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Agricultural Utilization of Unused Resources: Liquid Food Waste Material as a New Source of Plant Growth-Promoting Microbes

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Abstract: Organic amendment is important for promoting soil quality through increasing soil fertility and soil microbes. This study evaluated the effectiveness of using liquid food waste material (LFM) as a microbial resource, by analyzing the microbial community composition in LFM, and by isolating plant growth-promoting bacteria (PGPB) from the material. High-throughput sequencing of LFM, collected every month from May to September 2018, resulted in the detection of >1000 bacterial operational taxonomic units (OTUs) in the LFM. The results showed that *Firmicutes* was abundant and most frequently detected, followed by *Proteobacteria* and *Actinobacteria*. Of the culturable strains isolated from LFM, almost all belonged to the genus *Bacillus*. Four strains of PGPB were selected from the isolated strains, with traits such as indole acetic acid production and 1-aminocyclopropane-1-carboxylic acid deaminase activity. Lettuce growth was improved via LFM amendment with PGPB, and *Brassica rapa* showed significant differences in root biomass when LFM amendment was compared with the use chemical fertilizer. Field experiments using LFM showed slight differences in growth for *Brassica rapa*, lettuce and eggplant, when compared with the use of chemical fertilizer. LFM is a useful microbial resource for the isolation of PGPB, and its use as fertilizer could result in reduced chemical fertilizer usage in sustainable agriculture.

Keywords: bacterial community composition; liquid food waste materials (LFM); plant growth-promoting bacteria (PGPB); plant growth-promoting (PGP) traits

1. Introduction

Plant growth-promoting microorganisms (PGPM) are broadly accepted to enhance crop production [1]. Plant growth-promoting microorganisms enhance plant growth and development through a variety of functions, encompassing the increase of macro-nutrient availability to the host plant by assembly of growth-promoting chemicals [2], nitrogen fixation [3], solubilization of inorganic phosphate and mineralization of organic phosphate [4], production of different types of phytohormones-like organic compounds [5,6] and biological control of phytopathogens by synthesizing antibiotics and/or competing with harmful microorganisms [7,8]. Therefore, the continuous use of PGPM could lead to it replacing pesticides and chemical fertilizers [9].

On another front, the overuse of chemical fertilizers and continuous agricultural activities results in the deterioration of soil quality [10,11]. The associated loss of soil health, fertility and nutrient status leads to continuous input requirements. Crop nutrition needs can be met through the provision of inorganic as well as organic fertilizers and biofertilizers. Overreliance on inorganic fertilizers stretches the economics of the farming community, and also leads to consumption of available non-renewable

nutrient resources, compromises the potential plant-beneficial microbiome, and can have a severe environmental impact [12]. In contrast, the concerns around the use of organic fertilizers include that they are bulky, slow release, have inconsistent composition and can spread weed seeds, among other things. Therefore, sustainable solutions must be sought for crop production, while focusing on the utilization of all available resources. Organic waste production, which can be animal- or plant-based, including food leftovers, vegetable and fruit peels and market refuse, is a worldwide issue, and its disposal and treatment is increasingly important in developing countries [13]. The large amount of this waste that is produced is a major economic, social and environmental challenge [14], which is associated with extensive handling costs. There are great potential benefits to recycling and reusing this material in agriculture. Recent efforts have led to up to a 25% reduction in food waste in some parts of the world, however, in Japan, although food waste legislation has helped to reduce the volume of food waste produced, more needs to be done in addressing this issue, as reviewed by [15]. General waste, other than that generated by food processing industries and households, contains about 60% organic matter [16]. Hence, the separation of organic matter from general waste streams should be targeted, and treated as a resource rather than a problem [11,17].

