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Effect of Oak Tree Sawdust Fermentation Period on Peanut Seed Germination, Seedling Biomass, and Morphology

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Abstract: Peanut (*Arachis hypogaea* L.) seeds were germinated to investigate the effect of the fermentation period of oak tree sawdust on germination viability and seedling characteristics. Its germination rate, seedling weight, length, and total vigor index were assessed. The seeds were sown in oak tree sawdust fermented for 0, 30, 45, and 60 days. The germination rates of the seeds in fermented sawdust were significantly different. The seeds in the 45-day fermented sawdust produced the heaviest biomass weight (4.6 g) with the longest true leaf (1.7 cm) and hypocotyl (3.4 cm) resulting in the highest total vigor index (925.8). In contrast, seeds in 0-day fermented sawdust had the lowest total vigor index (18.3). Microbiome analysis showed that the microbial community in the sawdust changed as the fermentation progressed, indicating that the microbial community seems to affect seed germination physiology. Taken together, 45-day fermented sawdust is recommended for optimal peanut seed germination and seedling growth.

Keywords: cotyledon; epicotyl; peanut sprout; root soil; seed sowing

1. Introduction

Peanut sprouts are considered to be a good source of resveratrol, a natural phytoalexin phenolic compound, which is rarely found in most crops [1,2]. In addition, peanut sprouts have more carbohydrates, amino acids, minerals, fatty acids, and antioxidants but less fats than seeds [3]. Seed germination is an essential part of sprout growth, producing a new generation of plant [4]. Seed germination is affected by environmental factors such as temperature, light, pH, and soil [5]. Seeds normally require specific soil conditions to initiate seedling germination [6–8]. Oak tree sawdust consists of small fragments or discrete pieces of wood that are categorized as unworthy during the sawing process. However, oak tree sawdust can be used in the production of biofuel and packaging fillers and in insulation [9,10]. Fermented oak tree sawdust has been used as a topsoil alternative to germinate peanut seeds for sprout production. Thus, an oak tree sawdust cultivation system has been optimized for peanut seeds to enhance germination and sprout vigor [11,12].

In a previous study, seed sowing orientation was optimized to improve peanut seed germination rates, seedling biomass, and sprout morphology quality [11]. The fermentation of oak tree sawdust is costly and time-consuming. However, the sawdust fermentation

period has not been optimized for peanut seed germination and seedling growth, which are essential for the economical production of peanut sprouts.

Thus, in this study, we investigated the production of peanut sprouts in oak tree sawdust fermented for different periods. Specifically, we examined the effects of the oak tree sawdust fermentation period on the peanut seed germination rate, seedling growth, and seedlings.

2. Materials and Methods

2.1. Fermented Oak Tree Sawdust

The fermented oak tree sawdust used in this experiment was obtained from the Farmsko company (Cheongwon, Chungcheongbukdo, Korea). The oak trees were cut from November to February and fermented with 1~2 mm and 3~5 mm sawdust particles mixed 1:1. The sawdust consisted of more than 90% of oak tree and 10% of deciduous trees such as the chestnut tree and the cherry tree. In the crushing process, water was mixed with sawdust so that the moisture content was 55% or more, and then fermentation was performed for 30, 45, and 60 days in a fermentation house according to the company's protocol described in the Korea patent (#KR101235913B1).

2.2. Oak Tree Sawdust Cultivation for Seed Germination

Peanut (*A. hypogaea* L.) seeds were sown in oak tree sawdust trays (50 × 30 × 10 cm; Namyong Chemical, Hanam, Korea). The fermented oak tree sawdust used in this experiment was obtained from the Farmsko company (Cheongwon, Chungcheongbukdo, Korea). The oak trees were cut from November to February and fermented with 1~2 mm and 3~5 mm sawdust particles mixed 1:1.

Twenty seeds were sown in four different fermented sawdust trays as follows: unfermented sawdust (0), 30-day fermented sawdust (30), 45-day fermented sawdust (45), and 60-day fermented sawdust (60). The spacing between seeds, row spacing, and sowing depth was 4, 5, and 3 cm, respectively. The seed orientation was vertical with the hypocotyl end down. The trays were filled to a depth of 3 cm with oak tree sawdust, and the seeds were covered with a 3 cm layer of sawdust after sowing. The trays were under dark conditions and watered (2 L) every day in the morning and evening. In the darkroom, the temperature was 30 °C and the relative humidity was 85%. The growing environment of the peanuts could be changed. The temperature and humidity of the cultivation room were carefully maintained and checked every 12 h.

