agronomy

MDPI

Article

Dynamic and Migration Characteristics of Soil Free Amino Acids in Paddy Soil Applied with Milk Vetch

Jing Yang [1], Yi Lin [1], Christopher Rensing [1], Liming Zhang [1,2], Biqing Zhou [1,2], Shihe Xing [1,2,*] and Wenhao Yang [1,2,*]

[1] College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, China
[2] University Key Laboratory of Soil Ecosystem Health and Regulation of Fujian Provincial, Fuzhou 350002, China
* Correspondence: fafuxsh@126.com (S.X.); whyang@fafu.edu.cn (W.Y.)

Abstract: To explore the attribution factors and migration characteristics of free amino acids (FAAs) in paddy soils after green manure application during the entire growth period of rice. Amino acid analyzer, quantitative PCR, and high-throughput sequencing were used to analyze the effects of different application rates of milk vetch on FAAs in paddy soil under equal nitrogen, phosphorus, and potassium conditions. Soil FAAs concentration at different growth stages was highest at the seedling stage and lowest at the tillering stage. The concentration of threonine, alanine, valine, isoleucine, leucine, and phenylalanine was most abundant during the growth period, accounting for 59.42–76.46% of the respective FAAs pool. The application of milk vetch was shown to increase the soil FAAs concentration, especially glutamic acid, which increased by 368.17–680.78%, but the excessive application had an inhibitory effect. Soil bacteria were critical factors affecting soil FAAs dynamics, among which Bacteroidetes, Firmicutes, and Nitrospirae contributed 56.89% to the FAAs dynamics. FAAs displayed significant vertical profile characteristics, and the mobility of serine, glycine, and proline was high. Conclusively, the application of milk vetch was able to significantly change the concentration and composition of soil FAAs, which were affected by soil bacteria.

Keywords: free amino acids; composition; paddy soil; bacterial community; structural equation modeling

Citation: Yang, J.; Lin, Y.; Rensing, C.; Zhang, L.; Zhou, B.; Xing, S.; Yang, W. Dynamic and Migration Characteristics of Soil Free Amino Acids in Paddy Soil Applied with Milk Vetch. *Agronomy* **2022**, *12*, 2621. https://doi.org/10.3390/agronomy12112621

Academic Editor: Monika Mierzwa-Hersztek

Received: 8 September 2022
Accepted: 24 October 2022
Published: 25 October 2022

1. Introduction

More than 90% of total soil nitrogen (N) is organic and, within the organic forms of N, amino acids are the most abundant [1]. Free amino acids (FAAs) are usually present in soil solutions and pores, which are less abundant but more readily absorbed by plant roots and microorganisms [2]. FAAs are mainly generated by the hydrolysis of soil protein and peptides using extracellular enzymes [3], exudation and death from plant roots [4], and microbial turnover and excretion [5]. These amino acids cycle very rapidly in soil, can be selectively absorbed by plants and microorganisms [6], and in addition, easily migrate and are subsequently lost [7]. The concentration of FAAs in soil depends on the production and consumption of FAAs, and a higher concentration is probably conducive to FAAs captured by plants [8]. Therefore, determining the bioavailable concentration of FAAs in the soil is crucial to our understanding of plant nitrogen acquisition and the soil nitrogen cycle [5]. It was reported that FAAs concentration varied greatly when comparing different ecosystems, and the concentrations of amino acids in the boreal forest, agricultural soil, and alpine meadow soils were 438.00–4867.00 ng N g^{-1} dry soil [9], 12.87–48.93 ng N g^{-1} dry soil [10] and under the detection limit [4], respectively. However, most current studies focused on the size of the pool of FAAs in upland soils, while very limited information is currently available looking at the concentration and composition of FAAs in chronically flooded paddy soils with high anthropogenic disturbance.

Fertilizers are the key source of available nitrogen in farmland ecosystems, and different types of nitrogen fertilizers generate a different composition of soil amino acids. The addition

of urea causes important microbial community changes and leads to cell lysis of some microbes and subsequently leads to the release of amino acids into the soil [10]. Plant residues applied to soil were shown to release amino acids and easily degradable proteins after decomposition, but different plant residues have different C/N ratios, and the way and time of the release of amino acids to soil are different. In 10 days of aerobic culture of fresh alfalfa residue extracts, a large proportion (29–100%, depending on culture temperature) of soluble N containing a variety of amino acids can be released [11]. In contrast, the protein contained in poor-quality residues, such as straw, must be digested by enzymes to release the amino acids it contains [12]. Although more and more attention has been paid to soluble organic nitrogen, the research on amino acids in agricultural soil is still lacking, and the existing research mainly focuses on the concentration of exchangeable amino acids. Chinese milk vetch (Astragalus sinicus L., CMV), a winter-grown legume plant with high N_2-fixing ability, is commonly planted as an alternative N source for chemical fertilizer in rice cropping systems in Southern China [13]. However, the effect of the application of CMV on the concentration and composition of free amino acids in paddy soil is still unclear.

Soil microorganisms play a critical role in nitrogen cycling by breaking down organic matter into amino acids and other small molecules of organic nitrogen through the mineralization processes, which can be taken up by plants or microbes [14]. Then, amino acids were mineralized via microbial ammonification, nitrification, and denitrification processes to inorganic nitrogen, ammonium (NH_4^+), and nitrate (NO_3^-) [14]. We still know considerably less about the metabolism of microorganisms and how this affects the presence and/or consumption of amino acids. In very general terms, the dynamic variation of FAAs may be due to temporal variations in soil microorganisms. This is because the concentration of FAAs in the soil is affected by absorption [6], release [5], and release of extracellular enzymes by microorganisms [3]. The temporal variation of microbial N may determine the soil reservoir in FAAs [15]. Lipson et al. [16] reported that the decrease in microbial biomass in alpine soils is correlated to an increase in pools of FAAs. Microbial biomass is a strong competitor for amino acids, which can regulate FAAs content [17]. However, as far as we know, the specific microbial communities that cause the dynamic changes of soil amino acids and their roles are still poorly understood.

In this study, four different treatments (chemical fertilizer, low amount of CMV, medium amount of CMV, and high amount of CMV) were chosen to test the following three hypotheses: (i) Soil FAAs could be affected by the application of CMV and is correlated to the application rate. (ii) The concentration and composition of soil FAAs will vary with rice growth stages. (iii) The dynamics of FAAs in paddy soil are closely correlated to soil bacterial community structure.

2. Materials and Methods

2.1. Experimental Site and Design

The experimental area was located in Minhou County (119°08′05″ E, 26°22′40″ N), Fujian Province, which is situated in the transition zone between the middle subtropical zone and the south subtropical zone. The annual average temperature is 19.5 °C, the annual sunshine hours are 1812.5 h, the frost-free period is about 311 days, and the mean annual average precipitation is 1350 mm. The soil was composed of gray-yellow paddy soil with loamy clay and the main initial chemical properties were as follow: pH 5.53, total organic matter17.65 g kg^{-1}, alkali-hydrolyzed nitrogen 29.95 mg kg^{-1}, available phosphorus 20.85 mg kg^{-1}, and available potassium 96.65 mg kg^{-1}.

Four equal quantities of nitrogen treatments were set up in this experiment, including chemical fertilizer (CK), low amount of CMV (15,000 kg hm^{-2}, CL), medium amount of CMV (30,000 kg hm^{-2}, CM), and high amount of CMV (45,000 kg hm^{-2}, CH) (Figure 1). Three replicate experimental plots (3 m × 4 m in size) per treatment were designed in a random block arrangement. The fertilizers applied in the experiment were urea at 481.67 kg hm^{-2}, superphosphate at 900 kg hm^{-2}, and potassium chloride at 300 kg hm^{-2}, within which 50% of urea and potassium chloride were used as base fertilizer on the 9th

day after CMV overturning and 50% those fertilizers were applied at the tillering stage of rice growth on the 25th day after CMV overturning, and all of the superphosphate was applied as the base fertilizer. The variety of CMV was Minzi No.7, with 90% water content, 752.75 g kg^{-1} of total organic matter, 30.94 g kg^{-1} of total nitrogen, 5.91 g kg^{-1} of total phosphorus, 32.47 g kg^{-1} of total potassium, 82.35 mg kg^{-1} of an acid-hydrolyzed amino acid (acid-hydrolyzed amino acid compositions shown in Figure S1), 193.40 g kg^{-1} of protein. Fresh CMV was harvested at the full flowering stage and immediately distributed evenly in the corresponding plots according to the application rates of each treatment (0, 15,000, 30,000, 45,000 kg hm^{-2}) and tilled into the topsoil. The insufficient part of nitrogen, phosphorus, and potassium in the treatment with CMV should be supplemented with chemical fertilizer when applying base fertilizer. Irrigation was carried out before rice planting and a certain flooded layer was maintained.

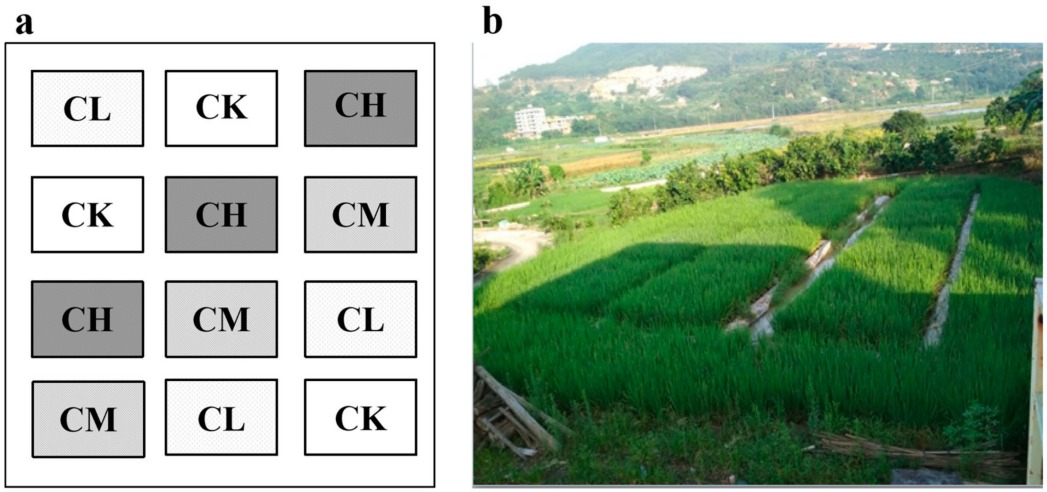

Figure 1. Distribution map (**a**) and field map (**b**) of the test plot. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV.

