseppi

Received: 8 April 2022
Accepted: 26 April 2022
Published: 19 May 2022

1. Introduction

Myriophyllum aquaticum (*M. aquaticum*) is a commonly used aquatic macrophyte for the purification of various water bodies, which has a strong ability to absorb the nutrients, especially nitrogen and phosphorus. Thus, in aquatic environments, *M. aquaticum* can rapidly grow at the expense of such eutrophic nutrients [1–3]. In addition, some research has shown that, in effluents with nitrogen concentrations not higher than 20 mg L^{-1}, *M. aquaticum* can both absorb nitrogen and phosphorus from sediments through their roots and utilize nutrients from the water through their stems and leaves. Nitrogen and phosphorus are absorbed and used for plant growth, while some organic compounds and heavy metals that are toxic to *M. aquaticum* may be degraded or immobilized in their bodies [4–6]. This macrophyte can store a variety of essential nutrients at high concentrations within the tissues, such as proteins with a relatively balanced amino acid composition, various trace elements, and vitamins and essential fatty acids. Therefore, these properties make it an ideal food for farming livestock [4–7].

However, the water content of *M. aquaticum* is usually above 90%, making it susceptible to spoilage during long-term preservation, a problem that needs to be addressed during its conversion into poultry feed. It is well known that silage can be preserved for a long time and is a major component of food for livestock in agriculture and farming livestock. Therefore, making silage from *M. aquaticum* may be a viable solution to this problem.

Thus, we hypothesized that the inoculation of lactic acid bacteria (LAB) could promote the fermentation of *M. aquaticum* into silage and also improve the quality and nutrient composition of silage.

A large quantity of research has shown that the lactic acid bacteria were the main microbial population that attached to the surface of silage and promoted its fermentation [8–10]. The addition of lactic acid bacteria inoculants can ensure the sufficient amount of lactic acid bacteria for fermentation in the early stage of ensiling, and enable the silage to enter the lactic acid fermentation stage as soon as possible [11]. *Lactobacillus buchneri* consumes more nutrients in silage during ensiling. However, *Lactobacillus buchneri* is more resistant to fungi that can degrade lactic acid, so it can improve the aerobic stability of silage and feed quality. Therefore, the loss of feed nutrients can be compensated by improving the production [12–14].

In the present study, *M. aquaticum* was harvested from a constructed wetland and processed into high-quality silage through reducing the water content for forage purposes. In order to improve the quality of *M. aquaticum* silage, two lactic acid bacteria (LAB) inoculants, *Lactobacillus buchneri* (LB) and *Lactobacillus plantarum* (LP), were fermented with the processed *M. aquaticum* silage. The aim of this study is to investigate the effects of LP and LB on the fermentation quality and the bacterial and fungal communities in *M. aquaticum* silage during ensiling. This work was conducted with the perspective of exploring a rational pathway for future applications in animal forage.

2. Materials and Methods

2.1. Silage Preparation

The *M. aquaticum*, which is used for silage experiments, was harvested in the ecological ditches and wetlands of the experimental station of the Chinese Academy of Sciences in Changsha County, Changsha City, Hunan Province, China. The water on the surface of the *M. aquaticum* was dried, and the *M. aquaticum* was chopped to 2 cm–3 cm pieces, to ensure that the raw materials were clean, with no mildew deterioration. The chopped *M. aquaticum* was treated with the following: (1) distilled water (control), (2) *Lactobacillus buchneri* CICC 20295 (LB, China Center of Industrial Culture Collection), (3) *Lactobacillus plantarum* CICC 20242 (LP, China Center of Industrial Culture Collection), and (4) combined LB with LP (FH). The LB (LP) was used at a rate of 1×10^6 CFU per gram of fresh weight by dissolving in 10 mL of distilled water and then sprayed on the *M. aquaticum* (about 600 g) to make sure that the inoculants and raw material were evenly mixed, and mixed LB and LP were added to the FH-treated group (1×10^6 CFU g^{-1} FW) and also evenly sprayed. The control group was added with 10 mL of distilled water. After thoroughly mixing, some polyethylene bags were applied to pack *M. aquaticum* forages and then the bags were vacuum sealed. The silage bags were stored at an environmental temperature of 26 ± 3 °C for 60 days.

2.2. Chemical Component and Fermentation Characteristics Analyses

Triplicate bags of all treated silages were sampled on days 1, 7, 21, 45, and 60. Dry matter (DM) was determined in triplicate by oven drying for 48 h at 65 °C. A mixture of 20 g of silage and 180 mL of distilled water was stored at −20 °C for 24 h; the filtered aqueous extract was used for the estimation of pH, lactic acid content, acetic acid content, propionic acid content, and butyric acid content. Organic acid, including lactic acid, acetic acid, propionic acid, and butyric acid, were determined by high-performance liquid chromatography (HPLC) and analyzed by the Shimadzu LC-20AD high-performance liquid chromatography system (Shimadzu, Japan). Crude protein (CP) was determined by the Kjeldahl method and analyzed by FOSS 2300 Auto Kjeldahl Nitrogen Analysis (FOSS, Denmark); the neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by the Van Soest method and analyzed by an Ankom A200i semi-automatic fiber analyzer (Ankom, Macedon, NY, USA). All the methods described above were performed according to Ke et al. [15].

2.3. DNA Extraction, Amplification, and Sequencing

After 60 days of ensiling, we collected all the samples, then stored them at −20 °C for further analysis. The isolation of microorganism cells was performed, as described. Briefly, 25 g of silage sample was mixed with 50 mL of sterile saline solution, and shaken at 140 rpm for 15 min, and then filtered with 2 layers of gauze. Then, 40 mL of each filtrate was centrifuged at 8000× g min^{-1} for 5 min to collect microorganism cells [16]. According to the manufacturer's standards, total DNA was extracted by the application of the FastDNA SPIN for the soil kit (MP Biomedicals, Solon, OH, USA). The concentration and purity of extracted DNA were determined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, CA, USA), and the quality of extracted DNA was evaluated by 1% agarose gel electrophoresis. All extracted DNA samples were frozen at −20 °C for further analysis.