2.3. Seedling Weight and Tissue Length Measurements

Peanut seedlings were removed from the trays after eight days of cultivation. All seedlings were washed with tap water and blotted dry on a paper towel (Yuhan Kimberly, Seoul, Korea). Seedlings that germinated from each fermentation period (0, 30, 45, and 60 days) were then observed. The seed germination rate, seedling length (hypocotyl, true leaf with epicotyl, and root), and weight of the germinated seedlings were measured.

2.4. Vigor Index Measurement

To determine the best oak tree sawdust fermentation period for peanut seedling cultivation, the vigor index was obtained using two criteria: germination rate and tissue length (hypocotyl, true leaf with epicotyl, and root). The vigor index was calculated as follows: seed germination rate × the length of each tissue part [12].

2.5. Morphological Analysis

Freshly harvested peanut seedlings from four different fermentation groups (0, 30, 45, 60 days) were placed on a black cotton flannel eight days after sowing. The seedling morphology was visually observed and photographed using a camera (Galaxy S7 edge) (Samsung, Seoul, Korea).

2.6. PCR Amplification and Illumina Sequencing

A PCR was conducted with primers for the V3 to V4 regions of the 16S rRNA gene in DNA obtained from the fermented oak tree sawdust. This protocol was modified according to a previous study [13]. For bacterial amplification, primers of 341F (5'-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGG TATCTAATCC-3') were used. The PCR amplifications were performed as follows: initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation at 72 °C for 5 min. Then, secondary amplification for attaching the Illumina NexTera barcode was conducted with an i5 forward primer (5'-AATGATACGGCGACCACCGAGATCTACA C-XXXXXXXX-TCGTCGGCAGC GTC-3'; X indicates the barcode region) and an i7 reverse primer (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-AGTCTCGTGGGCT C GG-3'). The condition of the secondary amplification was equal to the former one except the amplification cycle was set to 8. The PCR products were separated by 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). After purification of the PCR products using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), their equal concentrations were pooled together to remove short fragments (non-target products) with an Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size were determined on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. After pooling the mixed amplicons, the sequencing was carried out at Chunlab, Inc. (Seoul, Korea), with an Illumina MiSeq Sequencing system (Chunlab, Seoul, Korea) according to the manufacturer's instructions.

2.7. Statistical Analysis

All values are presented as the mean \pm SD. The statistical significance was determined using Graph Pad Prism software version 5 (GRAPH PAD software Inc., San Diego, CA, USA).

2.8. Miseq Pipeline Method

The processing of the raw reads started with a quality check and the filtering of low quality (<Q25) reads by trimmomatic 0.321. After a QC pass, paired-end sequence data were merged together using PandaSeq2. The primers were then trimmed with Chun Lab's in-house program at a similarity cut off of 0.8. Sequences were denoised using Mothur's 3 pre-clustering program, which merges sequences and extracts unique sequences allowing up to 2 differences between sequences. The EzTaxon database was used for Taxonomic Assignment using BLAST 2.2.224 and pairwise alignment 5 was used to calculate similarity. The UCHIME 6 and the non-chimeric 16S rRNA database from EzTaxon were used to detect chimera on reads that contained a less than 97% best hit similarity rate. Sequence data was then clustered using CD-Hit7 and UCLUST 8, and an alpha diversity analysis was carried out.

3. Results

3.1. Effect of Oak Tree Sawdust Fermentation Period on Peanut Seed Germination Rate

Seedling emergence times and seed germination rates varied among the oak tree sawdust groups (Figure 1). In the 45-day fermented sawdust and 60-day fermented sawdust groups, radicle emergence started four days after sowing, whereas it started after six days in the unfermented and 30-day fermented sawdust groups. Seeds in the 45-day fermented and 60-day fermented sawdust groups emerged significantly faster than in other groups. Peanut seeds in the unfermented and 30-day fermented sawdust groups had 20 and 40% germination rates, respectively, whereas seeds in the 45-day fermented and 60-day fermented sawdust groups had 95 and 85% germination rates, respectively (Figure 1). It is indicated that the optimal condition for seed germination rates was 45-day fermented (Figure 1).