Organic waste contains fatty acids, proteins and carbohydrates [18,19] among other constituents, which can be utilized as a source of crop nutrition. The application of organic waste materials in agriculture has been reported to reduce runoff, improve soil structure and increase soil biological activity [20]. In addition, some research has showed that local effective microorganisms (LEM) are a beneficial inoculant for the nitrogen mineralization of organic materials [21,22]. Therefore, the better management of organic waste materials could lead to preservation of soil quality and sustainable crop production [9]. Previous studies have explored the potential of the utilization of food and organic wastes in domestic, agricultural and industrial applications [11,23–25]. Among the variety of waste processing and manipulation procedures prior to their application in agriculture, most have had associated physical, chemical or biological problems. In the effective utilization of food waste, quick manipulation, easy operation and little or no reduction in the nutritional composition of the waste products are all considered publicly acceptable, and could increase the waste's potential for wide application and dissemination. Under this scenario, nutrient retention can be ensured, and minimal damage to the plant-beneficial microbes present in the food waste would be achieved. A food waste recycling facility started operating in 2014 in Kai-City, Yamanashi Prefecture, Japan, which collects food waste from school restaurants in the vicinity, processes the waste using lactic acid fermentation, and supplies the final product in liquid form to farmers (Kai City Biomass [26]). The food waste recycling facility has a structure divided into four phases. In the first phase, food wastes and water, along with an inoculum of microorganisms, such as lactic acid bacteria, are added, and the mixture is agitated and gradually moved to phases 2, 3 and 4. During that time, the pH drops to 3 and the temperature rises above 50 °C to promote fermentation. This liquid food waste material (LFM) has been used as a crop nutrient source by many farmers in the area. Although LFM has been mainly employed for use in agriculture and/or for energy production, the microbiological potential of the plant growth-promoting microbes in LFM has not been studied, to the best of our knowledge. In this study, we explored the microbial community composition of the final form of the recycled waste materials, and studied the ecology of those microorganisms, while also investigating the plant growth-promoting traits of the culturable bacterial isolates.

2. Materials and Methods

2.1. Liquid Food Waste Materials

The LFM was obtained in March 2017 and March 2018 for pot and field experiments, respectively, and in May, June, July, August and September 2018 for bacterial composition analysis (and in October for isolation of microbes), from the Biomass Center at Kai City, Yamanashi, Japan. A portion (50 mL) of the material each month was stored at −80 °C for DNA extraction and high-throughput sequencing.

The Kai City facility produces LFM from residues of local school-provided lunches using the lactic acid fermentation process at the rate of approximately 90 L/day^{-1}. The total carbon and nitrogen of the final form of fertilizer were 31.7% and 1.41%, respectively. The C/N ratio of LFM was 22.4. In addition, the pH of LFM was 3.42 because of the lactic acid fermentation process. Electrical conductivity (EC), nitrate–nitrogen (NO_3^--N), ammonium-N (NH_4^+-N) and available phosphate (Trough-P) were at the values of 6.65 mS/cm^{-1}, 0.95 mg/L^{-1}, 14.8 mg/L^{-1} and 0.69 mg/L^{-1}, respectively.

2.2. Assay of Liquid Food Waste Material (LFM) Utilization

An incubation experiment was carried out to assess the mineralization of NO_3^--N from the LFM according to a modified Soil Environmental Analytical Method, 1997. A total of 100 mL of the LFM material was weighed and mixed with 300 g of soil obtained from University of Yamanashi (UofY) Research Farm (hereinafter referred to as UofY farm soil); soil type is gray lowland soil (pH 6.79 ± 0.33; EC (mS/cm^{-1}) 0.11 ± 0.08; NO_3^--N 23.1 ± 3.13 mg/kg^{-1}; available phosphate 421 ± 86.8 mg/kg^{-1}). The pots were covered by aluminium foil and incubated for 14 weeks at 25 °C. Since rapeseed cake is used as an organic fertilizer, it was used as a control for nitrogen release after application to soil. The NO_3^--N content was measured via the alkali reduction diazo dye method (Soil Environmental Analytical Method, 1997).

2.3. Isolation of Bacteria from LFM

Bacterial isolation from LFM was performed through the dilution plating technique. A total of 1 mL of LFM and 4.0 mL of sterile distilled water was placed in a test tube and mixed thoroughly using a vortex mixer. Subsequently, 50-µL dilutions were taken from the first tube and spread onto Reasoner's 2A agar (R2A) media (Eiken Chemical Co. Ltd., Tochigi, Japan) using a disposable spreader; plates of each dilution were incubated at 25 °C for 3 days. Colonies appearing after 3 days were re-streaked until a single pure colony type per plate was achieved.