The PCR amplification and bioinformatic analysis of the samples were performed by Shanghai Personal Biotechnology Co., Ltd. According to the description of Chen et al. and Tian et al. [17,18], the bacterial 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and the fungal ITS gene was amplified using primers ITS1F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2-2043R (5′-GCTGCGTTCTTCATCGATGC-3′). The two-terminal (paired-end) sequencing of community DNA was performed using the Illumina platform. The quality filter, cluster, and analysis of the 16S and ITS rRNA gene sequencing data was determined by the DADA2 method and analyzed by QIIME2 software. The data were analyzed using the free online Personalbio cloud platform (www.personalbio.cn accessed on 1 January 2022).

2.4. Statistical Analyses

The SAS software (SAS version 9.0, SAS Institute, Inc., Cary, NC, USA) was used to analyze the data of silage fermentation and biochemical composition by one-way analysis of variance. The Tukey's multiple range test was used for multiple comparisons, and significance was declared if $p < 0.05$.

3. Results

3.1. The Chemical Composition and Quality of Silage

On the first day of ensiling, the pH value of LB was slightly higher than that of CK (Figure 1), but the pH values of FH and LP were significantly lower than that of CK ($p < 0.05$). On the 7th day of ensiling, the pH value of all treatments gradually decreased. On the 21st day of ensiling, the pH values of FH and LB were significantly lower than that of the CK ($p < 0.05$), while the pH value of LP was slightly lower than that of the CK, but there was no significant difference ($p > 0.05$). On the 60th day of ensiling, the pH of LP and LB decreased to the minimum, with an increase in the fermentation time, and the pH levels of all treatments were significantly lower than the CK ($p < 0.05$). From the 21st day to the 60th day of ensiling, the pH value of FH basically remained unchanged. During the whole ensiling process, the lactic acid content of the FH group was higher than that of the CK, except the FH on the 60th day ($p < 0.05$). The content of lactic acid in LP reached the highest on the 60th day of ensiling, and the content of lactic acid in LB and FH reached the highest on the 21st day of ensiling, which increased by 131.2%, 47.5%, and 29.3%, compared to CK, respectively. The content of acetic acid in LB increased at first, and then decreased in the whole ensiling process. The content of acetic acid in LP and FH decreased at first, and then increased in the ensiling process; the content of acetic acid in the bacteria-added group was higher than the control group ($p < 0.05$). In comparison to the CK, the acetic acid content in LB, LP, and FH increased by 41.6%, 109.1%, and 116.8%, respectively. On the 60th day, after ensiling, the propionic acid content in LB was slightly lower than that in CK ($p > 0.05$), and propionic acid content in LP and FH was significantly lower than that in CK ($p < 0.05$); compared to the CK, propionic acid content in LB, LP, and FH decreased by 11.5%, 23.4%, and 28.0%, respectively. The content of butyric acid in LP was 54.7% higher than that in CK ($p < 0.05$); the content of butyric acid in LB and FH was lower than that in

CK ($p < 0.05$). The content of butyric acid in LB and FH was 39.1% and 64.1% lower than that in CK, respectively.

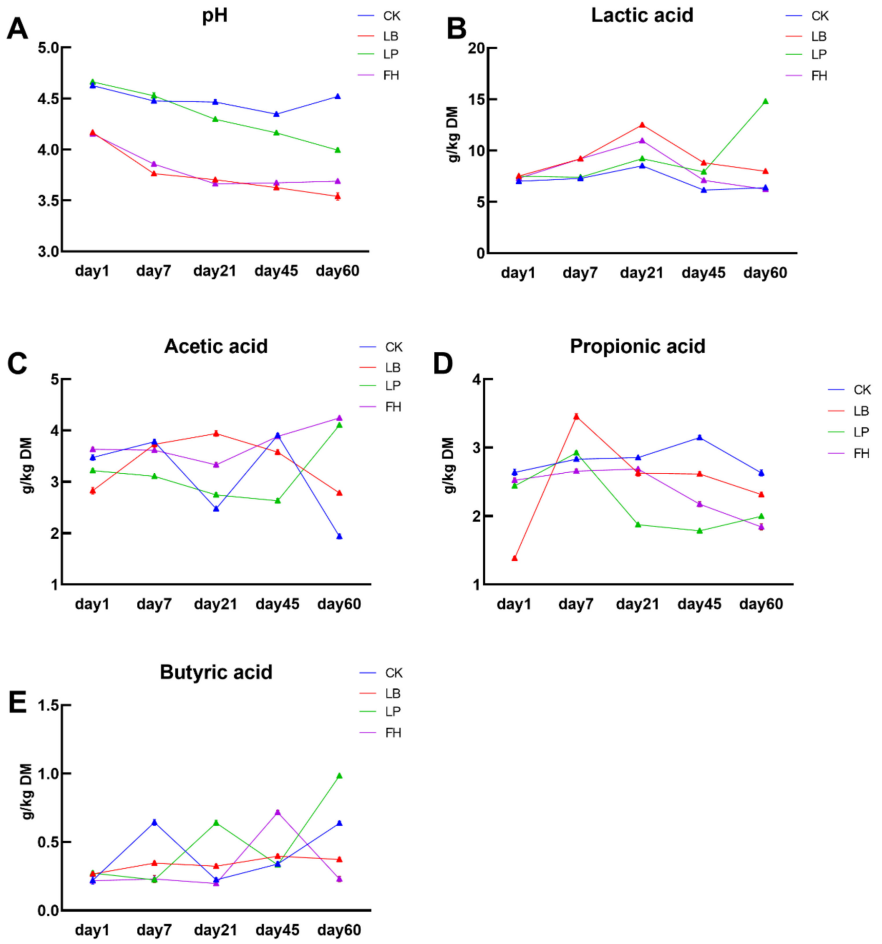

Figure 1. Changes in fermentation quality of *M. aquaticum* silage during ensiling. (**A**) pH value; (**B**) lactic acid content; (**C**) acetic acid content; (**D**) propionic acid content; and (**E**) butyric acid content. CK, control; LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; and FH, combination of LB and LP.