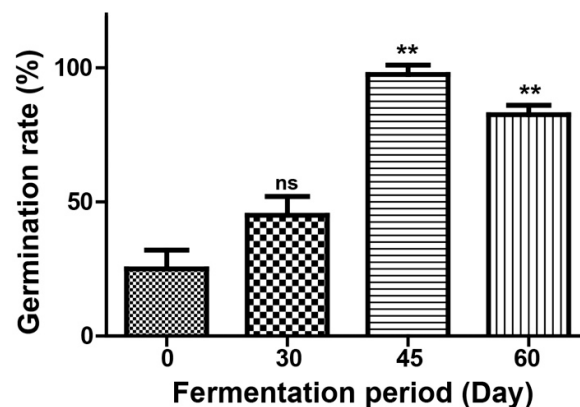


Figure 1. Effect of fermentation period [unfermented oak tree sawdust (0), 30-day fermented oak tree sawdust (30), 45-day fermented oak tree sawdust (45), and 60-day fermented oak tree sawdust (60)] on the seed germination rate. Each fermentation period was statistically compared with 0 day. The asterisks indicate significant difference (** $p < 0.01$). 'ns' indicates 'not significant'.

3.2. Effect of Oak Tree Sawdust Fermentation Period on Peanut Seedling Weight

All seedlings were uprooted and weighed eight days after sowing. The mean seedling weight of the 45-day fermented sawdust group (4.6 g) was significantly higher than that of the unfermented sawdust group (2.5 g). The mean seedling weights in the 30- and 60-day fermented oak tree sawdust groups were 3.6 g and 3.4 g, respectively (Figure 2).

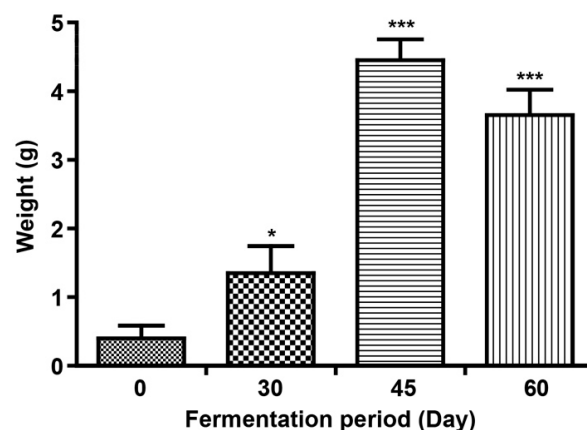


Figure 2. Effect of fermentation period [unfermented sawdust (0), 30-day fermented sawdust (30), 45-day fermented oak tree sawdust (45), and 60-day fermented oak tree sawdust (60)] on seedling weight. Each fermentation period was statistically compared with 0 day. The asterisks indicate significant difference (* $p < 0.05$ and *** $p < 0.001$).

3.3. Effect of Oak Tree Sawdust Fermentation Period on the Lengths of True Leaf with Epicotyl, Hypocotyl, and Root in Seedlings

True leaf seedlings began to emerge after 30 days in the fermented sawdust. The mean length of the true leaf with an epicotyl of seedlings in 45-day fermented sawdust (1.7 cm) was longer than that of seedlings in 30-day fermented sawdust (0 cm) and 60-day fermented sawdust (1.1 cm) (Figure 3a). The mean hypocotyl length of seedlings in 45-day fermented sawdust (3.4 cm) was significantly longer than those in unfermented sawdust (0.2 cm), 30-day fermented sawdust (1.0 cm), and 60-day fermented sawdust (2.6 cm) (Figure 3b). Root lengths for the 30-day fermented sawdust (1.5 cm) and 45-day fermented sawdust (4.6 cm) groups were significantly longer than those of the seedlings in unfermented sawdust (0.7 cm) (Figure 3c). The mean root length of the seedlings in 60-day fermented sawdust (3.9 cm) was slightly less than those in 30 and 45-day fermented sawdust groups (Figure 3c).