2.2. Soil Sampling

In this experiment, a multi-point sampling method was used to collect mixed samples of topsoil (0–20 cm) in each experimental plot with soil drill at 0d (background soil, B), 10d (seedling stage, S), 38d (tillering stage, T), 80d (flowering stage, F) and 122d (maturity stage, M) respectively after CMV application, according to the decomposition rule of rapid decomposition in early stage and gradually slowing down in late-stage and rice growth stage. Soil samples at 0–20 cm, 20–40 cm, and 40–60 cm in each plot were collected at the background and rice maturity stage. Soil samples at 0–20 cm, 20–40 cm, and 40–60 cm were successively extracted with a soil drill after a PVC pipe isolated the surface water. The mixed fresh soil samples were divided into three parts: one part was used for the analysis of soil FAAs and relative biochemical properties, another part was stored in −80 °C refrigerators for bacterial community analysis, and the other part was air-dried and sieved for determination of physical and chemical properties.

2.3. Analysis of Soil Chemical Properties

Soil pH was determined by a pH meter (PHS-3E, INESA Scientific Instrument Co., Ltd., Shanghai, China) in a 1:2.5 soil/water suspension. Soil organic matter (SOM) was measured by the potassium dichromate oxidation method [18]. Soil urease and protease activities were estimated by indophenol blue colorimetry and Folin colorimetry, respectively [19].

2.4. Analysis of Soil FAAs

Soil FAAs concentration and composition were analyzed using a water extraction-automatic amino acid analyzer method [7,20]. In brief, 10 g fresh soil was placed in a

100 mL triangular flask, 50 mL ultra-pure water was added and cultured in a 70 °C constant temperature oscillator for 18 h, then shaken for 5 min, and subsequently filtered through a 0.45 mm filter membrane. 10 mL filtrate and 0.25 g sulfosalicylic acid to was taken to deproteinate and centrifuged at 5000 r min^{-1} for 5 min. After adjusting the pH to 2.2, the filtrate was filtered with a 0.45 μm filter. 1 mL filtrate was absorbed and determined by an automatic amino acid analyzer (Biochrom 30+, Biochrom LTD., Cambridge, UK). A mixture of amino acids at the known concentration (Sigma Chemical Co., Milan, Italy) was used as the external standard (Standard spectra and concentrations are shown in Figure S2 and Table S1), and quantitative and qualitative analyses were carried out according to the peak time and peak area after gradient elution.

2.5. Analysis of Soil Bacterial Biomass and Community

2.5.1. Quantitative Fluorescent-PCR

The copy number of soil bacteria was determined by quantitative fluorescent-PCR analysis (Allwegene Company, Beijing, China), and the bacterial copy number was used to characterize the soil bacterial biomass. In brief, the soil DNA was extracted from 0.25 g of freeze-dried soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the instructions in the manual. The purity and quality of DNA have been checked on 0.8% agarose gels. The 16S rRNA gene of soil bacteria was amplified by real-time fluorescence, and the copy number of the 16S rRNA gene of the bacteria was determined. Fluorescence quantification was carried out by a two-step method. The specific conditions were 94 °C pre-denaturation for 5 min, (94 °C 30 s, 55 °C 30 s, 72 °C 30 s) × 30 cycles, and extension at 72 °C for 10 min after the end of the cycle. The standard gene was extracted as the standard for the preparation of the standard curve and the quantitative standard curve was established. These gene standards were generated from synthetic gene plasmid cloning vectors (Integrated DNA Technologies, Inc., Coralville, IA, USA) transformed into One ShotTM TOP10 Competent Escherichia coli (Life Technologies, Carlsbad, CA, USA) with the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany) which uses the pCRTM4-TOPO® TA vector. The cloned plasmids were subjected to amplification with primers and product sizes were verified with gel electrophoresis. The PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) and quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) [21].

2.5.2. Analysis of Bacterial Community Structure

Soil bacterial community structure was determined by high-throughput sequencing analysis (Allwegene Company, Beijing, China). DNA was extracted by using the MoBio Laboratories DNA kit. The V3-V4 region of 16S rRNA genes was amplified by PCR using the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) [22]. The PCR was carried out on a Mastercycler Gradient (Eppendorf, Germany) with the following program: 95 °C for 5 min, 32 cycles of 95 °C for 45 s, 55 °C for 50 s, and 72 °C for 45 s. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) before quantification and sequencing. After sequencing, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline (Version 2.6). The sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97% [23] to generate rarefaction curves and calculate the richness and diversity indices. The Ribosomal Database Project (RDP) Classifier tool was used to classify all sequences into different taxonomic groups [21].

2.6. Structural Equation Modeling Analysis

Structural equation modeling (SEM) includes confirmatory factor analysis and regression or path analysis [24,25], which allows for both the direct and indirect theoretical causal relationships between inter-correlated variables to be tested, and for potential multivariate relationships to be identified [26]. SEM analysis was used to analyze the main impact factors and their pathways affecting FAAs in paddy soil. In our study, six observable variables

(pH, SOM, protease, urease, bacterial biomass, bacterial community), and FAAs dynamics in paddy soil in 0–20 cm soil layer after CMV application were used to construct the SEM (Figure S3). According to the path significance level (Tables S2 and S3) and model fit index (Table S4), the modified model (Figure S4) and path coefficient were obtained (Table S5). In the model, the Maximum Likelihood method was used to estimate the parameters, and *p*-values and chi-square test ($\chi 2$) were used to assess the general model fit because the respective *p*-values (*p*-values > 0.05) associated with the model chi-square are used to judge the fit between model and data [27]. Several indices are also used to evaluate the ideal model, including the relative fit index (RFI), root mean square error of approximation (RMSEA), normed fit index (NFI), tucker-lewis index (TLI); comparative fit index (CFI) and incremental fit index (IFI). Except for RMSEA which is less than 0.05, the value of these indices close to 1 indicates a good fit [26,28].

2.7. Statistical Analysis

All statistical analysis and correlation analyses were performed using SPSS 19.0 or Excel 2007, and data plots were obtained using SigmaPlot 12.0 and R 3.5.1 software. Analysis of variance (ANOVA) and Least significant difference (Duncan, $p < 0.05$) analysis were used to separate the means with significant differences. The SEM was used to study the impact path and effect of driving factors on FAAs dynamics by AMOS 21.0, the Mantel test was used to explore the impact of the bacterial community on FAAs dynamics by PASSaGE 3.0 and the variance partition analysis (VPA) was used to quantify the contribution rate of the major bacterial communities to FAAs by the "Vegan" program in R 3.5.1 statistical software.

3. Results

3.1. Dynamics of FAAs Concentration

The temporal variations in the concentration of FAAs displayed a similar pattern under different fertilization treatments (Figure 2). The concentration of FAAs increased rapidly under different fertilization treatments and reached a peak at the seedling stage. The FAA concentrations of CK, CL, CM, and CH treatments at the seedling stage were increased by 28.77%, 11.34%, 31.64%, and 38.58%, respectively, compared to the background soil. The concentration of FAAs of CK, CL, CM, and CH treatments decreased rapidly from the seedling stage to tillering stage, and tillering stage only accounted for 22.94%, 50.57%, 49.95%, and 47.76% of the seedling stage, respectively. The concentration of FAAs under CK, CL, CM, and CH treatment gradually increased after the tillering stage and reached the second peak in the flowering stage, and increased by 185.06%, 44.21%, 21.71%, and 26.92% respectively compared with the tillering stage. After the flowering stage, the concentration of FAAs decreased gradually, and the concentration of FAA in the treatments of CK, CL, CM, and CH at the maturity stage only accounted for 55.54%, 62.61%, 66.45%, and 65.82% of the flowering stage. The application of CMV increased soil FAAs concentrations, but the excessive application has a certain inhibitory effect. In both background soil and seedling stage, only CM treatment significantly increased compared with CK treatment. Compared to CK treatment, the FAAs concentration under CL, CM, and CH treatments significantly increased by 131.42%, 182.02%, and 133.30% at tillering stage, and increased by 31.99%, 44.06%, and 23.11% at the maturity stage. However, there was no significant difference among different treatments at the flowering stage.

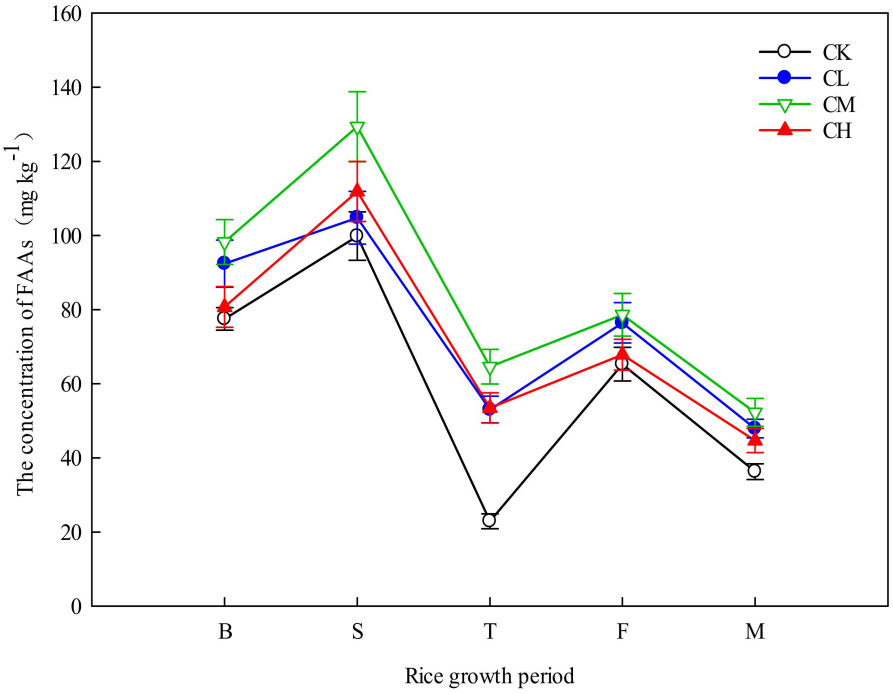

Figure 2. Dynamics of soil FAAs concentration under different fertilization treatments during different rice growth stages. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, B: background soil, S: seedling stage, T: tillering stage, F: flowering stage, M: maturity stage. The error line indicates a standard error (n = 3).

3.2. Dynamics of FAAs Composition

The FAAs composition under different fertilization treatments in different rice growing periods was diverse, with 15 neutral amino acids and 2 acidic amino acids detected (Figure 3). The concentration dynamics of neutral amino acids and acidic amino acids under different fertilization treatments followed a similar temporal pattern as that of the FAAs concentration, accounting for 95.74–100% and 0–4.26% of the FAAs pool respectively. Acidic amino acids were present only in the background, seedling stage, and flowering stage. The concentration of individual FAAs followed a similar pattern to that of the amino acid composition. During the different rice growth periods, different fertilization treatments had similar FAAs pools, in which threonine, alanine, valine, isoleucine, leucine, and phenylalanine were the most abundant, and accounted for 59.42–76.46% of the amino acid pools. In general, CMV application could significantly increase the concentration of amino acid components, especially glutamic acid, which increased by 368.17–680.78%.