During the whole ensiling process, the dry matter content (DM) of all the treatments showed a trend of decreasing at first and then increasing (Figure 2). On the first day of ensiling, there was no significant difference between the DM content of each group and CK ($p > 0.05$). On the 7th and 21st days, the DM content of FH was the highest, significantly higher than that of the other treatments ($p < 0.05$); on the 45th day, the DM content of LP was significantly higher than that of the other treatments ($p < 0.05$). The DM content of CK was the highest, and was significantly higher than that of the other treatments ($p < 0.05$). The crude protein (CP) content of the CK was the lowest during ensiling, and the change in CP in each treatment was not significant. On the 60th day, the CP content of the LP was the highest, which was 8.9% higher than that of the CK, and the content of CP in LB and FH was 4.0% and 3.7% higher than that in CK. During ensiling, the neutral detergent fiber (NDF) content in LP increased at first, and then decreased; NDF content in the other three treatments increased after 60 days ensiling; the content of NDF in the LP and FH groups was significantly lower than that in the control group ($p < 0.05$); and the content of NDF in the LB group was slightly higher than that in the control group ($p > 0.05$). The content of NDF in FH was the lowest, 19.9% lower than that in CK, and the content of NDF in the LP group was 12.6% lower than that in CK. The content of acid detergent fiber (ADF) in LB and FH showed an increasing trend, while the ADF content in LP showed an increasing

and then decreasing trend after 60 days; compared to the CK, the ADF content in LB, LP, and FH decreased by 2.4%, 23.0%, and 16.3%, respectively.

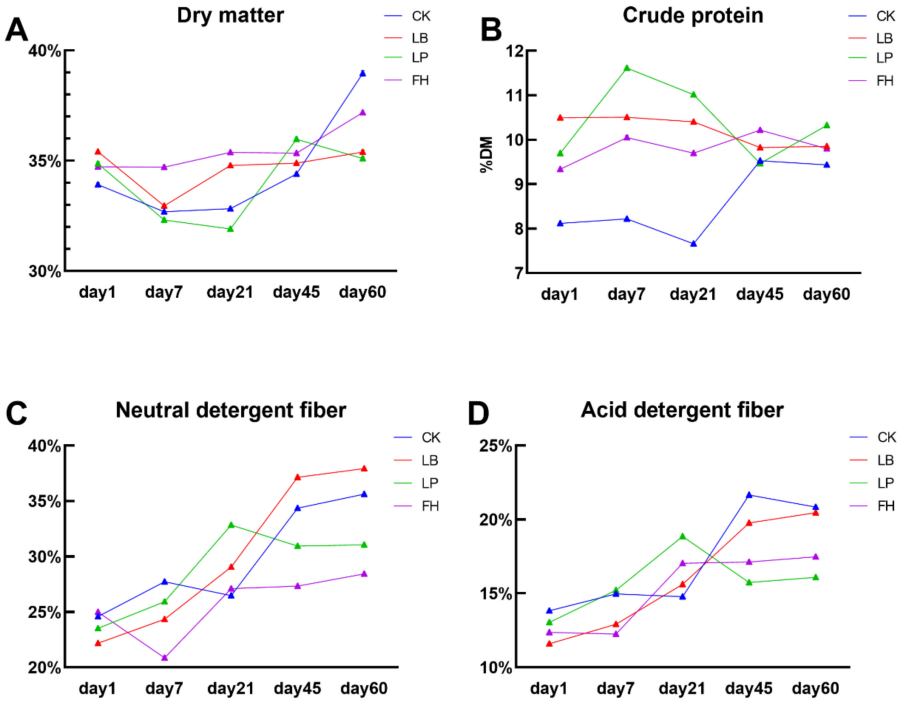

Figure 2. Changes in the chemical composition of *M. aquaticum* silage during ensiling. (**A**) Dry matter content; (**B**) crude protein content; (**C**) neutral detergent fiber content; and (**D**) acid detergent fiber content. CK, control; LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; and FH, combination of LB and LP.

3.2. Microbial Diversity in M. aquaticum Silage

The alpha diversity of bacteria in *M. aquaticum* silage is shown in Table 1. Based on the Good's coverage values, the sequencing adequately captured most of the bacterial communities. The diversity and richness of bacteria (Table S1) decreased in the LB- and FH-treated silages, compared to those in the control silages. This may be explained by the lower pH, and anaerobic and acidic environment could lead to the decrease in aerobic epiphytic bacteria [16]. Similarly, Bai et al. [19] found that silages inoculated with LB had decreased bacterial diversity due to the predominant genus (*Lactobacillus*). Ogunade et al. [20] also reported that, when the relative abundance of dominant bacteria increased, the microbial community was less diverse.

Table 1. Alpha diversity of bacteria in *M. aquaticum* silage after 60 days of ensiling.