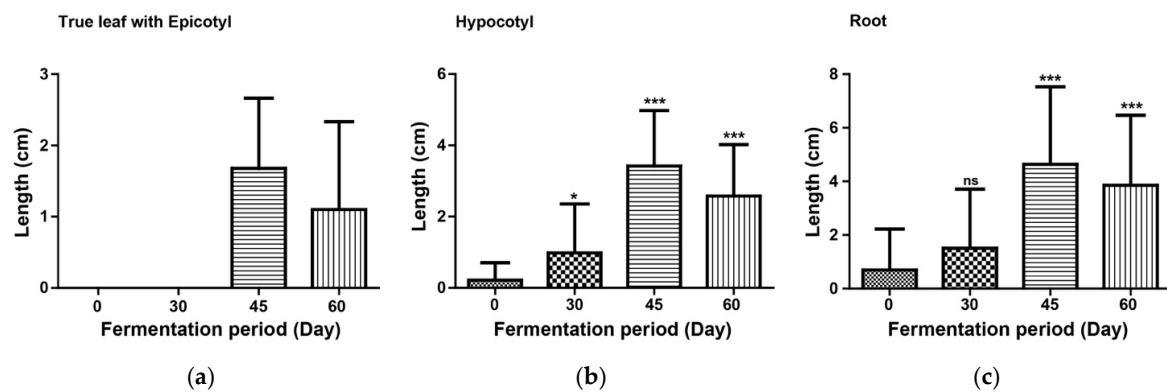


Figure 3. Effect of the fermentation period [unfermented sawdust (0), 30-day fermented sawdust (30), 45-day fermented oak tree sawdust (45), and 60-day fermented oak tree sawdust (60)] on seedling length. (a): True leaf with epicotyl; (b): Hypocotyl; (c): Root. Each fermentation period was statistically compared with 0 day. The asterisks indicate significant difference (ns $p > 0.05$, * $p < 0.05$, and *** $p < 0.001$).

3.4. Effect of Oak Tree Sawdust Fermentation Period on Vigor Index

The vigor index of seedlings was calculated to determine the sawdust fermentation period that resulted in the most efficient biomass production (Table 1) (Figure 4). Among the four groups, the 45-day fermentation period produced the highest total vigor index (925.8), whereas the 0-day fermentation period produced the lowest total vigor index (18.3). The total vigor index of the 45-day fermentation period group was significantly higher than that of the 60-day fermentation period group. In general, the fermented sawdust groups had higher total vigor index values than the non-fermented sawdust group (Figure 5).

Table 1. Calculation of Vigor Index (VI) and total VI on seedlings each fermentation period of oak tree sawdust.

Fermentation Period ^z	Germination Rate (%)	Length (cm)			Vigour Index (VI) ^y			Total ^x
		True Leaf with Epicotyl	Hypocotyl	Root	True Leaf with Epicotyl	Hypocotyl	Root	VI (True Leaf with Epicotyl) + VI (Hypocotyl) + VI (Root)
0	20	0	0.2	0.7	0 ± 0	4.3 ± 1.1	14 ± 3.5	18.3 ± 4.6
30	40	0	1.0	1.5	0 ± 0	39.2 ± 6.2	60.4 ± 9.8	99.6 ± 16
45	95	1.7	3.4	4.6	159.6 ± 10.5	325.4 ± 16.5	440.8 ± 30.7	925.8 ± 57.7
60	85	1.1	2.6	3.9	93.5 ± 11.7	218.9 ± 13.8	328.1 ± 24.8	640.5 ± 50.3

^z Fermentation period of oak tree sawdusts; 0: Unfermented sawdust, 30: 30-day fermented sawdust, 45: 45-day fermented sawdust, 60: 60-day fermented sawdust. ^y Vigour Index (VI) = Germination Rate (%) × Length (cm). ^x Total = VI (True leaf with epicotyl) + VI (Hypocotyl) + VI (Root).

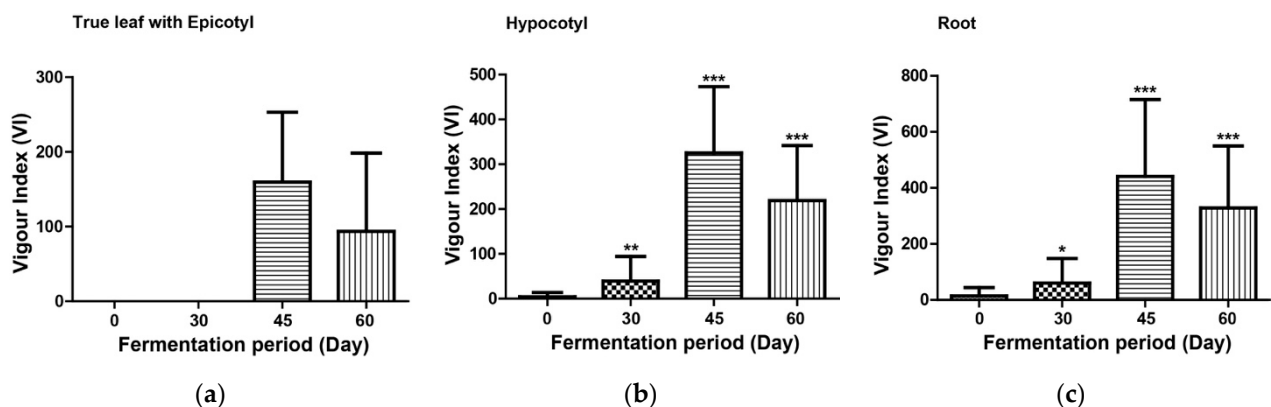


Figure 4. Effect of the fermentation period [unfermented sawdust (0), 30-day fermented sawdust (30), 45-day fermented oak tree sawdust (45), and 60-day fermented oak tree sawdust (60)] on vigor index (VI). (a): True leaf with epicotyl; (b): Hypocotyl; (c): Root. Each fermentation period was statistically compared with 0 day. The asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