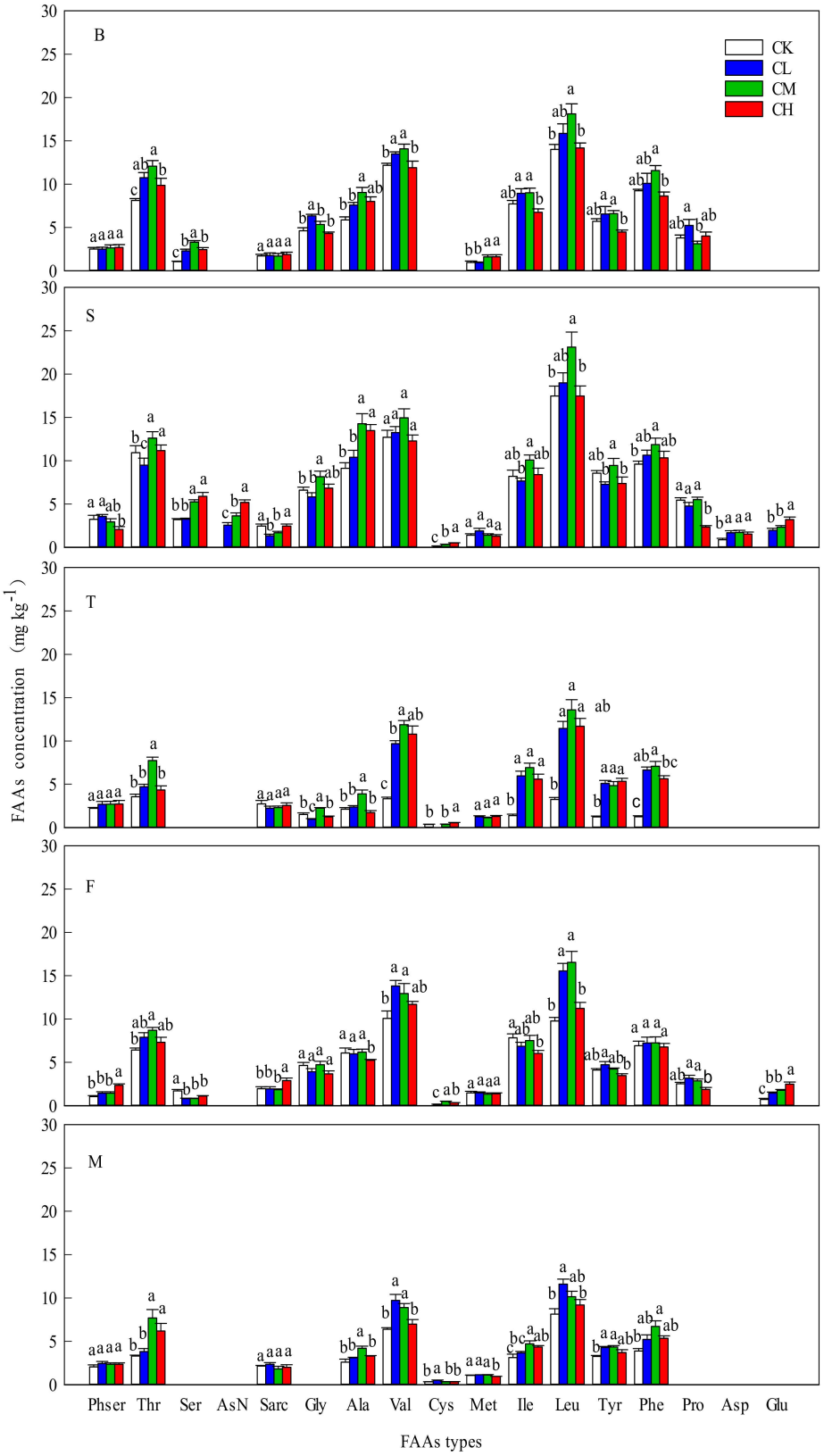

Figure 3. Dynamics of soil FAAs composition under different fertilization treatments during the rice growth stage. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, B: background soil, S: seedling stage, T: tillering stage, F: flowering stage, M: maturity stage. The error line indicates a standard error (n = 3). Lowercase letters represent significant differences between treatments ($p < 0.05$, Duncan test).

3.3. Profile Distribution of FAAs

The concentration and type of soil FAAs in the background and maturity stage decreased with increasing soil layers (Figure 4). In the 0–20 cm soil layer, the FAAs concentration was background > maturity stage, while in the 20–40 and 40–60 cm soil layer, the FAAs concentration was maturity stage > background, indicating that FAAs displayed a downward migration trend. The application of CMV increased the accumulation of FAAs in the soil profile. Compared to CK, the application of CMV increased the FAAs concentration in 0–20 cm, 20–40 cm, and 40–60 cm soil layers by 9.21–22.86%, 25.90–44.19%, and 7.05–13.92%, respectively, during the different rice growth stages. More types of FAAs migrated to the 20–40 cm soil layer. Compared to the background, the FAAs increased serine, glycine, and proline in the 20–40 cm soil layer and increased proline in the 40–60 cm soil layer at the maturity stage, indicating that serine, glycine, and proline displayed strong migration.

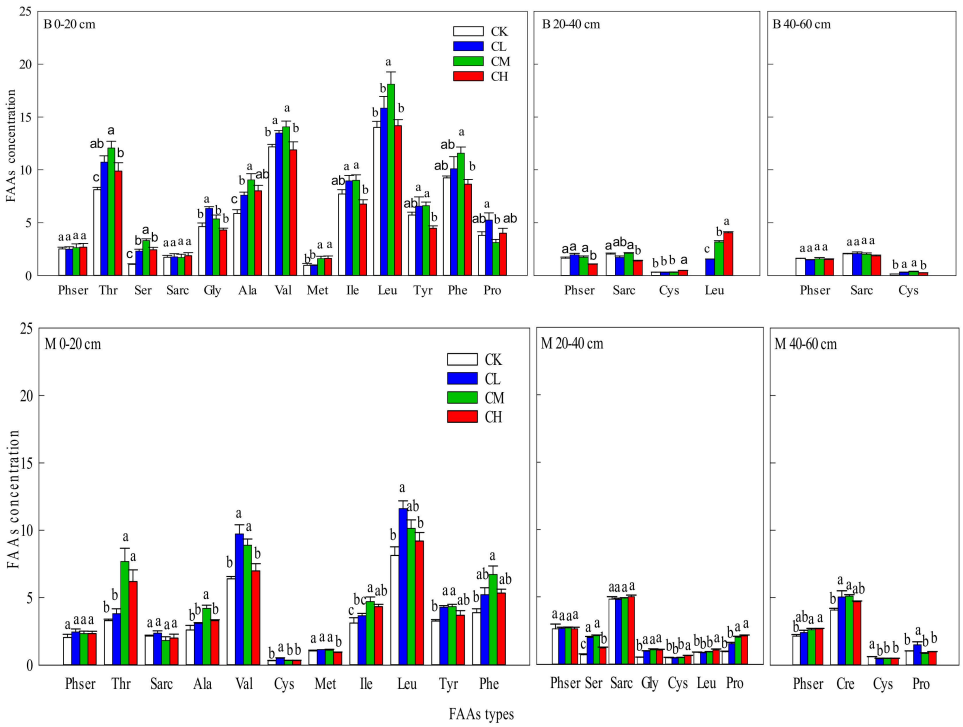

Figure 4. FAAs Profile distribution of different fertilization treatments in the rice growth stage. CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, Note: B: background soil, M: maturity stage. The error line indicates a standard error (n = 3). Lowercase letters represent significant differences between treatments ($p < 0.05$, Duncan test).

3.4. Dynamics of Soil Bacterial Community during the Rice Growth Stage

3.4.1. Soil Bacterial Biomass and Diversity

The copy number of soil bacteria and Chao1 index under different fertilization treatments in different rice growing stages increased from the background, decreased after the seedling stage and reached a low point in the tillering stage, then increased rapidly to the highest point in the flowering stage and then decreased rapidly (Figure 5). The copy number of soil bacteria at the maturity stage only accounted for 24.74–28.68% of the background soil ($p < 0.05$). The application of CMV can increase the copy number of soil bacteria and Chao1 index, but the excessive application has a certain inhibitory effect. The mean value of soil bacterial copy number and Chao1 index in rice growth period under different application rates of CMV increased by 13.95–25.14% and 2.41–4.97% compared with CK treatment, respectively. However, there was no significant difference in the Shannon index between different fertilization treatments during the different rice growth periods.

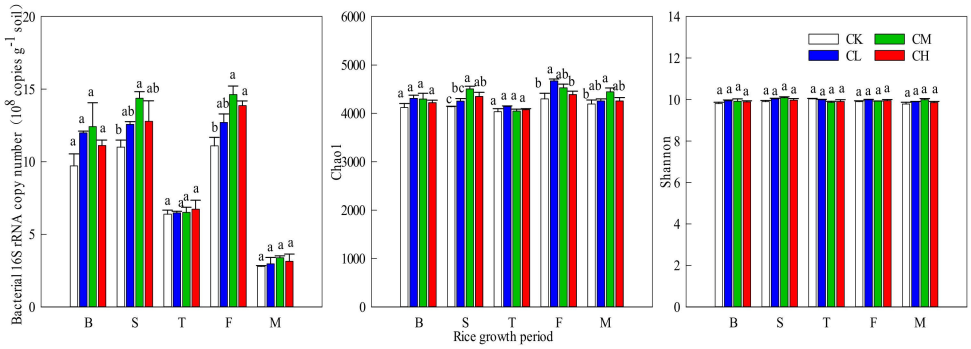

Figure 5. Dynamics of the copy number of soil bacteria and alpha diversity under different fertilization treatments during the different rice growth stages. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, B: background soil, S: seedling stage, T: tillering stage, F: flowering stage, M: maturity stage. The error line indicates a standard error (n = 3). Lowercase letters represent significant differences between treatments ($p < 0.05$, Duncan test).

3.4.2. Soil Bacterial Community Structure

The phyla Chloroflexi, Acidobacteria, and Proteobacteria occupied 69.75–73.96% of the bacterial sequences obtained from different fertilization treatments and were followed by Actinobacteria (2.98–5.96%), Planctomycetes (3.06–4.47%), Bacteroidetes (2.18–3.87%), Nitrospirae (1.60–3.70%), Verrucomicrobia (1.88–3.36%), Latescibacteria (1.33–2.62%) and Firmicutes (0.71–3.97%) (Figure 6a). The composition and structure of the bacterial community were shown to be significantly changed at different growth stages of rice. PCA analysis showed that the contribution rate of the first axis and the second axis was 13.20% and 8.59% respectively (Figure 6b). The treatments were separated along the first axis reflecting the different growth periods of rice. Different treatments in the same growth stage were clustered together, and different growth periods were far apart, especially the tillering period and other growth periods were far apart, indicating that the bacterial community structure at the tillering stage was significantly different from other growth stages.