Samples	Sequence Number	OTUs	Shannon	Chao1	Simpson	Good's Coverage
CK	48,481	60	6.185	1126.34	0.911	0.991
LP	46,958	51	6.333	1033.33	0.917	0.992
LB	42,065	43	4.362	589.6	0.819	0.996
FH	47,440	46	5.002	684.28	0.848	0.995

The relative abundances of bacteria at the phylum and genus levels in *M. aquaticum* silage after 60 days of ensiling are shown in Figure 3. At the phylum level, after 60 d of fermentation, the main phylum in the control silage were Proteobacteria (56.9%), Bacteroidetes (27.6%), Firmicutes (13.1%), and Cyanobacteria (1.3%). In contrast, in treatments inoculated with lactic acid bacteria, Firmicutes (83.4% in LP, 68.2% in LB, and 59.2% in FH) domi-

nated the epiphytic flora of the silage. Firmicutes are acid hydrolytic microorganisms and Proteobacteria can digest organic matter; they both play important roles during ensiling. These results were analogous to the previous observations of other common silages, such as maize and alfalfa [19,21–25]. At the genus level, Lactobacillus was the most dominant in LP (80.9%), LB (61.4%), and FH (55.3%). It is well known that Lactobacillus has an important role in reducing pH and enhancing lactic acid content; it becomes a predominant genus in silages. Similarly, when mixed high-moisture amaranth and rice straw was ensiled with Lactobacillus plantarum and/or cellulase, Lactobacillus was the dominant genera in all treatments [26].

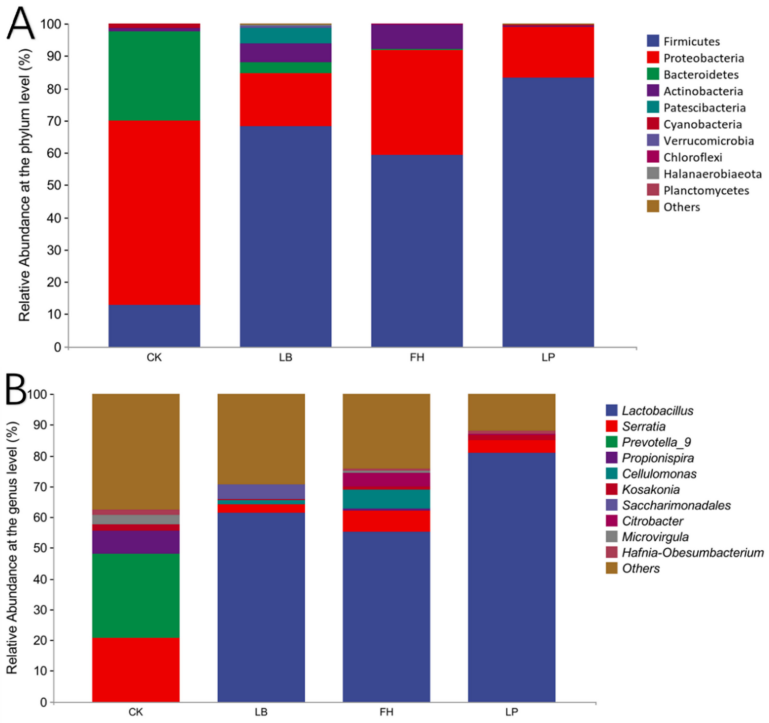

Figure 3. The epiphytic bacterial community in *M. aquaticum* silage after 60 d of ensiling. The bacterial communities are shown at the phylum level (**A**) and the genus level (**B**). CK, control; LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; and FH, combination of LB and LP.

The alpha diversity of fungi in *M. aquaticum* silage is shown in Table 2. Based on the Good's coverage values, the sequencing had adequately captured most of the fungal communities. The diversity and richness of fungi (Table S2) in the silage were increased in the treatment with the addition of lactic acid bacteria inoculants, compared to the control group. This was in agreement with the results of Liu et al. [27] that the fungal richness and diversity of silage inoculated with LAB was significantly higher than that of uninoculated silage. However, a different result was obtained by Vu et al. [28], who showed an increase in fungal richness in elephant grass silage under natural conditions, and no difference in fungal richness and diversity between silage with and for added inoculants.

Table 2. Alpha diversity of fungi in *M. aquaticum* silage after 60 days of ensiling.

Samples	Sequence Number	OTUs	Shannon	Chao1	Simpson	Good's Coverage
CK	63,721	69	3.488	169.16	0.681	0.999
LP	56,737	78	4.809	321.02	0.848	0.999
LB	72,852	67	5.594	198.84	0.937	0.999
FH	71,732	76	3.837	222.81	0.883	0.999

The relative abundances of fungi at the phylum and genus levels in *M. aquaticum* silage after 60 days of ensiling are shown in Figure 4. At the phylum level, after 60 d of fermentation, the main phylum in the control silage were Basidiomycota (55.6%), Ascomycota (29.4%), Mortierellomycota (0.8%), and Mucoromycota (0.1%). Similarly, the LP-treated silage was predominated by Basidiomycota (56.6%) and Ascomycota (27.9%), while the abundance of Ascomycota was highest in FH-treated silage (83.6%) and followed by LB-treated silage (63.0%). The abundance of Basidiomycota was observed in FH-treated silage (12.5%) and LB-treated silage (21.8%). Zhang et al. [29] also found that Ascomycota had a high abundance, and then was followed by Basidiomycota in silages. However, Liu et al. [27] found that Ascomycota had the highest abundance at the phylum level in the control silage after 60 days of ensiling, whereas Basidiomycota was still the dominant phylum in the LAB-treated silage. This result was different from our finding that Basidiomycota was the dominant phylum in the control silage of *M. aquaticum*. At the genus level, the abundance of *Saitozyma* was higher in LP-treated silage (48.9%) and silage treated with CK (45.4%), than that in LB-treated silage (14.8%) and FH-treated silage (9.3%). *Saitozyma* was reported to suppress acetic acid fermentation and is an integral component of soil and epiphytic plants [30–32]. Although more information on the fungal microbiota of silage is lacking, the roles of these fungi in forage ensiling are not known.