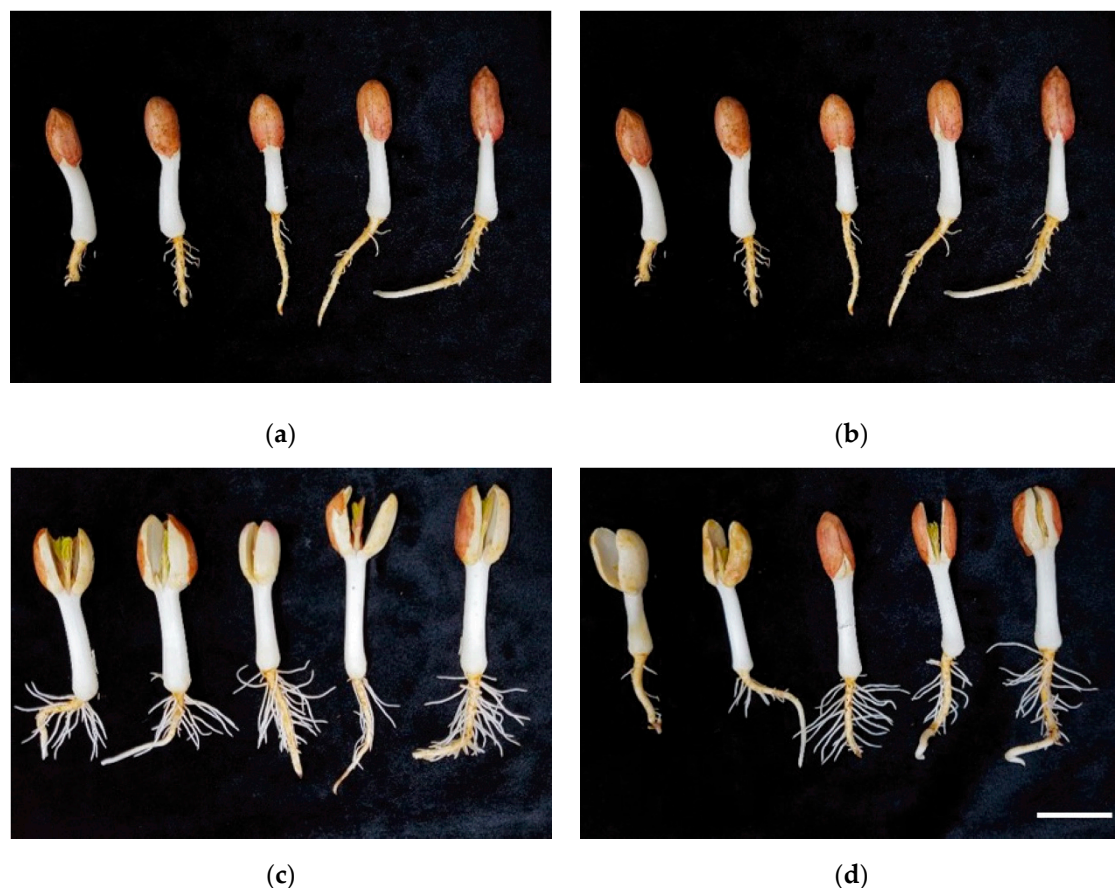


Figure 5. Effect of the fermentation period on the phenotype of the germinated seedling. (a): (unfermented oak tree sawdust); (b): (30-day fermented oak tree sawdust); (c): (45-day fermented oak tree sawdust); (d): (60-day fermented oak tree sawdust). Scale bar = 2 cm.

3.5. Effect of Oak Tree Sawdust Fermentation Period on Seedling Morphology

Among the four fermentation period groups, the seedlings in unfermented sawdust had the shortest hypocotyl and root (Figure 5a). The true leaf and epicotyl were not observed in unfermented sawdust (Figure 5a). The epicotyl and the root of seedlings in the 30 day fermentation period group were slightly longer than seedlings in unfermented sawdust (Figure 5a,b). The true leaf and epicotyl were observed in seedlings in the 45- and 60-day fermentation period groups (Figure 5c,d). The hypocotyls of seedlings in the 45-day fermentation period group were slightly thicker than those in the 60-day fermentation period group (Figure 5c,d). The root hairs were relatively short in unfermented and 30-day fermented sawdust (Figure 5a,b). However, the root hairs in the primary root were the longest and most abundant in the 45-day fermentation period group (Figure 5).