2.4. DNA Extraction and PCR Amplification for Culturable Bacteria

DNA was extracted from the isolated strains using the ZR Fungal/Bacterial DNA MiniPrep Kit™ (Zymo Research Corp., Irvine, CA, USA). 16S rRNA gene sequencing was carried out for identification of the strains. Extracted DNA from isolated strains was mixed with prior to PCR amplification. The universal primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 1378R (5′-TGTGCAAGGAGCAGGGAC-3′) were used to amplify the 16S rRNA gene on a TaKaRa PCR Thermal Cycler Dice® Series Gradient (Takara, Shiga, Japan). The PCR amplification conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min [27]. The amplification mixture for PCR (total volume: 25 µL) contained 1 µL of DNA template, 1 µL of each primer, 9.5 µL of sterilized distilled water and 12.5 µL of GoTaq Green Master mix (Promega, Madison, WI, USA). The amplification products (5 µL) were subjected to electrophoresis on a 1% (*w/v*) agarose gel in tris-acetate-ethylenediaminetetraacetic acid buffer at 100 V for 25 min, and visualized by GelRed™ staining (1:20,000 dilution; Biotium, Fremont, CA, USA). The DNA sequences obtained were compared with those previously reported in the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/), and the nearest neighbor was noted. The sequences of numbers 2, 4, 6 and 11 were submitted to DNA Data Bank of Japan (DDBJ).

2.5. High-Throughput DNA Sequencing

DNA was isolated from the stored LFM samples using the FastDNA™ Spin Kit for Soil (MP Biomedicals Japan, Tokyo, Japan). The DNA concentration was measured using a nano-spectrophotometer and DNA was diluted to 1 ng/µL^{-1} using sterile water. The V4 region of the 16S rRNA gene was amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with additional barcode sequences. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs

Japan Inc., Tokyo, Japan). The quality and quantity of PCR products was assessed by mixing equal volumes of a loading buffer (containing SYBR green) with PCR products and electrophoresing the samples on 2% (*w/v*) agarose gel. Samples with a bright main strip between 400 and 450 bp were chosen for further experiments. The PCR products were mixed in equal density ratios. Thereafter, the mixed PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The libraries—250 bp paired-end reads generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina (New England Biolabs Japan Inc.,Tokyo, Japan) and quantified via Qubit and quantitative PCR—were sequenced on an Illumina HiSeq 2500 platform. Quality control was performed at each step of the procedure. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) [28]. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags [29] according to the QIIME (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality control process [30]. The tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) [31] to detect chimera sequences (http://www.drive5.com/usearch/manual/chimera_formation.html). Next, the chimera sequences were removed [32], and the effective tags were finally obtained. Sequence analysis was performed via Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using all the effective tags [33]. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was used against the small subunit rRNA database of SILVA (http://www.arb-silva.de/) [34] for species annotation at each taxonomic rank (Threshold: 0.8–1) [35]. The phylogenetic relationship of the representative sequences of all OTUs was obtained by using MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/) for rapid comparison of multiple sequences [36]. The abundance of OTUs was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses were all performed based on this output normalized data.

The reads were submitted to the DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html) under Bioproject, and are available under accession number DRA010367.

2.6. Plant Growth-Promoting Traits of Isolates

Indole acetic acid production: The isolated strains were tested for indole-3-acetic acid (IAA) production. Cultures of each isolate were grown at 25 °C for 4 days in IAA production media (2 g beef extract, 3 g $CaCO_3$, 30 g glucose, pH 7 in 1 L of distilled water) with or without 1 mM (final concentration) tryptophan. The cultures were centrifuged at 10,000 g for 10 min. IAA production was measured in 300 µL of supernatant using 1.2 mL of Salkowski's reagent [37,38]; absorbance was measured at 535 nm in a spectrophotometer, and the concentration was estimated from a standard curve. Control/blank samples were prepared without bacterial inoculation.