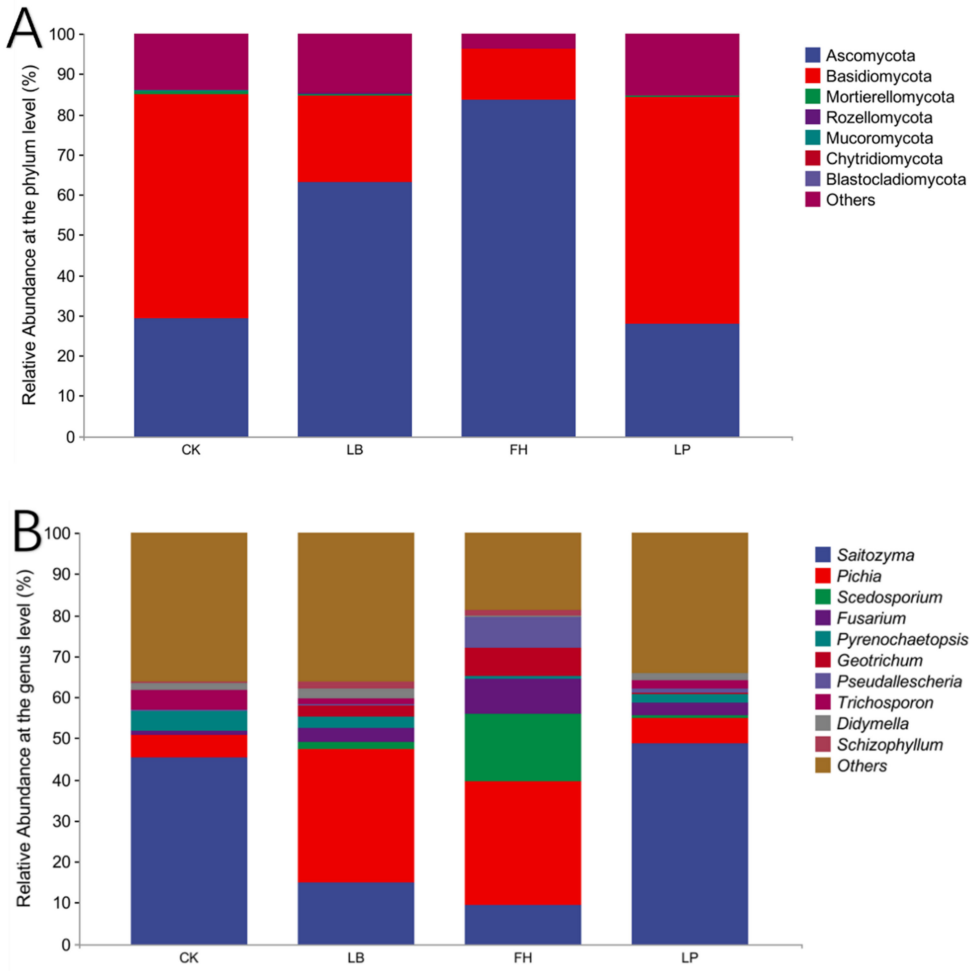

Figure 4. The epiphytic fungal community in *M. aquaticum* silage after 60 d of ensiling. The bacterial communities are shown at the phylum level (**A**) and the genus level (**B**). CK, control; LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; and FH, combination of LB and LP.

4. Discussion

However, fresh *M. aquaticum* with water content between 85% and 93% is not easy to preserve for a long period of time and easily goes bad, while the activity of lactic acid bacteria was inhibited when the moisture content was too low, which affected the fermentation quality of the silage. Therefore, the experiment controlled the water content of *M. aquaticum* between 75~80% for silage. Lactic acid bacteria were added to increase the abundance of lactic acid bacteria on the surface of *M. aquaticum*, which made the fermentation rapidly proceed. A large amount of lactic acid was generated in the fermentation, resulting in a rapid decrease in pH, which is conducive to inhibiting the degradation of crude protein and reducing the decomposition rate of protein in feed, which can be directly digested and absorbed by animals. Zielińska et al. [33] reported that added *Lactobacillus plantarum* and *Lactobacillus buchneri* on alfalfa silage could improve the quality of alfalfa silage. Arasu et al. [34] found that, when *Lactobacillus plantarum* was added to rye, lolium multiflorum, and barley silages, the silage quality improved, and the *L. plantarum* strain has good antibiotic resistance and meted the safety standard of animal feed. The lactic acid inoculant used in this experiment is the lactic acid bacteria preparation of *M. aquaticum* silage, which has a higher adaptability to *M. aquaticum*. Braman W L et al. [35] also reported that the lactic acid bacteria additive could improve the fermentation quality of corn silage, and cause the silage to have a low pH, and a high content of volatile fatty acid and lactic acid. The amount and composition of organic acids reflect the fermentation of carbohydrates in the silage during the ensiling process. The higher the ratio of lactic acid to total organic acids, the more the lactic acid bacteria become the dominant group in the fermentation process, while the lower the butyric acid content, the more the harmful bacteria, such as Clostridium, were inhibited. In this study, the organic acid, pH, and butyric acid contents were significantly higher in the treatment with the addition of inoculant, compared to CK, and the lactic acid content and lactic acid to total acid ratio were also significantly higher. Sucu E et al. [36] showed that the addition of lactic acid bacteria improved the quality and nutritional value of rye silage, specifically by reducing the pH, butyric acid, and ammonia nitrogen content of the silage, increasing the content of lactic acid and neutral detergent fiber, and inhibiting the activity of microorganisms, such as molds. Jatkauskas J et al. [37] found that the addition of lactic acid bacteria to alfalfa, lolium perenne, and red clover mixed with ryegrass and timothy reduced the loss of nutrients in feed and improved the silage quality and aerobic stability of silage. The main components of NDF include cellulose, lignin, and hemicellulose, while cellulose and lignin are the main components of ADF. The content of NDF and ADF in the animal forage affected the dry-matter intake and digestibility of the feed. The content of NDF and ADF in the treatment with lactic acid bacteria was significantly lower than that of CK, while the content of CP was increased and much higher than that of conventional green feed and grain crops, which could be an important supplement to the protein source in animal forage.