3.6. Bacterial Communities of 0, 30, 45, and 60-Day Fermented Oak Tree Sawdust

The microbiota distribution and composition in 0, 30, 45, and 60-day fermented oak tree sawdust were determined by Illumina MiSeq sequencing analysis and Pyrosequencing (Figure 6). The bacterial communities were dramatically changed by the fermentation periods (Figure 6). In 30-day fermented sawdust, *Dyella ginsengisoli* and *Streptomyces cattleya* group were the relative majority. In 45-day fermented sawdust, *Ethanoligenesis_uc*, and *Paraburkholderia_uc* became the relative majority. In 60-day fermented sawdust, the microbiota were dominated by the *Acetobacter pasteurianus* group and *Alicyclobacillus kakegawawensis*. The current data showed a variation in the relative abundance and diversity of the microbial species in the fermented sawdust samples.

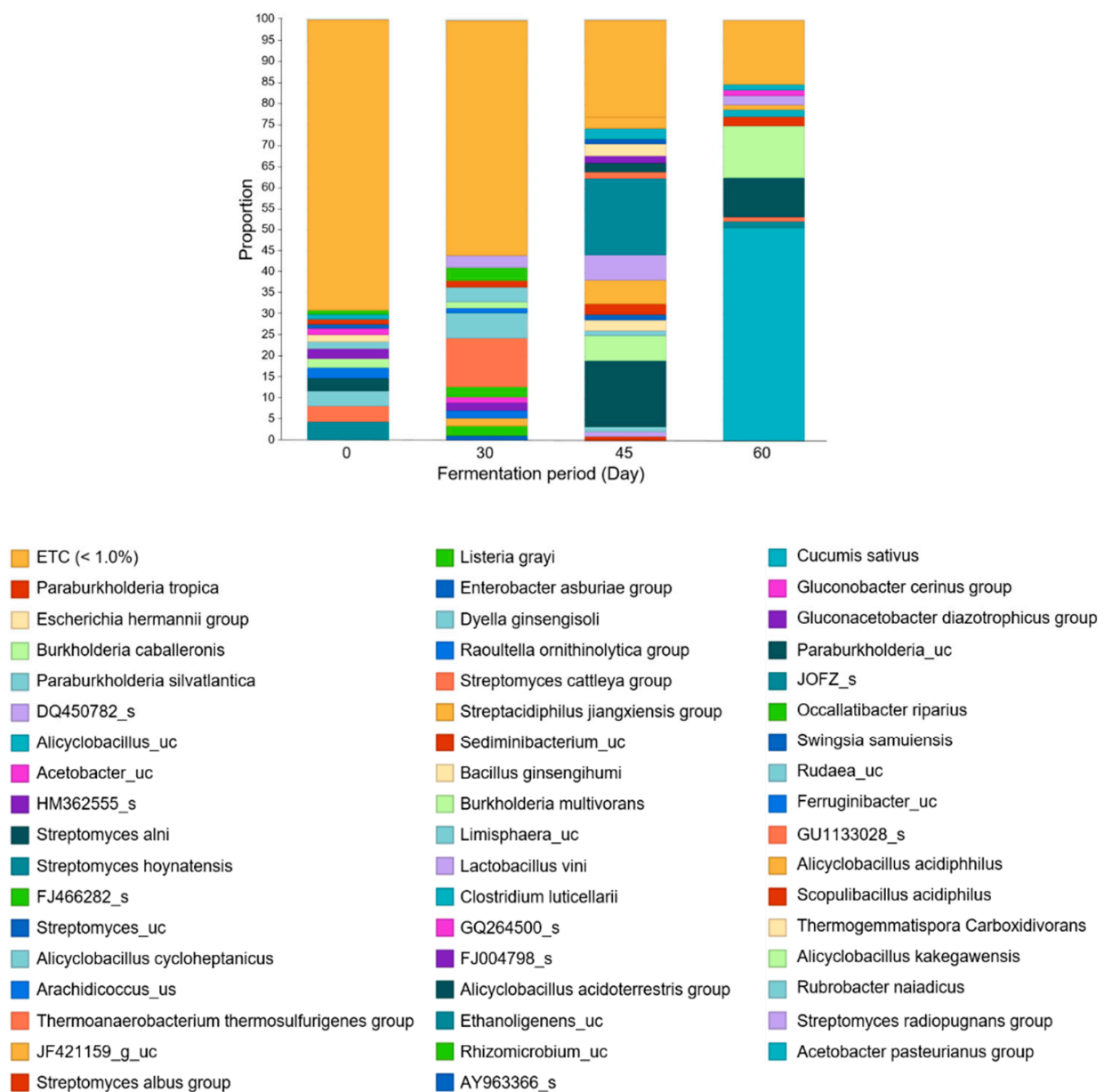


Figure 6. Taxonomic classification of microbial reads retrieved from 0, 30, 45, 60-day fermented oak tree sawdust using the EzTaxon database. Microbial analysis of 0, 30, 45, and 60-day fermented oak tree sawdust conducted using a 454 GS FLX titanium next-generation sequencing (NGS) system (Roche, Branford, CT) according to the manufacturer's instructions (Lee and Eom, 2016). The relative proportion of microbiota was displayed using an EzTaxon database. Each color indicates a unique species.

3.7. Composition of 0, 30, 45, and 60-Day Fermented Oak Tree Sawdust-Associated Bacterial Communities

The MiSeq pipeline method was subsequently conducted to identify members of the bacterial community at the phylum level together with the species (Figure 7). In non-fermented oak tree sawdust, the microbiota were dominated by *Proteobacteria* (59.1%). In 30-day fermented oak tree sawdust, *Acidobacteria* (29.9%) and *Proteobacteria* (32.7%) became the relative majority. In 45-day fermented oak tree sawdust, *Firmicutes* (51.8%) and *Acidobacteria* (34.3%) became the majority. In 60-day fermented oak tree sawdust, the microbiota were dominated by *Proteobacteria* (55.3%) and *Firmicutes* (44.2%). The bacterial communities were different along with the oak tree sawdust fermentation period.