1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and nitrogen fixation: DNA was extracted using ZR Bacterial/Fungal DNA MiniPrep Kit™ (Zymo Research Corp., Irvine, CA, USA). The PCR amplification conditions for ACC deaminase and *nif*H genes were as follows: 1 cycle of 95 °C for 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min using a T100™ Thermal Cycler (Bio-rad, Hercules, CA, USA). The PCR mixture (total volume: 25 µL) contained 1 µL of DNA template, 1 µL of 10 mM primers (Po1F (5′ TGCGAYCCSAARGCBGACTC 3′) and Po1R (5′ ATS GCC ATCATY TCR CCG GA 3′) [39] for *nif*H genes; ACCF (5′ GCCAARCGBGAVGACTGCAA 3′) and ACCR (5′ TGCATSGAYTTGCCYTC 3′) [40] for ACC deaminase), 12.5 µL of GoTaq® Green Master Mix and 9.5 µL sterilized distilled water. The PCR amplification products were checked via 1.0% of agarose gel electrophoresis, staining and visualization.

Siderophore production: A slightly modified Chrome Azurol S (CAS) method was used for determination of siderophore production by bacterial isolates [41,42]. A total of 100 mL of medium was prepared as follows: 7.3 mg of hexadecyl trimetly ammonium bromide, 6.04 mg of CAS, 3.04 g of piperazine-1,4-bis(2-ethanesulfonic acid) and 1 mL of 1 mM $FeCl_3 \cdot 6H_2O$. A quantity of 10 mL of the siderophore production medium was applied over the surface of agar plates containing cultivated microorganisms. The blue CAS agar changed to light yellow or orange if siderophores were produced by the bacteria; the siderophore production was evaluated by the following index: + color change, − no color change and ++ color change detected over the entire medium.

Phosphate solubilization: The medium developed by Pikovskaya [43] was used for qualitative estimation of calcium phosphate solubilization by the isolates. Selected strains were inoculated into the media and incubated for 7 days at 25 °C. Zones of clearance around the bacterial colonies were indicative of phosphate solubilization; the results were compiled on the basis of the following index: − No clear zone, ± detectable clear zone but very weak activity, + detectable clear zone.

2.7. Pot Experiments

Two pot experiments were conducted using UofY farm soil. The first compared selected bacterial isolates with an uninoculated control to evaluate the role of specific isolates, and the second compared LFM with an untreated control and a fertilizer control to determine the role of LFM in plant growth promotion. For the first experiment, 11 bacterial isolates, that were selected based on PGP traits, were compared with an uninoculated control in a *Lactuca sativa* var. crispa (lettuce) growth trial. Pots (size: 100 cm^2; Fujiwara Seisakusho, Ltd., Tokyo, Japan) were filled with 300 g of soil (dry weight) and soil moisture was maintained at 60% of water holding capacity daily. A suspension of each bacterial strain (grown for 48 h (stationary phase) at 25 °C with shaking in PDB medium) was applied to the pots while the same volume of uninoculated PDB was applied as the control. Subsequently, a lettuce seedling germinated on a petri dish was transferred to each pot. The plants were grown for five weeks, harvested, and the dry weight of aboveground and belowground parts was recorded after being put into the dry oven set at 60 °C. From the results of the pot experiment using lettuce, four isolates (numbers 2, 4, 6 and 11), which showed the maximum growth-enhancement of lettuce, were selected (data not shown), and then these strains were tested under similar growth conditions for *Brassica campestris* (brassica) and the same parameters were recorded. In the second experiment, the response to LFM was compared with that of chemical fertilizer and control treatments. To achieve this goal, 100 mL of LFM and chemical fertilizer (HYPONeX Japan Corp., Ltd., Osaka, Japan. Liquid Fertilizer, N:P:K = 6:10:5) was mixed with soil to achieve a final concentration of 200 mg/kg^{-1} soil N, while there was no amendment in the control pots. Similar growing conditions and parameters were recorded as for the first experiment.