In the present study, the complex microbial communities of the raw materials were gradually replaced by lactic acid bacteria during anaerobic fermentation, and the microbial diversity sharply reduced after successful fermentation. Similarly, Guan H et al. [38] found that the microbial diversity of LAB-treated silage decreased after ensiling. This suggested that there was a difference between the epiphytic LAB found on the raw materials and LAB. Consequently, although they have successfully become the dominant microorganisms during the fermentation process, they did not produce sufficient lactic acid to reduce the pH, which could inhibit the growth of other undesirable microorganisms. In the present study, the most dominant phylum in CK was Proteobacteria, while for LAB-treated silage, Firmicutes were the dominant phylum. This could indicate that aerobic microorganisms grow and take advantage of oxygen during the early period of fermentation, and, afterwards, anaerobes start to increase. LAB dominate in the fermentation process and play a key role in inhibiting the growth of harmful microorganisms by the production of lactic acid and reducing the pH; as a result, the nutrients of the forage can be preserved [39]. Fungi and their toxic secondary metabolites—mycotoxins—have been shown to cause a health

risk to animals [40]. Even though the fermentation process inhibits the growth of most of the microorganisms related to silage spoilage, some species of filamentous fungi, such as *A. fumigatus* and *P. niveus*, can tolerate the low pH levels and low availability of oxygen during storage [41,42]. Therefore, these fungi could exit the silage, reducing the dry matter content and nutrients of the forage. We found that the most dominating genus in CK and LP was *Saitozyma*, and the abundance of *Saitozyma* decreased because of the application of LB to the forage. While several previous reports found the existence of *Saitozyma* in silage, the roles of these fungi in forage ensiling need further research [43].

5. Conclusions

The application of *Lactobacillus buchneri* (LB) and *Lactobacillus plantarum* (LP) improved the fermentation and nutrition of *M. aquaticum* silage. Both LB and LP increased the abundance of *Lactobacillus*, but decreased the abundance of *Serratia* and *Prevotella_9* in *M. aquaticum* silage after 60 days of fermentation. Both LB and LP increased the diversity and richness of fungi. These results show that the inoculation of lactic acid bacteria improves the fermentation quality and nutrition of *M. aquaticum* silage. The present study provides support for further research and the production of *M. aquaticum* silage, demonstrating the potential of *M. aquaticum* for conversion into animal forage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12105139/s1, Table S1: Summary of sequence quantity on 16S rRNA gene. Table S2: Summary of sequence quantity on ITS rRNA gene.

Author Contributions: Conceptualization, S.X. and X.Z.; methodology, Q.S.; software, Q.S.; formal analysis, Q.S.; investigation, Q.S.; resources, S.X. and X.Z.; data curation, Q.S.; writing—original draft preparation, Q.S.; writing—review and editing, B.S., Z.Q., H.Z., J.G., and Q.S.; visualization, Q.S.; supervision, Z.Q. and S.X.; project administration, Z.Q. and S.X.; funding acquisition, S.X. and Z.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article. The raw sequence data reported in this study are available on request from the corresponding authors, Shengjun Xu and Zhi Qian.

Acknowledgments: The authors would like to thank the Key Laboratory of Environmental Biotechnology of Research Center for Eco-Environmental Sciences for the technical support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Hussner, A.; Meyer, C.; Busch, J. The Influence of Water Level and Nutrient Availability on Growth and Root System Development of *Myriophyllum aquaticum*. *Weed Res.* **2009**, *49*, 73–80. [CrossRef]
2. Zhang, S.; Liu, F.; Xiao, R.; He, Y.; Wu, J. Nitrogen removal in *Myriophyllum aquaticum* wetland microcosms for swine wastewater treatment: 15N-labelled nitrogen mass balance analysis. *J. Sci. Food Agric.* **2017**, *97*, 1511–1520. [CrossRef] [PubMed]
3. Zhang, H.; Xu, G.; Zeng, Z.; Li, Y.; Qiu, M.; Zeng, H. Nitrogen removal of a *Myriophyllum elatinoides* purification system for treating piggery wastewater in karst region. In Proceedings of the International Conference on Sustainable Energy and Environment Engineering, Zhuhai, China, 12–13 November 2016.
4. Polomski, R.F.; Taylor, M.D.; Bielenberg, D.G.; Bridges, W.C.; Klaine, S.J.; Whitwell, T. Nitrogen and Phosphorus Remediation by Three Floating Aquatic Macrophytes in Greenhouse-Based Laboratory-Scale Subsurface Constructed Wetlands. *Water Air Soil Pollut.* **2009**, *197*, 223–232. [CrossRef]
5. Liu, F.; Zhang, S.; Wang, Y.; Li, Y.; Xiao, R.; Li, H.; He, Y.; Zhang, M.; Wang, D.; Li, X.; et al. Nitrogen removal and mass balance in newly-formed *Myriophyllum aquaticum* mesocosm during a single 28-day incubation with swine wastewater treatment. *J. Environ. Manag.* **2016**, *166*, 596–604. [CrossRef] [PubMed]