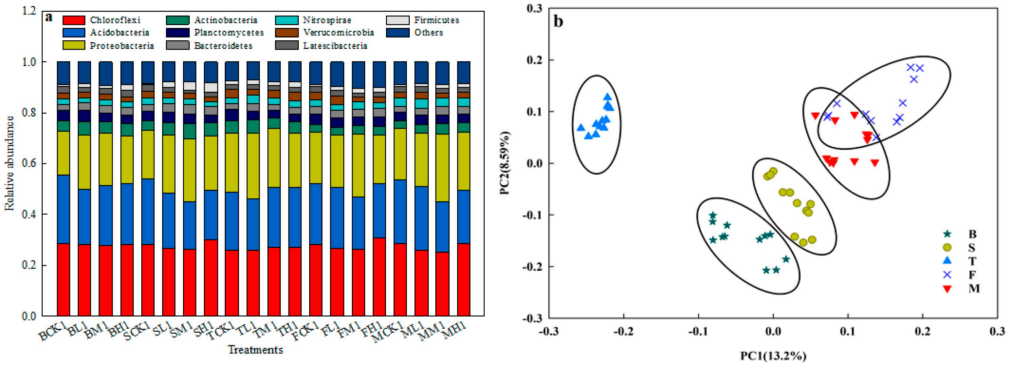

Figure 6. Relative abundance of bacterial taxa at the phylum level (**a**) and PCA plot of soil bacterial community distribution (**b**) under different fertilization treatments during the different rice growth stages. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, B: background soil, S: seedling stage, T: tillering stage, F: flowering stage, M: maturity stage.

3.5. Impact Factors of FAAs Dynamics during the Rice Growth Stage

3.5.1. Influence Factors and Paths of FAAs Dynamics

In this study, pH, SOM, urease, protease, bacterial biomass, and bacterial community indicators were measured (Figure 7) as impact factors of FAAs. The reliability coefficients and the overall fit indices of the second fitting fall within an acceptable range, with

χ^2/df = 0.500, RFI = 0.971, RMSEA = 0, NFI = 0.996, TLI = 1.031, CFI = 1.000, IFI = 1.004, indicating that the model was successful. The SEM analysis showed that pH, SOM, protease, bacterial biomass, and bacterial community accounted for 83% of the variations in soil FAAs dynamics during the rice growth stage (Figure 8). Soil pH, SOM, protease, bacterial biomass, and bacterial community had direct effects on FAAs, and their standard path coefficients were −0.43, 0.52, 0.71, 0.49, and −0.72, respectively. Meanwhile, pH and SOM could indirectly affect FAAs through protease, bacterial biomass, and bacterial community, and the standardized indirect effect was 0.65 and 0.12. Soil bacterial biomass and bacterial community could indirectly affect FAAs through protease, and the standardized indirect effect was 0.22.

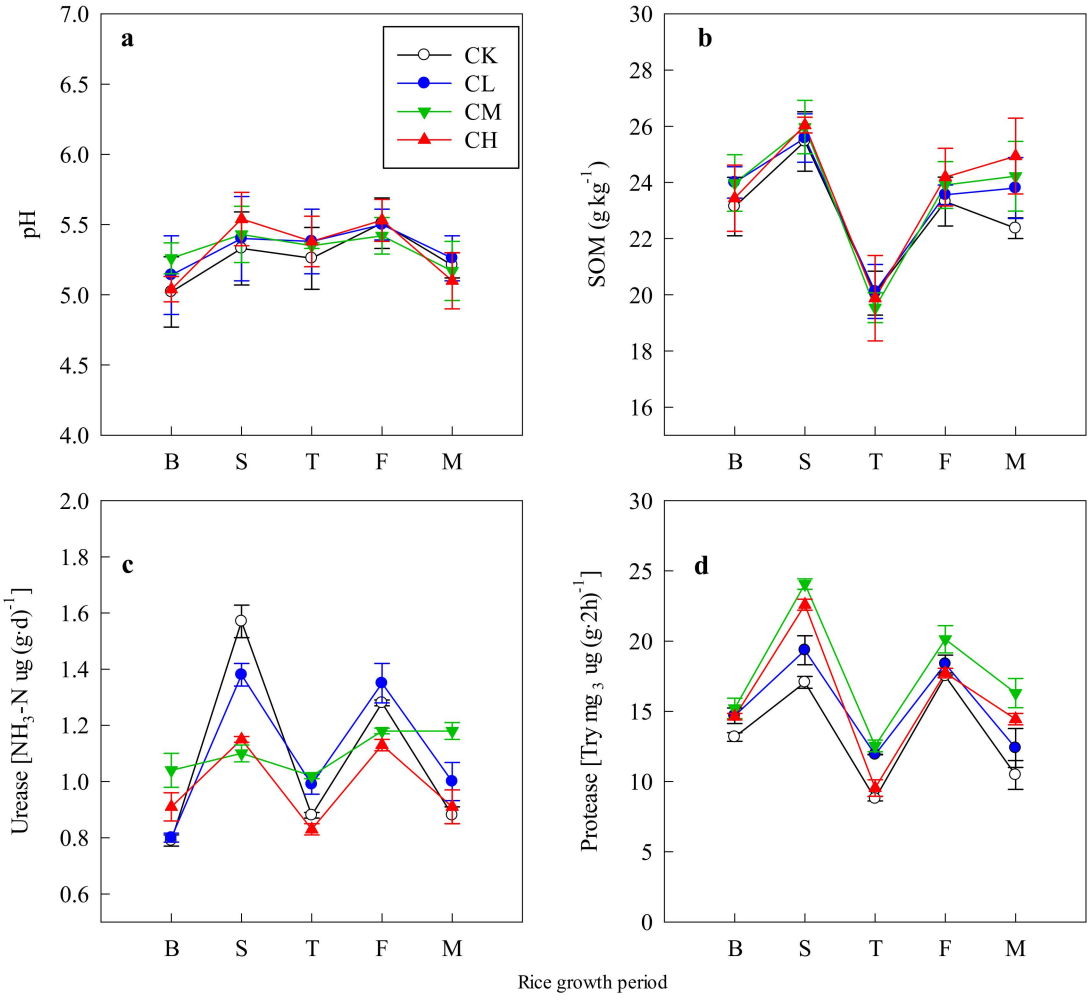

Figure 7. Soil pH (**a**), SOM (**b**), urease activities (**c**), and protease activities (**d**) under different fertilization treatments during different rice growth stages. Note: CK: chemical fertilizer, CL: the low amount of CMV, CM: the medium amount of CMV, CH: the high amount of CMV, B: background soil, S: seedling stage, T: tillering stage, F: flowering stage, M: maturity stage. The error line indicates a standard error (n = 3).

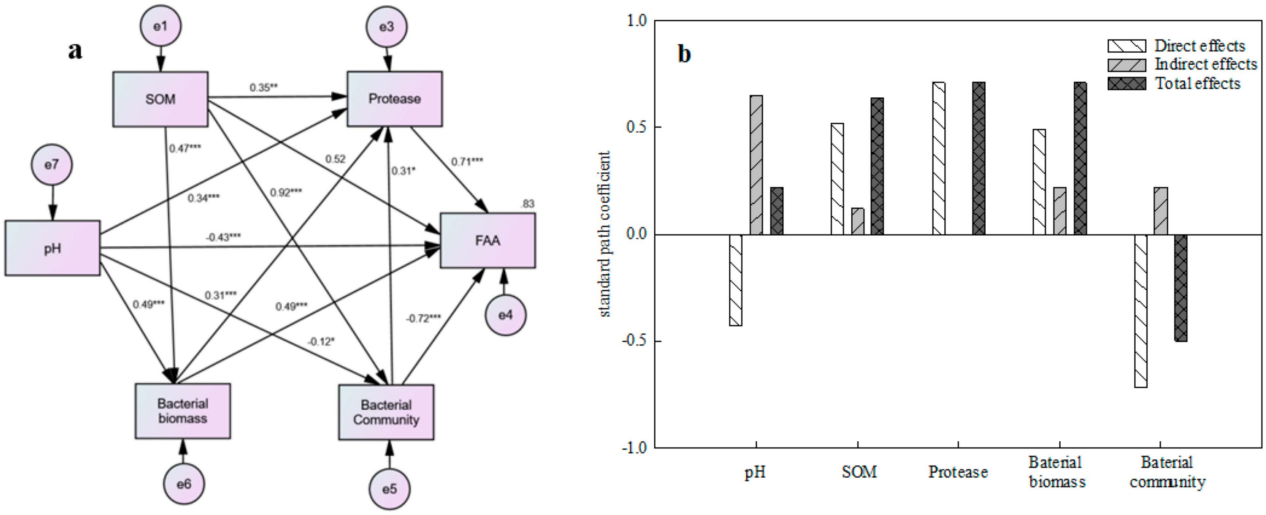

Figure 8. Structural equation modeling (**a**) and standard path coefficient (**b**) of impact factors on soil FAAs during the rice growth stage. Note: The values next to the arrow are standard path coefficients (also known as regression coefficients), *, **, *** Significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, e1–e6: errors 1–6.

3.5.2. Key Bacterial Communities of FAAs Dynamics

The results of Mantel test results showed that Bacteroidetes, Firmicutes, and Nitrospirae had a significant influence on FAAs dynamics of different fertilization treatments during the rice growth stage, and the correlation coefficients were 0.40, 0.30, and −0.26 respectively. The VPA analysis showed that the relative abundance of Bacteroidetes, Firmicutes, and Nitrospirae contributed 22.99%, 22.18%, and −0.42% of the variation of SON content, respectively (Figure 9). The contribution rate of the interaction of the three major effects to SON content variation was 17.27%, and the total contribution rate of each variable and its interaction with SON concentration variation was 56.89%.