2.8. Field Experiment Using LFM

A field experiment was conducted comparing chemical fertilizer with LFM at the University of Yamanashi Research Farm (35°60′39.5″ N, 138°57′82.9″ E). The field experimental plots (4 m × 2 m) were treated with LFM and chemical fertilizer. In this field, the chemical fertilizer plots have been continuously treated with chemical fertilizer, and cow compost was applied every two years in all subplots until the year before the study. Soil chemical properties were as follows: pH (H_2O) 7.0, EC 0.12 (mS/cm^{-1}), ex-Ca 2940 mg/kg^{-1}, ex-Mg 874 mg/kg^{-1}, ex-K 381 mg/kg^{-1}, CEC 14 ($cmolc/kg^{-1}$), Trough-P 344 mg/kg^{-1}, NH_4-N 5.8 mg/kg^{-1}, NO_3-N 59.4 mg/kg^{-1}. Two replicates were prepared for each of the test vegetables: *Brassica rapa* var., *Lactuca sativa* var. crispa (lettuce) and *Solanum melongena* (eggplant). LFM was input at the rate of 200 kg/ha^{-1}, 200 kg/ha^{-1} and 75 kg/ha^{-1} to the final concentration of soil N for brassica, lettuce and eggplant, respectively, whereas chemical fertilizer input was 200 kg/ha^{-1} soil N for all crops. *Brassica rapa* and lettuce were planted at 20 to 30 plants/plot, and eggplant was cultivated at 9 plants/plot. *Brassica rapa* was grown for 29 days and plant height was measured upon harvest. Lettuce was grown for 56 days; plant height and dry weight of the edible

part were measured at harvest. The eggplants were harvested when the fruits grew to a suitable size (around 120 g/fruit); the quantity and weight of the fruits were measured. Fruit harvest began on 12 August 2018 and continued until 29 September 2018.

2.9. Statistical Analysis

Analysis of variance (ANOVA) was carried out to determine the statistical effects of treatments in Statistix 8.0 (Analytical Software, Tallahassee, FL, USA) followed by pairwise comparison of treatment means using Tukey's honestly significant difference (HSD) test and multiple comparisons through Dunnett's test. For the veracity of sequencing data analysis, raw data was merged and filtered to obtain clean data. Effective data was used for operational taxonomic unit (OTUs) clustering. The clustering analysis was applied, and a clustering tree was constructed to study the similarities among different samples. The unweighted pair–group method with arithmetic means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage, and was conducted using QIIME software (Version 1.7.0).

3. Results

3.1. Isolation of Plant Growth Promoting Bacteria (PGPB)

The bacterial isolation from the LFM was performed via standard methods, through the serial dilution plating technique. Various different strains appeared in the media. The number of culturable bacteria in the LFM was 3.5×10^4 colony forming units/mL^{-1} of LFM. After isolation, 31 strains were randomly selected, and 11 out of those 31 strains were examined for plant growth-promoting (PGP) traits. The sequence of strain numbers 2, 4, 6 and 11 (approximately 940 nt; GenBank accession No. LC553393, LC553394, LC553395, LC553396) was compared with other bacterial nucleotide sequences in GenBank. All strains exhibited a high sequence similarity with *Bacillus* spp.

3.2. Identification of Culturable Bacteria

All 31 isolates that were identified belonged to genus *Bacillus* (Figure S2); these were type A—closely related to *Bacillus velezensis* strain FZB42 (frequency: 3.3%), type B—closely related to *Bacillus amyloliquefaciens* strain MPA 1034 (frequency: 56.7%), type C—closely related to *Bacillus vallismortis* strain NRRL B-14890 (frequency: 26.7%), type D—closely related to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28 (frequency: 3.3%), type E—closely related to *Bacillus wiedmannii* strain FSL W8-0169 (frequency: 3.3%), type F—closely related to *Bacillus velezensis* strain NTGB-29 (frequency: 3.3%), and type G—closely related to *Bacillus vallismortis* strain DSM 11031 (frequency: 3.3%).