6. Li, X.; Zhang, M.; Liu, F.; Li, Y.; He, Y.; Zhang, S.; Wu, J. Abundance and Distribution of Microorganisms Involved in Denitrification Insediments of A *Myriophyllum elatinoides* Purification System for Treating Swine wastewater. *Environ. Sci. Pollut. Res.* **2015**, *22*, 17906–17916. [CrossRef]
7. Sytsma, M.D.; Anderson, L.W.J. Nutrient limitation in *Myriophyllum aquaticum*. *J. Freshw. Ecol.* **2011**, *8*, 165–176. [CrossRef]
8. Oliveira, A.S.; Weinberg, Z.G.; Ogunade, I.M.; Cervantes, A.A.; Arriola, K.G.; Jiang, Y.; Kim, D.; Li, X.; Gonçalves, C.M.M.; Vyas, D.; et al. Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *J. Dairy Sci.* **2017**, *100*, 4587–4603. [CrossRef]
9. Mohammadzadeh, H.; Khorvash, M.; Ghorbani, G.R. Ensiling corn silage with different levels of a multi-species lactic acid bacteria inoculant. *Anim. Prod. Sci.* **2014**, *54*, 165–171. [CrossRef]
10. Wang, Y.; Chen, X.; Wang, C.; He, L.; Zhou, W.; Yang, F.; Zhang, Q. The bacterial community and fermentation quality of mulberry (*Morus alba*) leaf silage with or without *Lactobacillus casei* and sucrose. *Bioresour. Technol.* **2019**, *293*, 122059. [CrossRef]
11. Konca, Y.; Beyzi, S.B.; Ayasan, T.; Kaliber, M.; Kiraz, A.B. The Effects of Freezing and Supplementation of Molasses and Inoculants on Chemical and Nutritional Composition of Sunflower Silage. *Asian-Australas. J. Anim. Sci.* **2016**, *29*, 965–970. [CrossRef]
12. Gallo, A.; Bernardes, T.F.; Copani, G.; Fortunati, P.; Giuberti, G.; Bruschi, S.; Bryan, K.A.; Nielsen, N.G.; Witt, K.L.; Masoero, F. Effect of inoculation with Lactobacillus buchneri LB1819 and Lactococcus lactis O224 on fermentation and mycotoxin production in maize silage compacted at different densities. *Anim. Feed Sci. Technol.* **2018**, *246*, 36–45. [CrossRef]
13. Gandra, J.R.; Oliveira, E.R.; Gandra, E.R.D.S.; Takiya, C.S.; Goes, R.H.T.B.D.; Oliveira, K.M.P.; Silveira, K.A.; Araki, H.C.; Orbach, N.D. Inoculation of Lactobacillus buchneri alone or with Bacillus subtilis and total losses, aerobic stability, and microbiological quality of sunflower silage. *J. Appl. Anim. Res.* **2016**, *45*, 609–614. [CrossRef]
14. Rabelo, C.H.S.; Basso, F.C.; Lara, E.C.; Jorge, L.G.O.; Härter, C.J.; Mesquita, L.G. Effects of Lactobacillus buchneri as a silage inoculant and as a probiotic on feed intake, apparent digestibility and ruminal fermentation and microbiology in wet hers fed low-dry-matter whole-crop maize silage. *Grass Forage Sci.* **2017**, *73*, 67–77. [CrossRef]
15. Ke, W.C.; Ding, W.R.; Xu, D.M.; Ding, L.M.; Zhang, P.; Li, F.H.; Guo, X.S. Effects of addition of malic or citric acids on fermentation quality and chemical characteristics of alfalfa silage. *J. Dairy Sci.* **2017**, *100*, 8958–8966. [CrossRef] [PubMed]
16. Keshri, J.; Chen, Y.; Pinto, R.; Kroupitski, Y.; Weinberg, Z.G.; Sela, S. Microbiome dynamics during ensiling of corn with and without Lactobacillus plantarum inoculant. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 4025–4037. [CrossRef]
17. Chen, W.; Xu, R.; Chen, J.; Yuan, X.; Zhou, L.; Tan, T.; Fan, J.; Zhang, Y.; Hu, T. Consistent responses of surface- and subsurface soil fungal diversity to n enrichment are mediated differently by acidification and plant community in a semi-arid grassland. *Soil Biol. Biochem.* **2018**, *127*, 110–119. [CrossRef]
18. Tian, J.; He, N.; Hale, L.; Niu, S.; Yu, G.; Liu, Y.; Blagodatskaya, E.; Kuzyakov, Y.; Gao, Q.; Zhou, J. Soil organic matter availability and climate drive latitudinal patterns in bacterial diversity from tropical to cold-temperate forests. *Funct. Ecol.* **2017**, *32*, 61–70. [CrossRef]
19. Bai, J.; Xu, D.; Xie, D.; Wang, M.; Li, Z.; Guo, X. Effects of antibacterial peptide-producing Bacillus subtilis and Lactobacillus buchneri on fermentation, aerobic stability, and microbial community of alfalfa silage. *Bioresour. Technol.* **2020**, *315*, 123881. [CrossRef]
20. Ogunade, I.M.; Jiang, Y.; Pech Cervantes, A.A.; Kim, D.H.; Oliveira, A.S.; Vyas, D. Bacterial diversity and composition of *Alfalfa silage* as analysed by Illumina Miseq sequencing: Effects of Escherichia coli O157:H7 and silage additives. *J. Dairy Sci.* **2018**, *101*, 2048–2059. [CrossRef]
21. Romero, J.J.; Zhao, Y.; Balseca-Paredes, M.A.; Tiezzi, F.; Gutierrez-Rodriguez, E.; Castillo, M.S. Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. *J. Dairy Sci.* **2017**, *100*, 1812–1828. [CrossRef]
22. Ni, K.; Minh, T.T.; Tu, T.T.; Tsuruta, T.; Pang, H.; Nishino, N. Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 1385–1394. [CrossRef] [PubMed]
23. Wang, Y.; Wang, C.; Zhou, W.; Yang, F.Y.; Chen, X.Y.; Zhang, Q. Effects of wilting and Lactobacillus plantarum addition on the fermentation quality and microbial community of Moringa oleifera leaf silage. *Front. Microbiol.* **2018**, *9*, 1817. [CrossRef] [PubMed]
24. Xu, Z.; Zhang, S.; Zhang, R.; Li, S.; Kong, J. The changes in dominant lactic acid bacteria and their metabolites during corn stover ensiling. *J. Appl. Microbiol.* **2018**, *15*, 675–685. [CrossRef] [PubMed]
25. Zhao, X.; Liu, J.; Liu, J.; Yang, F.; Zhu, W.; Yuan, X.; Hu, Y.; Cui, Z.; Wang, X. Effect of ensiling and silage additives on biogas production and microbial community dynamics during anaerobic digestion of switchgrass. *Bioresour. Technol.* **2017**, *241*, 349–359. [CrossRef]
26. Mu, L.; Xie, Z.; Hu, L.; Chen, G.; Zhang, Z. Cellulase interacts with Lactobacillus plantarum to affect chemical composition, bacterial communities, and aerobic stability in mixed silage of high-moisture amaranth and rice straw. *Bioresour. Technol.* **2020**, *315*, 123772. [CrossRef]
27. Liu, B.; Huan, H.; Gu, H.; Xu, N.; Shen, Q.; Ding, C. Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresour. Technol.* **2019**, *273*, 212–219. [CrossRef]
28. Vu, V.H.; Li, X.; Wang, M.; Liu, R.; Zhang, G.; Liu, W.; Xia, B.; Sun, Q. Dynamics of fungal community during silage fermentation of elephant grass (Pennisetum purpureum) produced in northern Vietnam. Asian-Austral. *J. Anim Sci.* **2019**, *32*, 996–1006.