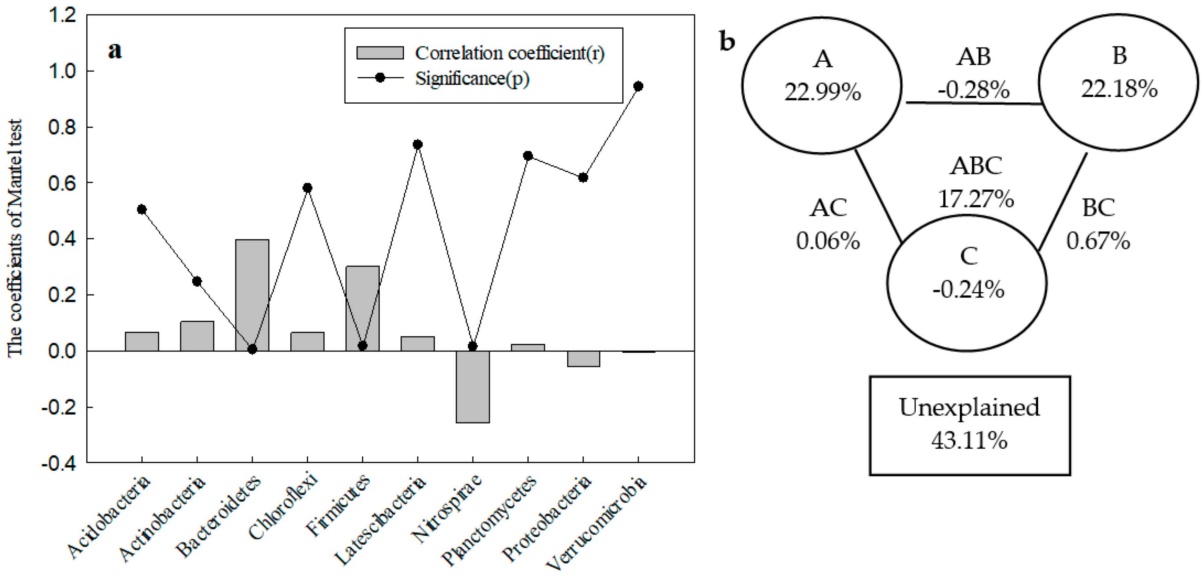

Figure 9. Mantel test (**a**) and variance partition analysis (**b**) between bacterial phyla and soil FAAs dynamics of different fertilization treatments during the rice growth stage. Note: A: Nitrospirae, B: Bacteroidetes, C: Firmicutes.

4. Discussion

4.1. Effect of CMV Treatments on Soil FAAs Concentration Dynamics

The production and degradation of FAAs in the soil is a dynamic process, influenced by the presence of plants, soil microorganisms, and enzyme activity. The content of FAAs in soil with CMV increased rapidly to peak value and then quickly decreased to the lowest value, thereafter gradually increasing to a second peak value at the flowering stage and then gradually decreasing (Figure 2). This result is consistent with the following four stages of rapid decomposition, the rapid decline of decomposition rate, accelerated decomposition, and slow decomposition after CMV application [29]. The FAAs concentration in the soil reached a peak at the rice seedling stage, indicating that the turnover rate of CMV was extremely fast, which may be due to the low C/N value of CMV (14.11). Moreover, the rapid decomposition of CMV after overturning to was shown to produce a large amount of FAAs. Furthermore, the addition of fresh organic matter not only directly provides a large amount of energy and nutrients for soil microorganisms, but also provided nutrients and energy for the reproduction of soil microorganisms through the priming effect [30]. In addition, the addition of fresh organic matter improved soil protease activity, bacterial biomass, and diversity (Figures 5 and 7), thereby promoting the decomposition of macromolecular organic matter into FAAs. The FAAs concentration in the soil quickly dropped to a minimum from the seedling stage to tillering stage. The reason was partly due to this stage belonging to a period of rapid decline in the decomposition of CMV and the source of FAAs decreasing [29]. On the other hand, this stage was a flourishing stage for rice growth, and the enhanced nutrient uptake ability of rice roots, which competed with microorganisms for inorganic nitrogen and small-molecule organic nitrogen [31], leading to a reduction in bacterial biomass (Figure 4) and protease activity (Figure 7), which led to a decrease in soil FAAs content. Along with the above observations, we also found that the soil FAAs concentration at the maturity stage under different fertilization treatments was lower than that of the background soil (Figure 2). This may be because the background soil under aerobic conditions, which is conducive to the mineralization of organic nitrogen, releases more amino acids [32]. At the same time, the maturity soil is flooded and the number of bacteria is therefore reduced (Figure 5), which in turn is not conducive to the production of FAAs. Aerobic systems generally display a more metabolically active microbial community than anaerobic systems [11]. In addition, amino acids are highly mobile and tend to migrate to the bottom layer (Figure 4), resulting in a decrease in the concentration of amino acids in surface soil at the maturity stage.

The application of CMV can continuously bring a large amount of nitrogen-containing organic matter into the soil and significantly increase the soil FAAs content. The protein and amino acid concentrations of the tested CMV were 193.4 g kg^{-1} and 82.35 mg kg^{-1}, respectively, and the Fourier infrared spectroscopy results showed that the main components of the tested CMV were carbohydrates, phenolics, protein, lignin, and aliphatic compounds, etc. (Figure S1). These nitrogen-containing substances were depolymerized into small molecule FAAs by depolymerization catalyzed by microorganisms and proteases [33], so the concentration of FAAs in the soil was higher than the concentration under CK treatment. In addition, the application of fresh green manure increased soil active organic carbon and nitrogen, thereby changing the soil bacterial activity and community composition, promoting the decomposition of CMV and the secretion of rice roots [28], thereby contributing to the increase of FAAs concentration. The results of this study showed that a high dose application of CMV was not conducive to the accumulation of FAAs, mainly because excessive application of CMV to the soil produced a large number of organic acids and inhibited the growth of microorganisms [34]. In addition, excessive application of CMV was shown to reduce soil Eh, and produce a large amount of reducing substances such as Fe^{2+}, Mn^{2+}, H_2S, etc. [35], leading to a decrease in bacterial biomass (Figure 5), which is consistent of the experimental results of Cheng et al.'s [36] studying the effect of applying different concentrations of CMV is having on soluble organic nitrogen.

4.2. Effect of CMV Treatments on Soil FAAs Composition Dynamics

The different concentrations of acidic amino acids and neutral amino acids displayed similar time dynamics as did different concentrations of FAAs in paddy soil, which was in part consistent with the results that amino acids have temporal dynamics in the temperate grassland [17], temperate forest [2] and alpine meadow soil [4]. In these studies, in addition to neutral amino acids, both acidic and basic amino acids also accounted for a distinct proportion of the soil amino acid pools. However, in our study, only neutral amino acids and acidic amino acids were detected under different fertilization treatments during the different rice growth periods (Figure 3). The different compositions of individual amino acid pools in these studies may be ascribed to the difference between the free and the exchangeable pool of amino acids [37]. The free amino acid pool consists mainly of acidic and neutral amino acids that have been extracted by water or weak salts, whereas the exchangeable amino acid pool has been shown to include a larger proportion of basic amino acids since these were absorbed into the soil solid phase and could only be extracted with strong salts [17]. The difference in soil pH also led to a difference in amino acid composition in different studies [4]. The test site in our study was located in the subtropical zone, where desilication and iron-rich aluminization in the process of soil formation resulted in the acidity of the tested paddy soil (pH value is 5.02–5.51). Under acidic conditions, the chemical stability of basic amino acids was low and these were easily degradable, so no basic amino acids were detected in the tested soil [38].

The pool of FAAs was dominated by threonine, alanine, valine, isoleucine, leucine, and phenylalanine under four fertilization treatments during different rice growth periods. The finding that similar amino acids dominated under four different fertilization treatments is predicted to be due to soil amino acids originating from similar biochemical processes [9]. Hence, except for valine, these amino acids have previously been shown to be among the most abundant amino acids in vegetable soil and alpine meadow soil [4,10]. Alanine and leucine are abundant in microbial cell walls [4,15], and threonine is secreted into the soil by rice roots [39]. The presence of large amounts of tryptophan in paddy soil is somewhat unusual and may be related to the decomposition of organic matter. Increases in the abundance of glycine, alanine, and threonine have been found during organic matter decomposition and are thought to be linked to their abundance in recalcitrant structures [40]. Gonzalez Perez et al. [10] showed that the production and mineralization of phenylalanine were correlated to the presence of isoleucine and leucine, and the variation trend was similar. Glycine was also shown to be one of the most abundant amino acids in agricultural soils, and its concentration increased during the process of organic decomposition [1]. Glycine is believed to be abundant in bacterial, fungal, and plant cell walls [40]. However, in this study, the concentration of glycine was higher in the background soil and seedling stage, but was not detected in the maturity stage which may be correlated to the strong migration of glycine (Figure 4).

The decomposition of organic matter is one of the direct sources of soil amino acids [41]. This study showed that the application of CMV was able to significantly increase the acidic FAAs in soil. This is due to the high amounts of acidic amino acids (aspartic acid and glutamic acid) in CMV (Figure S1). After being applied to the soil, the amount of aspartic and glutamic acid in the soil was significantly increased under the catalytic activity mediated by microorganisms and proteases [33]. Moreover, another reason for the increased presence might be that the addition of exogenous organic materials increased soil microbial biomass [42]. Soil FAAs were secreted and autolyzed products of microorganisms, and their cell walls have been shown to be rich in glutamine, glutamate, aspartic acid, and asparagines [43]. In addition, a high concentration of acidic amino acids was found to correlate with the organic matter concentration and improved the availability of nitrogen [9]. Studies have shown that polar amino acids have a high degree of mineralization [44], and the concentration of polar glutamic acid and aspartic acid increased after applying CMV, which increased the availability of nitrogen.

4.3. Effect of CMV Treatments on Soil FAAs Migration

FAAs displayed significant vertical distribution characteristics in the soil profile. CMV application was shown to significantly improve the concentration and composition of FAAs in all soil layers, especially in the 0–20 cm soil layer (Figure 4). This finding is mainly caused by the distribution of CMV and rice roots in the 0–20 cm soil layer [2,45]. The tested CMV contained a large amount of protein (193.4 g kg^{-1}) and acid-hydrolyzed amino acid (82.35 mg kg^{-1}), which were mainly accumulated in the surface soil after being applied to the soil, providing abundant nutrients and energy for microbes in the 0–20 cm soil layer, thus improving the mineralization rate of organic material [28]. In addition, the application of CMV is beneficial to the growth of rice and promotes the increase of root exudates [46], which have been shown to be one of the important sources of soil FAAs [47]. On the other hand, the vertical profile characteristics of FAAs are correlated to the adsorption of FAAs by soil. Fischer et al. [48] had previously shown that the mobility of FAAs in the soil is affected by the process of soil adsorption. The test soil in this study was clay with an abundance of surface charges, which displayed a strong absorption of FAAs [49], leading to a relatively weak infiltration of FAAS. Moreover, the plough pan of paddy soil is 20 cm below the surface, which has an intercept effect on water infiltration [50], so only part of the FAAs migrated to the bottom.

Different types of soil FAAs have different properties, which makes their migration characteristics in soil different. This study showed that glycine, serine, and proline displayed strong mobility (Figure 4), which may be correlated to the adsorbed amount of amino acids in soil. As neutral amino acids, glycine, serine, and proline were not easily adsorbed by soil colloids and displayed strong mobility in the soil profiles [51]. Glycine is the amino acid with the smallest molecular weight and the simplest structure in soil, therefore making it easy to migrate in the soil profile [52], while serine, as a hydrophilic amino acid, can easily be dissolved in the soil solution and migrate to the deep soil under the influence of gravity [53]. Proline, as one of the most water-soluble amino acids [54], easily migrates downward with soil water and can migrate to the 40–60 cm soil layer during the growth period of rice. In addition, the distribution of amino acids in the profile is closely correlated to their properties. The degree of mineralization of polar amino acids is higher than that of non-polar amino acids [44]. Therefore, the degree of mineralization of proline in the tested soil as a non-polar amino acid is lower and its turnover rate is slower, thus becoming one of the main components of amino acid migration in the soil profile.