3.3. PGP Traits

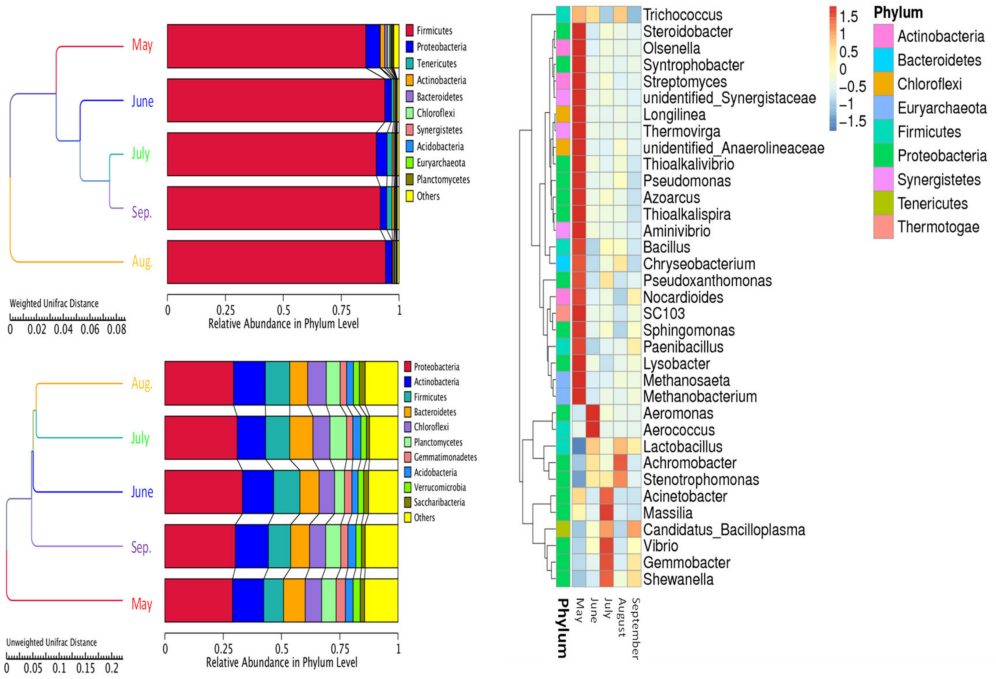
Because of the importance of microbial IAA production in influencing the root architecture and initial plant growth, IAA production was examined for 14 of the 31 isolates. Strain numbers 2, 4, 6 and 11 were positive for IAA production with tryptophan (Table 1). Amplification of the *nifH* gene confirmed N fixation potential in strain number 6, whereas amplification of the ACC deaminase gene was positive for all four selected strains (Table 1). Only strain number 11 showed zones of clearance on the Pikovskaya agar plates, indicating the phosphate solubilization ability of this strain (Table 1). Strain number 11 was also positive for siderophore production, with complete color change from blue to yellow, when compared with other non-siderophore-producing strains and the control that had a negative reaction.

Table 1. Plant growth-promoting traits, where + indicates the possession of the following trait, and – indicates the lack of the trait.

Strain No.	Indole-3-Acetic Acid (IAA)	Phosphate Solubilization	Nitrogen Fixation	1-Aminocyclopropane-1-Carboxylic Acid Deaminase	Siderophore
1	–	–	–	–	–
2	+	–	–	+	–
3	–	–	–	–	+
4	+	–	–	+	–
5	-	–	–	+	–
6	+	–	+	+	–
7	–	+	–	–	–
8	–	+	–	–	–
9	–	–	-	+	+
10	–	+	–	–	–
11	+	+	–	+	+

3.4. High-Throughput DNA Sequencing of LFM

In total, 192,355 reads were obtained; the average number of observed species per sample was 1013 ± 170 (max: 1306, min: 892), and the coefficient of variation was 0.17. The average bacterial composition was shown via the integration of the clustering results and the relative abundance of each sample by phylum (Figures 1a,b and S3). *Proteobacteria* were most frequently detected, followed by *Firmicutes* and *Actinobacteria*. The species composition at the phylum level was different in August when compared with that from the other months (Figure 1a). At the genus level, when the top 10 genera were compared between different months, the genera composition of the LFM in May was different from that from the other months (Figures 1a,b and S3). This is because there were few *Lactobacillus* spp. at this time, and the fermentation was in the early stages.



(**a**) (**b**)

Figure 1. (**a**) Unweighted pair–group method with arithmetic means cluster tree based on unweighted and weighted unifrac distance. This was displayed with the integration of clustering results and the relative abundance of each sample by phylum. (**b**) The top 10 taxa at the genus level were selected to form the distribution histogram of relative abundance.

3.5. Incubation Study of LFM Utilization

The release of NO_3^--N and phosphate was used to determine the potential for nutrient provision from LFM. The release of NO_3^--N started from 14 days in the LFM, and approximately 200 mg/kg^{-1} of NO_3^--N had accumulated by the end of 14 weeks of incubation (Figure S1a). Phosphate availability followed the same trend as that of NO_3^--N release (Figure S1b). Available phosphate was rapidly released from rapeseed cake, whereas no phosphate was detected from LFM until week 2. From week 3, the availability of phosphates increased slightly in the LFM.