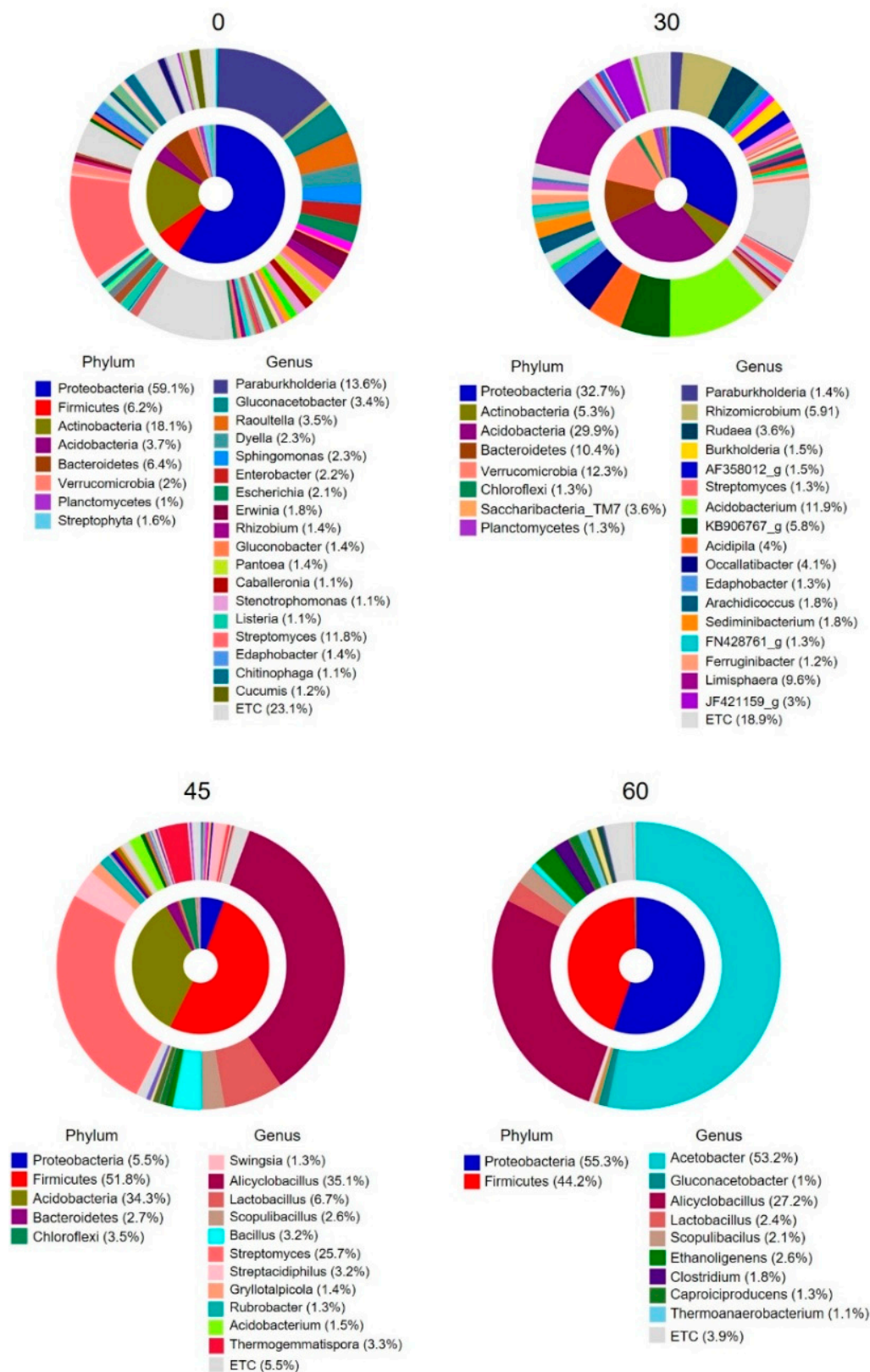


Figure 7. Bacterial composition profiles in 0, 30, 45, 60-day fermented oak tree sawdust group. Microbial analysis of the 0, 30, 45, and 60-day fermented oak tree sawdust conducted using a 454 GS FLX titanium next-generation sequencing (NGS) system (Roche, Branford, CT) according to the manufacturer's instructions (Lee and Eom, 2016). Levels of phyla and genera of each bacteria with more than 1% of their proportion in 0, 30, 45, 60-day fermented oak tree sawdust was displayed (inner area: Phylum, outer area: Genus).

4. Discussion and Conclusions

In this study, we investigated the effect of the fermentation period of oak tree sawdust on peanut seed germination, seedling tissue biomass, and peanut sprout morphology. The 45-day fermented oak tree sawdust yielded the highest seed germination rate, the longest hypocotyls, true leaf with epicotyl, and root length, resulting in the highest total seedling vigor index. The results indicate that oak tree sawdust fermentation affects peanut seed germination and the growth and physiology of seedlings.

It is crucial to determine the optimal soil conditions if efficient germination and complete seedling tissues are to be accomplished [14,15]. For instance, seed germination occurs when proper surface soil water conditions are maintained [7,14,15]. In a previous study, 60-day fermented oak tree sawdust was used to determine the optimal peanut seed orientation to achieve a high seedling vigor index [11]. However, the sawdust fermentation period has not been optimized for peanut seed germination. In the current study, seed germination rates with different fermentation periods were significantly different, indicating that peanut seed germination was affected by sawdust fermentation. The 45-day-oak tree sawdust fermentation period resulted in the highest seedling growth. The highest seedling growth and the complete morphology of the peanut sprouts were achieved when 45-day fermented sawdust was used, whereas unfermented sawdust showed the lowest growth level with poor morphology. Furthermore, the primary root lengths of the 30, 45, and 60-day fermentation period groups were