4.4. Factors Driving Soil FAAs Dynamics

Previous studies have shown that pH, SOM, enzyme activities, and bacterial community were important factors affecting soil FAAs dynamics [4,10,55]. In this study, the results of SEM analysis showed that pH, SOM, protease, bacterial biomass, and bacterial community accounted for 83% of the variations in soil FAAs dynamics (Figure 8), which were the important factors affecting the variations in soil FAAs dynamics during the rice growth period.

The pH value is one of the main factors affecting nitrogen conversion. The pH value was shown to affect the absorption of amino acids by soil colloids and directly affect the concentration of free amino acids in soil [48]. The results of the SEM in this study showed that pH had a direct influence on the soil FAAs concentration, and the standardized path coefficient was −0.43, which may be correlated to the variable charge in amino acids [41,56]. Amino acids are amphoteric electrolytes, which usually carry a positive charge in acidic soil. CMV contains high ash alkali, which can neutralize soil acidity and improve soil pH when applied to acidic soil [57]. With an increase in pH (Figure 7), amino acids usually carry a net neutral charge or negative charge. They were shown to interact weakly with soil colloids and were easily lost from the soil, leading to a decrease in soil FAAs content. Fischer et al. [48] found that about 10% of amino acids were adsorbed to the soil solid phase, and this value strongly varied depending on the soil pH. Soil pH is considered to be a primary control of microbial activity and enzyme kinetics, as it strongly influences

both nutrient availabilities and enzyme denaturation and folding [58,59]. The results of the structural equation in this study also showed that the pH could indirectly affect FAAs concentrations through protease, bacterial biomass, and bacterial community structure, which might be due to pH affecting the decomposition of soil soluble organic matter to produce FAAs by affecting soil bacterial enzyme activity [60]. Li et al. [61] also showed that soil pH could indirectly affect the mineralization of soil organic nitrogen by affecting microbial biomass and enzyme activity.

Soil organic matter is an important source of soil FAAs and is closely correlated to FAAs concentration [48]. The results of the SEM in this study showed that organic matter is an important factor affecting the FAAs concentration after applying CMV with a total effect of 0.64. This is predicted to be due to an increase in soil organic matter content after CMV addition [62], which can increase soil microbial enzyme activities [28], promote the decomposition and transformation of soil organic matter, and increase the source of FAAs [63]. This is consistent with Kieloaho et al. [64] who found that after OM decomposition, released organic N forms, including proteinaceous material, can be degraded into smaller units that can be utilized by the majority of soil organisms and plants. In addition, the increase in soil organic matter content was shown to improve the adsorption of FAAs and reduce their migration [48].

Soil enzyme activity is the main driving factor of organic matter decomposition and nitrogen transformation in soil, which can directly affect the availability of soil nitrogen [65,66]. The application of CMV has been shown to release enzymes into the soil, provide energy and nutrients for soil microorganisms, improve soil enzyme activity, and promote the decomposition of organic matter [67]. Similarly, in our study, soil protease activity increased significantly after the application of CMV (Figure 7), and protease had an important influence on the soil SON concentration, with path coefficients of 0.71. This is mainly because protease plays a decisive role in the process of protein hydrolysis and conversion, and is an important enzyme in the process of hydrolysis of soil organic nitrogen into amino acids [68–70]. Generally, complex organic polymers with large molecular weights have been shown to be released after CMV application into the soil, which was difficult to be utilized by microorganisms. Therefore, large molecular weight organic nitrogen needs to be depolymerized by enzymes to release small molecular weight organic nitrogen (e.g., amino acids and amino sugars) before they can be absorbed by plants [71].

Soil microbes play an important role in the nitrogen cycle by driving global soil nitrogen mineralization and availability [61]. Our study showed that soil bacterial biomass increased significantly after the application of CMV, which is similar to a previous study that showed that turning over CMV can create good organic substrates and carbon resources for soil microorganisms to promote microbial growth and significantly increase soil microbial biomass [45,72]. Microbial decomposition of the soil SOM is considered to be a major factor controlling the amount of soluble OM retained in soil [73]. The results of the SEM in this study showed that the bacterial biomass could directly affect the soil FAAs content, and the path coefficient was 0.49, which was mainly due to the fact that bacteria were the producers of FAAs generated by the decomposition of organic matter [74]. Secondly, bacterial secretion and death were determined to be one of the sources of FAAs, with the most abundant component of bacterial cells being proteins consisting of amino acid chains [75]. The cell walls of microorganisms contain a large proportion of alanine, aspartate, and glutamic acid [43], and a considerable amount of amino acids will be released after bacterial apoptosis [76]. The amino acids in soil are mainly derived from the hydrolysis of proteins by extracellular enzymes [69]. The SEM results of this study also indicated that bacteria can indirectly affect the soil FAAs concentration by affecting the protease activity, with a path coefficient of 0.22. Similar findings were also reported by Hofmockel et al. [77] who found that extracellular enzymes (protease) produced by the microbial community were one of the important factors in the rate of proteolysis.

The composition of the microbial community was closely correlated to the decomposition of organic matter [78]. Gao et al. [79] showed that the functional microbes which play

important roles in the soil nitrogen (N) cycle changed after years of application of Chinese milk vetch, thus affecting organic nitrogen mineralization. The results of the SEM in this study showed that the bacterial community structure could directly impact the soil FAAs concentration, and also indirectly influence the FAAs concentration through the catalytic activities of proteases. This was due to bacteria not only being the source of amino acids but also absorbing amino acids to meet the needs of life. Therefore, the influence on the amino acid pool is a comprehensive action of the two processes [80]. Interestingly, the direct influence path coefficient of bacterial community structure on FAAs was −0.72, indicating that the bacterial community structure was closely correlated to FAAs mineralization, and amino acid mineralization was an important amino acid consumption pathway [81]. Different bacterial community compositions had a direct influence on FAAs dynamics. The results of our studies showed that the phylum of Nitrospirae, Bacteroidetes, and Firmicutes significantly affected FAAs dynamics, and their contribution rates were 22.99%, 22.18%, and −0.42%, respectively. Nitrospirae phylum was one of the main bacteria affecting soil nitrification, which was shown to reduce the concentration of ammonium nitrogen in the soil and promote the mineralization of amino acids [32,82]. The genus of *Cytophaga* and *Flavobacterium* in the Bacteroidetes phylum and *Bacillus* genus in the Firmicutes phylum were reported to be the dominant proteolytic bacteria in many soils, and they all secreted metalloproteinases to promote the formation of FAAs [83]. The results showed that neutral metallopeptidase secreted by *Bacillus subtilis* and alkaline serine protease secreted by *Bacillus subtilis* was shown to be proteases that control the degradation of peptides in the paddy soil [84]. Therefore, bacteria may directly affect the mineralization of FAAs through Nitrospirae, and indirectly affect the production of FAAs through Bacteroidetes and Firmicutes controlling protease activity.

5. Conclusions

As one of the important nitrogen sources for plants and microorganisms, FAAs have an important impact on soil nitrogen supply and the nitrogen cycle. This study provides new insights into FAAs pools in paddy soil and the effects of CMV application on FAAs dynamics and profile differentiation. Overall, the concentration and composition of FAAs in paddy soil were significantly affected by the input of green manure and the growth period of rice. Soil FAAs displayed temporal dynamics during the rice growth period, with the pool size increasing from the background to the peak level in the seedling stage and then rapidly decreasing to the lowest level in the tillering stage, then increasing gradually to a second peak in the flowering stage and then decreasing gradually. The application of CMV was shown to increase soil FAAs concentrations and types, but the excessive application was shown to have an inhibitory effect. Therefore, 30,000 kg hm^2 was determined to be a more appropriate application amount in paddy soil. Four different treatments shared six dominant amino acids in the different rice growth periods. Neutral amino acids, especially serine, glycine, and proline displayed a strong migration. The relationships between FAAs and environmental factors in the farmland ecosystem are multifaceted and complex, mainly being affected by pH, SOM, protease, bacterial biomass, and bacterial community. The phylum of Bacteroidetes, Firmicutes, and Nitrospirae were the main bacteria that were shown to affect the dynamics of FAAs, and the total contribution rate of the three bacteria reached 56.89%.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112621/s1, Figure S1:Acidolysis amino acids content (a) and infrared spectra(b) of the tested Chinese milk vetch; Table S1: Concentration of amino acid standard; Figure S2: Standard spectrum of free amino acids; Figure S3: Conceptual model demonstrating the relationship between FAAs and its impact factors; Table S2: Estimates of conceptual model coefficients for FAAs and its impact factors; Table S3: Estimates of modified model coefficients for FAAs and its impact factors; Table S4: Fitting coefficients of the FAAs and its impact factors model; Figure S4: Modified model for FAAs and its impact factors; Table S5: Total effects of factors contributing to the variability of FAAs content.

Author Contributions: Conceptualization, J.Y., W.Y. and S.X.; methodology, J.Y. and W.Y., investigation, J.Y. and Y.L., data curation, L.Z. and B.Z., writing—original draft preparation, J.Y., writing—review and editing, C.R., W.Y. and S.X., funding acquisition, W.Y. and S.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (41671490), the Science and Technology Innovation Fund Project of Fujian Agriculture and Forestry University (KFb22073XA, KFb22121XA), the Science and Technology Innovation Platform Project of Fujian Provincial Education Department (KJg21008A) and the Scientific Research Foundation of the Graduate School of Fujian Agriculture and Forestry University (324-1122yb080).