29. Zhang, L.; Zhou, X.K.; Gu, Q.C.; Liang, M.Z.; Mu, S.L.; Zhou, B.; Huang, F.; Lin, B.; Zou, C.X. Analysis of the correlation between bacteria and fungi in sugarcane tops silage prior to and after aerobic exposure. *Bioresour. Technol.* **2019**, *291*, 121835. [CrossRef]
30. Hou, J.; Nishino, N. Bacterial and Fungal Microbiota of Guinea Grass Silage Shows Various Levels of Acetic Acid Fermentation. *Fermentation* **2022**, *8*, 10. [CrossRef]
31. Surussawadee, J.; Khunnamwong, P.; Srisuk, N.; Limtong, S. *Papiliotrema siamense* f.a.; sp. nov.; a yeast species isolated from plant leaves. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 3058–3062. [CrossRef]
32. Vujanovic, V. Tremellomycetes yeasts in kernel ecological niche: Early indicators of enhanced competitiveness of endophytic and mycoparasitic symbionts against wheat pathobiota. *Plants* **2021**, *10*, 905. [CrossRef] [PubMed]
33. Zielińska, K.; Fabiszewska, A.; Stefańska, I. Different aspects of Lactobacillus inoculants on the improvement of quality and safety of alfalfa silage. *Chil. J. Agric. Res.* **2015**, *75*, 298–306. [CrossRef]
34. Arasu, M.V.; Jung, M.W.; Da, H.K.; Ilavenil, S.; Jane, M.; Park, H.S.; Al-Dhabi, N.A.; Jeon, B.T.; Choi, K.C. Enhancing Nutritional Quality of Silage by Fermentation with Lactobacillus plantarum. *Indian J. Microbiol.* **2014**, *54*, 396–402. [CrossRef] [PubMed]
35. Braman, W.L.; Kurtz, J.E.; Bryan, K.A. Effect of fermented corn silage density and bacterial inoculants on corn silage pH and fermentation end products. *J. Anim Sci.* **2016**, *94*, 27. [CrossRef]
36. Sucu, E.; Esra, C.A. Effects of lines and inoculants on nutritive value and production costs of triticale silages. *R. Bras. Zootec.* **2016**, *45*, 355–364. [CrossRef]
37. Jatkauskas, J.; Vrotniakiene, V.; Lanckriet, A. The effect of different types of inoculants on the characteristics of alfalfa, ryegrass and red clover/ryegrass/timothy silage. *Zemdirb. Agric.* **2015**, *102*, 95–102. [CrossRef]
38. Guan, H.; Yan, Y.H.; Li, X.L.; Li, X.M.; Shuai, Y.; Feng, G.Y.; Ran, Q.F.; Cai, Y.M.; Li, Y.; Zhang, X.Q. Microbial communities and natural fermentation of corn silages prepared with farm bunker-silo in Southwest China. *Bioresour. Technol.* **2018**, *265*, 282–290. [CrossRef]
39. Ávila, C.L.S.; Carvalho, B.F. Silage fermentation—updates focusing on the performance of microorganisms. *J. Appl. Microbiol.* **2020**, *128*, 966–984. [CrossRef]
40. Dunière, L.; Sindou, J.; Chaucheyras-Durand, F.; Chevallier, I.; Thévenot-Sergentet, D. Silage processing and strategies to prevent persistence of undesirable microorganisms. *Anim. Feed Sci. Technol.* **2013**, *182*, 1–15. [CrossRef]
41. Alonso, V.A.; Pereyra, C.M.; Keller, L.A.M.; Dalcero, A.M.; Rosa, C.A.R.; Chiacchiera, S.M.; Cavaglieri, L.R. Fungi and mycotoxins in silage: An overview. *J. Appl. Microbiol.* **2013**, *115*, 637–643. [CrossRef]
42. Wambacq, E.; Vanhoutte, I.; Audenaert, K.; De Gelder, L.; Haesaert, G. Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: A review. *J. Sci. Food Agric.* **2016**, *96*, 2284–2302. [CrossRef] [PubMed]
43. Storm, I.; Rasmussen, R.R.; Rasmussen, P.H. Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. *Toxins* **2014**, *6*, 2256–2269. [CrossRef] [PubMed]