significantly different. The length and number of the lateral roots of seedlings in the 45-day fermentation period group were longer and higher, respectively, compared to those of the 30-day fermentation period group (Figure 5). Seedlings in unfermented sawdust had short roots with less lateral hairs, short hypocotyl, no true leaf, and weak morphology. The 45-day fermentation period is recommended to enhance the extensive root system with lateral roots, which play a role in soil water and mineral uptake and thereby act as a soil anchoring system [16].

In general, oak tree sawdust, which is regarded as waste, can be obtained during wood processing. Sawdust waste is a subject of environmental concern worldwide, especially since sawdust provides useful goods and services [17]. Sawdust is made up of three major components: cellulose, hemicellulose, and lignin [18,19]. Lignin is the most recalcitrant and protects cellulose and hemicellulose from enzymatic attack by certain microorganisms [17,20]. Tree sawdust is fermented by a group of microorganisms that produce enzymes for organic substrate decomposition [21]. According to previous studies, sawdust is quickly decomposed by bacteria at the beginning of fermentation into simple compounds such as glucose and xylose; later, only complex compounds such as lignin and cellulose remain, which are difficult to break down [22]. The enzymes of actinomyces play a significant role in the decomposition of recalcitrant materials in fermentation, since coarse debris such as trunk or bark cannot easily be decomposed.

In our study, it is speculated that sawdust fermentation changed the microbial community to decompose the sawdust and to inhibit the growth of pathogenic microbes [23] (Figures 6 and 7), enhancing the optimal biological and mechanical sawdust conditions for peanut seed germination and seedling growth physiology [24–26]. The microbiome in fermented sawdust can affect the seed germination and seedling growth. This information is useful to understand the optimal design of microbial communities for peanut sprout cultivation in the fermented oak tree sawdust system.

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