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to acknowledge Wenqi Guo and Xiumei Lei for their assistance in the bioassay experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rovira, P.; Kurz-Besson, C.; Hernàndez, P.; Coûteaux, M.M.; Vallejo, V.R. Searching for an indicator of N evolution during organic matter decomposition based on amino acids behaviour: A study on litter layers of pine forests. *Plant Soil* **2008**, *307*, 149–166. [CrossRef]
2. Gao, L.; Hill, P.W.; Jones, D.L.; Guo, Y.F.; Gao, F.; Cui, X.Y. Seasonality is more important than forest type in regulating the pool size and composition of soil soluble N in temperate forests. *Biogeochemistry* **2020**, *150*, 279–295. [CrossRef]
3. Jan, M.T.; Roberts, P.; Tonheim, S.K.; Jones, D.L. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. *Soil Biol. Biochem.* **2009**, *41*, 2272–2282. [CrossRef]
4. Feng, Y.L.; Wang, J.; Yuan, K.; Zong, W.Z.; Guo, D. Vegetation affects pool size and composition of amino acids in Tibetan alpine meadow soils. *Geoderma* **2018**, *310*, 44–52. [CrossRef]
5. Hill, E.J.; Jones, D.L.; Paterson, E.; Hill, P.W. Hotspots and hot moments of amino acid N in soil: Real-time insights using continuous microdialysis sampling. *Soil Biol. Biochem.* **2019**, *131*, 40–43. [CrossRef]
6. Harrison, K.A.; Bol, R.; Bardgett, R.D. Preferences for different nitrogen forms by coexisting plant species and soil microbes. *Ecology* **2007**, *88*, 989–999. [CrossRef]
7. Nie, S.A.; Zhao, L.X.; Lei, X.M.; Sarfraz, R.; Xing, S.H. Dissolved organic nitrogen distribution in differently fertilized paddy soil profiles: Implications for its potential loss. *Agric. Ecosyst. Environ.* **2018**, *262*, 58–64. [CrossRef]
8. Jones, D.L.; Shannon, D.; Junvee-Fortune, T.; Farrar, J.F. Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biol. Biochem.* **2005**, *37*, 179–181. [CrossRef]
9. Werdin-Pfisterer, N.R.; Kielland, K.; Boone, R.D. Soil amino acid composition across a boreal forest successional sequence. *Soil Biol. Biochem.* **2009**, *41*, 1210–1220. [CrossRef]
10. Gonzalez Perez, P.; Zhang, R.; Wang, X.; Ye, J.; Huang, D. Characterization of the amino acid composition of soils under organic and conventional management after addition of different fertilizers. *J. Soil. Sediment.* **2015**, *15*, 890–901. [CrossRef]
11. El-Naggar, A.; Rasmussen, J.; Neergaard, A.; El-Araby, A.; Høgh-Jensen, H. Effect of plant species and temperature on amino acid release from plant material. *Agron. Sustain. Dev.* **2010**, *30*, 679–688. [CrossRef]
12. Geisseler, D.; Horwath, W.R. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. *Soil Biol. Biochem.* **2008**, *40*, 3040–3048. [CrossRef]
13. Chen, J.R.; Qin, W.J.; Chen, X.F.; Cao, W.D.; Qian, G.M.; Liu, J.; Xu, C.X. Application of Chinese milk vetch affects rice yield and soil productivity in a subtropical double-rice cropping system. *J. Integr. Agric.* **2020**, *19*, 2116–2126. [CrossRef]
14. Schimel, J.; Bennett, J. Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **2004**, *85*, 591–602. [CrossRef]
15. Weintraub, M.N.; Schimel, J.P. The seasonal dynamics of amino acids and other nutrients in Alaskan Arctic tundra soils. *Biogeochemistry* **2005**, *73*, 359–380. [CrossRef]
16. Lipson, D.A.; Schmidt, S.K. Links between microbial population dynamics and nitrogen availability in an alpine ecosystem. *Ecology* **1999**, *80*, 1623–1631. [CrossRef]
17. Warren, C.R.; Taranto, M.T. Temporal variation in pools of amino acids, inorganic and microbial N in a temperate grassland soil. *Soil Biol. Biochem.* **2010**, *42*, 353–359. [CrossRef]
18. Lu, R.K. *Methods of Soil Agricultural Chemical Analysis*; China Agricultural Science and Technology Press: Beijing, China, 2000. (In Chinese)
19. Guan, S.Y. *Soil Enzyme and Research Methods for Soil Enzyme*; Agricultural Press: Beijing, China, 1986. (In Chinese)
20. Yang, J.; Nie, S.A.; Yang, W.H.; Chen, C.R.; Zhang, L.M.; Zhou, B.Q.; Xing, S.H. Effects of various influencing factors of soil soluble organic nitrogen components under different long-term fertilization treatments in paddy soil. *Acta Pedol. Sinica* **2018**, *55*, 955–966. (In Chinese) [CrossRef]
21. Beach, N.K.; Noguera, D.R. Design and assessment of species-level qPCR primers targeting comammox. *Front. Microbiol.* **2019**, *10*, 36. [CrossRef]

22. Peris, M.M.; Eissa, N.; Charles, N.B. Antepartum Antibiotic Treatment Increases Offspring Susceptibility to Experimental Colitis: A Role of the Gut Microbiotas. *PLoS ONE* **2015**, *10*, e0142536. [CrossRef]
23. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon readss. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef] [PubMed]
24. Liu, Y.; Pan, X.Z.; Wang, C.K.; Li, Y.L.; Shi, R.J. Can subsurface soil salinity be predicted from surface spectral information? -From the perspective of structural equation modelling. *Biosyst. Eng.* **2016**, *152*, 138–147. [CrossRef]
25. Soliman, A.; Bellaj, T.; Khelifa, M. An integrative psychological model for radicalism: Evidence from structural equation modeling. *Pers. Indiv. Differ.* **2016**, *95*, 127–133. [CrossRef]
26. Grace, J.B. *Structural Equation Modeling and Natural Systems*; Cambridge University Press: New York, NY, USA, 2006.
27. Eisenhauer, N.; Bowker, M.A.; Grace, J.B.; Powell, J.R. From patterns to causal understanding: Structural equation modeling (SEM) in soil ecology. *Pedobiologia* **2015**, *58*, 65–72. [CrossRef]
28. Yang, J.; Yang, W.H.; Wang, F.; Zhang, L.M.; Zhou, B.Q.; Sarfraz, R.; Xing, S.H. Driving factors of soluble organic nitrogen dynamics in paddy soils: Structure equation modeling analysis. *Pedosphere* **2020**, *30*, 801–809. [CrossRef]
29. Zhu, B.; Yi, L.X.; Hu, Y.G.; Zeng, Z.H.; Lin, C.W.; Tang, H.M.; Yang, G.L.; Xiao, X.P. Nitrogen release from incorporated 15N-labelled Chinese milk vetch (*Astragalus sinicus* L.) residue and its dynamics in a double rice cropping system. *Plant Soil* **2013**, *374*, 331–344. [CrossRef]
30. Kuzyakov, Y.; Friedel, J.K.; Stahr, K. Review of mechanisms and quantification of priming effects. *Soil Biol. Biochem.* **2000**, *32*, 1485–1498. [CrossRef]
31. Ma, Q.X.; Wu, L.H.; Wang, J.; Ma, J.Z.; Zheng, N.G.; Hill, P.W.; Chadwick, D.R.; Jones, D.L. Fertilizer regime changes the competitive uptake of organic nitrogen by wheat and soil microorganisms: An in-situ uptake test using 13C, 15N labelling, and 13C-PLFA analysis. *Soil Biol. Biochem.* **2018**, *125*, 319–327. [CrossRef]
32. Ma, H.; Pei, G.T.; Gao, R.; Yin, Y.F. Mineralization of amino acids and its signs in nitrogen cycling of forest soil. *Acta Ecol. Sinica* **2017**, *37*, 60–63. [CrossRef]
33. Zhou, Q.; Chen, J.; Xing, Y.; Xie, X.Y.; Wang, L.C. Influence of intercropping Chinese milk vetch on the soil microbial community in rhizosphere of rape. *Plant Soil* **2019**, *440*, 85–96. [CrossRef]
34. Macias-Benitez, S.; Garcia-Martinez, A.M.; Jimenez, P.C.; Gonzalez, J.M.; Moral, M.T.; Rubio, J.P. Rhizospheric organic acids as biostimulants: Monitoring feedbacks on soil microorganisms and biochemical properties. *Front. Plant Sci.* **2020**, *11*, 633. [CrossRef] [PubMed]
35. Liu, Y.; Lou, J.; Li, F.B.; Xu, J.M.; Yu, X.S.; Zhu, L.A.; Wang, F. Evaluating oxidation–reduction properties of dissolved organic matter from Chinese milk vetch (*Astragalus sinicus* L.): A comprehensive multi-parametric study. *Environ. Technol.* **2014**, *35*, 1916–1927. [CrossRef] [PubMed]
36. Cheng, H.D.; Lu, Y.H.; Nie, J.; Zhu, Q.J.; Nie, X.; Cao, W.D.; Gao, Y.J.; Liao, Y.L. Effects of reducing chemical fertilizer combined with Chinese milk vetch on soil carbon and nitrogen in paddy fields. *J. Agro-Environ. Sci.* **2020**, *39*, 1259–1270. (In Chinese) [CrossRef]
37. Cao, X.; Ma, Q.; Zhong, C.; Yang, X.; Zhu, L.; Zhang, J.; Jin, Q.Y.; Wu, L.H. Elevational variation in soil amino acid and inorganic nitrogen concentrations in Taibai Mountain, China. *PLoS ONE* **2016**, *11*, e0157979. [CrossRef]
38. Guo, X.C.; Cao, Y.S.; Xing, S.H. Composition of soft free amino acid and its difference in three plantations in Southern China. *J. Jiangxi Norm. Univ.* **2013**, *37*, 310–315. (In Chinese) [CrossRef]
39. Rae, I.C.M.; Castro, T.F. Root exudates of the rice plant in relation to akagare, a physiological disorder of rice. *Plant Soil* **1976**, *26*, 317–323. [CrossRef]
40. Jämtgård, S.; Näsholm, T.; Huss-Danell, K. Nitrogen compounds in soil solutions of agricultural land. *Soil Biol. Biochem.* **2010**, *42*, 2325–2330. [CrossRef]
41. Laudicina, V.; Palazzolo, E.; Badalucco, L. Natural Organic Compounds in Soil Solution: Potential Role as Soil Quality Indicators. *Curr. Org. Chem.* **2013**, *17*, 2991–2997. [CrossRef]
42. Yu, Q.G.; Hu, X.; Ma, J.W.; Ye, J.; Sun, W.C.; Wang, Q.; Lin, H. Effects of long-term organic material applications on soil carbon and nitrogen fractions in paddy fields. *Soil Tillage Res.* **2020**, *196*, 104483. [CrossRef]
43. Friedel, J.; Scheller, E. Composition of hydrolysable amino acids in soil organic matter and soil microbial biomass. *Soil Biol. Biochem.* **2002**, *34*, 315–325. [CrossRef]
44. Rothstein, D.E. Effects of amino-acid chemistry and soil properties on the behavior of free amino acids in acidic forest soils. *Soil Biol. Biochem.* **2010**, *42*, 1743–1750. [CrossRef]
45. Xie, Z.J.; Zhou, C.H.; Shah, F.; Lqbal, A.; Ni, G.R. The role of Chinese Milk Vetch as cover crop in complex soil nitrogen dynamics in rice rotation system of South China. *Sci. Rep.* **2018**, *8*, 12061. [CrossRef]
46. Huang, C.C.; Liu, S.; Li, R.Z.; Sun, F.S.; Zhou, Y.Z.; Yu, G.H. Spectroscopic evidence of the improvement of reactive iron mineral content in red soil by long-term application of swine manure. *PLoS ONE* **2016**, *11*, e0146364. [CrossRef] [PubMed]
47. Yang, S.H.; Peng, S.Z.; Xu, J.Z.; He, Y.P.; Wang, Y.J. Effects of water saving irrigation and controlled release nitrogen fertilizer managements on nitrogen losses from paddy fields. *Paddy Water Environ.* **2015**, *1*, 71–80. [CrossRef]
48. Fischer, H.; Meyer, A.; Fischer, K.; Kuzyakov, Y. Carbohydrate and amino acid composition of dissolved organic matter leached from soil. *Soil Biol. Biochem.* **2007**, *39*, 2926–2935. [CrossRef]

49. Zhu, C.; Wang, Q.; Huang, X.X.; Yun, J.N.; Hu, Q.L.; Yang, G. Adsorption of amino acids at clay surfaces and implication for biochemical reactions: Role and impact of surface charges. *Colloids Surface B* **2019**, *183*, 110458. [CrossRef]
50. Islam, M.M.; Saey, T.; De Smedt, P.; Van De Vijver, E.; Delefortrie, S.; Van Meirvenne, M. Modeling within field variation of the compaction layer in a paddy rice field using a proximal soil sensing system. *Soil Use Manag.* **2014**, *30*, 99–108. [CrossRef]
51. Hedges, J.I.; Hare, P.E. Amino acid adsorption by clay minerals in distilled water. *Geochim. Cosmochim. Acta* **1987**, *51*, 255–259. [CrossRef]
52. Dashman, T.; Stotzky, G. Adsorption and binding of amino acids on homoionic montmorillonite and kaolinite. *Soil Biol. Biochem.* **1982**, *14*, 447–456. [CrossRef]
53. Trevino, S.R.; Scholtz, J.M.; Pace, C.N. Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute more Favorably than the other Hydrophilic Amino Acids in RNase Sa. *J. Mol. Biol.* **2007**, *366*, 449–460. [CrossRef]
54. Jensen, K.T.; Lobmann, K.; Rades, T.; Grohganz, H. Improving co-amorphous drug formulations by the addition of the highly water soluble amino Acid, proline. *Pharmaceutics* **2014**, *6*, 416–435. [CrossRef] [PubMed]
55. Reeve, J.R.; Smith, J.L.; Carpenter-Boggs, L.; Reganold, J.P. Soil-based cycling and differential uptake of amino acids by three species of strawberry (Fragariaspp.) plants. *Soil Biol. Biochem.* **2008**, *40*, 2547–2552. [CrossRef]
56. Inselsbacher, E.; Wanek, W. An unexpected source of nitrogen for root uptake: Positively charged amino acids dominate soil diffusive nitrogen fluxes. *New Phytol.* **2021**, *30*, 99–108. [CrossRef]
57. Wang, Y.F.; Liu, X.M.; Butterly, C.; Tang, C.X.; Xu, J.M. pH change, carbon and nitrogen mineralization in paddy soils as affected by Chinese milk vetch addition and soil water regime. *J. Soil Sediments* **2013**, *13*, 654–663. [CrossRef]
58. Chen, J.; Wu, Q.; Li, S.; Ge, J.; Liang, C.; Qin, H.; Xu, Q.; Fuhrmann, J.J. Diversity and function of soil bacterial communities in response to long-term intensive management in a subtropical bamboo forest. *Geoderma* **2019**, *354*, 113894. [CrossRef]
59. Sinsabaugh, R.; Lauber, C.; Weintraub, M.; Ahmed, B.; Allison, S.; Crenshaw, C.; Contosta, A.; Cusack, D.; Frey, S.; Gallo, M.; et al. Stoichiometry of soil enzyme activity at global scale. *Ecol. Lett.* **2008**, *11*, 1252–1264. [CrossRef]
60. Min, K.; Lehmeier, C.A.; Ballantyne, F.; Tatarko, A.; Billings, S.A. Differential effects of pH on temperature sensitivity of organic carbon and nitrogen decay. *Soil Biol. Biochem.* **2014**, *76*, 193–200. [CrossRef]
61. Li, Z.L.; Tian, D.S.; Wang, B.X.; Wang, J.S.; Wang, S.; Chen, H.Y.H.; Xu, X.F.; Wang, C.h.; He, N.P.; Niu, S.L. Microbes drive global soil nitrogen mineralization and availability. *Glob. Chang. Biol.* **2019**, *25*, 1078–1088. [CrossRef]
62. Hu, R.; Chen, K.W.; Song, K.L.; Yin, X.; Zhou, C.H.; Ni, G.R. Effects of Long-term Return of Milk Vetch (Astragalus sinicus) on Soil Nutrients and Microorganism Communities in Double-rice Cropping Field. *Agric. Biotechnol.* **2021**, *10*, 127–129, 133.
63. Huang, Q.R.; Hu, F.; Huang, S.; Li, H.X.; Yuan, Y.H.; Pan, G.X.; Zhang, W.J. Effect of long-term fertilization on organic carbon and nitrogen in a subtropical paddy soil. *Pedosphere* **2009**, *19*, 727–734. [CrossRef]
64. Kieloaho, A.J.; Pihlatie, M.; Dominguez Carrasco, M.; Kanerva, S.; Parshintsev, J.; Riekkola, M.L.; Pumpanen, J.; Heinonsalo, J. Stimulation of soil organic nitrogen pool: The effect of plant and soil organic matter degrading enzymes. *Soil Biol. Biochem.* **2016**, *96*, 97–106. [CrossRef]
65. Cenini, V.L.; Fornara, D.A.; McMullan, G.; Ternan, N.; Carolan, R.; Crawley, M.J.; Clément, J.-C.; Lavorel, S. Linkages between extracellular enzyme activities and the carbon and nitrogen content of grassland soils. *Soil Biol. Biochem.* **2016**, *96*, 198–206. [CrossRef]
66. Noll, L.; Zhang, S.S.; Zheng, Q.; Hu, Y.; Wanek, W. Wide-spread limitation of soil organic nitrogen transformations by substrate availability and not by extracellular enzyme content. *Soil Biol. Biochem.* **2019**, *133*, 37–49. [CrossRef] [PubMed]
67. Tang, H.M.; Xiao, X.P.; Tang, W.G.; Lin, Y.C.; Wang, K.; Yang, G.L. Effects of winter cover crops residue returning on soil enzyme activities and soil microbial community in double-cropping rice fields. *PLoS ONE* **2014**, *9*, e100443. [CrossRef]
68. Mishra, S.; Di, H.; Cameron, K.; Monaghan, R.; Carran, A. Gross Nitrogen Mineralisation Rates in Pastural Soils and Their Relationships with Organic Nitrogen Fractions, Microbial Biomass and Protease Activity under Glasshouse Conditions. *Biol. Fertil. Soils* **2005**, *42*, 45–53. [CrossRef]
69. Noll, L.; Zhang, S.S.; Wanek, W. Novel high-throughput approach to determine key processes of soil organic nitrogen cycling: Gross protein depolymerization and microbial amino acid uptake. *Soil Biol. Biochem.* **2019**, *130*, 73–81. [CrossRef]
70. Reiskind, J.B.; Lavoie, M.; Mack, M.C. Kinetic studies of proteolytic enzyme activity of arctic soils under varying toluene concentrations. *Soil Biol. Biochem.* **2011**, *43*, 70–77. [CrossRef]
71. Yang, Z.P.; Zheng, S.X.; Nie, J.; Liao, Y.L.; Xie, J. Effects of Long-Term Winter Planted Green Manure on Distribution and Storage of Organic Carbon and Nitrogen in Water-Stable Aggregates of Reddish Paddy Soil Under a Double-Rice Cropping System. *J. Integr. Agric.* **2014**, *13*, 1772–1781. [CrossRef]
72. Tao, J.M.; Liu, X.D.; Liang, Y.L.; Niu, J.J.; Xiao, Y.H.; Gu, Y.B.; Ma, L.Y.; Meng, D.L.; Zhang, Y.G.; Huang, W.K. Maize growth responses to soil microbes and soil properties after fertilization with different green manures. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 1289–1299. [CrossRef]
73. Qualls, R.G.; Haines, B.L.; Swank, W.T.; Tyler, S.W. Retention of soluble organic nutrients by a forested ecosystem. *Biogeochemistry* **2002**, *61*, 135–171. [CrossRef]
74. Hossain, M.Z.; Sugiyama, S.i. Relative importance of soil microbes and litter quality on decomposition and nitrogen cycling in grasslands. *Ecol. Res.* **2020**, *35*, 912–924. [CrossRef]
75. Miltner, A.; Kindler, R.; Knicker, H.; Richnow, H.H.; Kästner, M. Fate of microbial biomass-derived amino acids in soil and their contribution to soil organic matter. *Org. Geochem.* **2009**, *40*, 978–985. [CrossRef]

76. Pan, J.H.; Wang, J.; Zhuang, S.Y. Amino acid nitrogen trends in paddy soils under long-term rice cultivation in southeast coast of China. *Catena* **2022**, *212*, 106044. [CrossRef]
77. Hofmockel, K.S.; Fierer, N.; Colman, B.P.; Jackson, R.B. Amino acid abundance and proteolytic potential in North American soils. *Oecologia* **2010**, *163*, 1069–1078. [CrossRef]
78. Xu, Z.w.; Yu, G.R.; Zhang, X.Y.; Ge, J.P.; He, N.P.; Wang, Q.F.; Wang, D. The variations in soil microbial communities, enzyme activities and their relationships with soil organic matter decomposition along the northern slope of Changbai Mountain. *Appl. Soil Ecol.* **2015**, *86*, 19–29. [CrossRef]
79. Gao, S.J.; Cao, W.D.; Zhou, G.P.; Rees, R.M. Bacterial communities in paddy soils changed by milk vetch as green manure: A study conducted across six provinces in South China. *Pedosphere* **2021**, *31*, 521–530. [CrossRef]
80. Philben, M.; Billings, S.A.; Edwards, K.A.; Podrebarac, F.A.; van Biesen, G.; Ziegler, S.E. Amino acid δ15N indicates lack of N isotope fractionation during soil organic nitrogen decomposition. *Biogeochemistry* **2018**, *138*, 69–83. [CrossRef]
81. Moe, L.A. Amino acids in the rhizosphere: From plants to microbes. *Am. J. Bot.* **2013**, *100*, 1692–1705. [CrossRef]
82. Daims, H.; Wagner, M. Nitrospira. *Trends Microbiol.* **2018**, *26*, 462–463.
83. Bach, H.J.; Munch, J.C. Identification of bacterial sources of soil peptidases. *Biol. Fertill. Soils* **2000**, *31*, 219–224. [CrossRef]
84. Watanabe, K.; Hayano, K. Source of soil protease based on the splitting sites of a polypeptide. *Soil Sci. Plant Nutr.* **1994**, *40*, 697–701. [CrossRef]