3.6. Pot Experiments

A pot experiment was conducted to examine the effect of selected strains on the growth of brassica and lettuce. In the initial experiment on lettuce, 11 strains were tested; 4 of these strains showed high activity when compared with the control. Therefore, strains 2, 4, 6 and 11 were tested on brassica, and showed significant differences in their growth-promoting effect (Figure 2). A pot experiment was also conducted to assess the effect of LFM on the growth of both brassica and lettuce. There was no significant difference in the growth characteristics of lettuce between the LFM and chemical fertilizer treatments (Figure 3a); however, brassica exhibited significant differences in its root biomass with LFM amendment (Figure 3b, Tukey's HSD, $p < 0.05$).

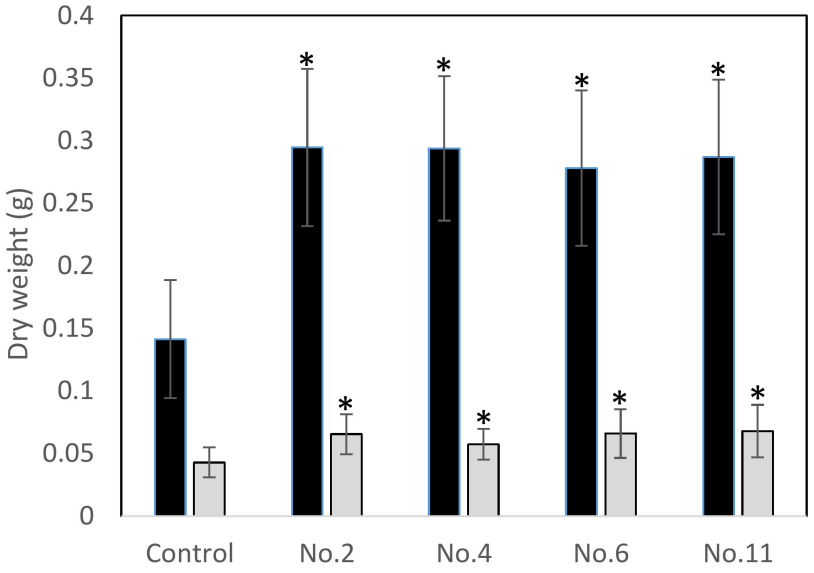
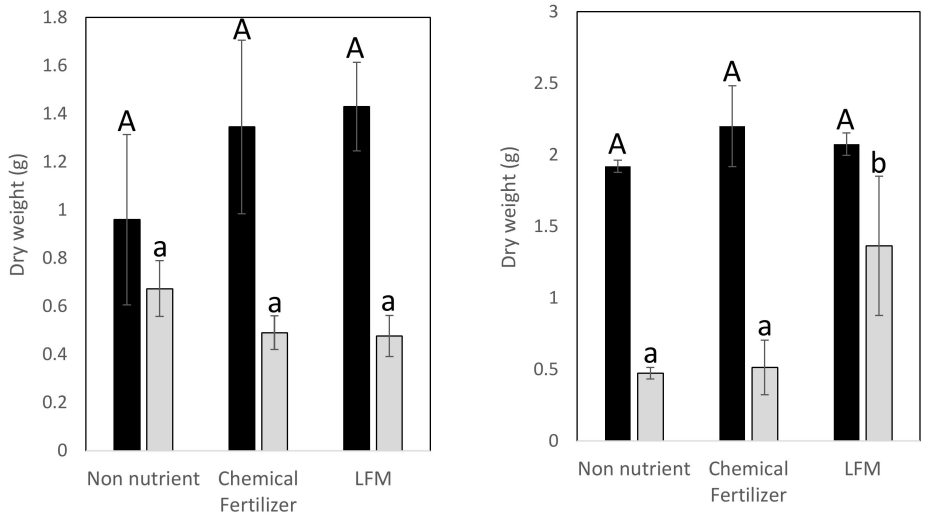


Figure 2. Growth response of *Brassica campestris* with plant growth-promoting bacteria selected in this study. Control (n = 7), strain number 2 (n = 10), strain number 4 (n = 10), strain number 6 (n = 7), strain number 11 (n = 10), Dunnett test ($p < 0.05$). The vertical bar indicates the standard error. * indicates significance differences between treatments when compared with the control ($p < 0.05$).

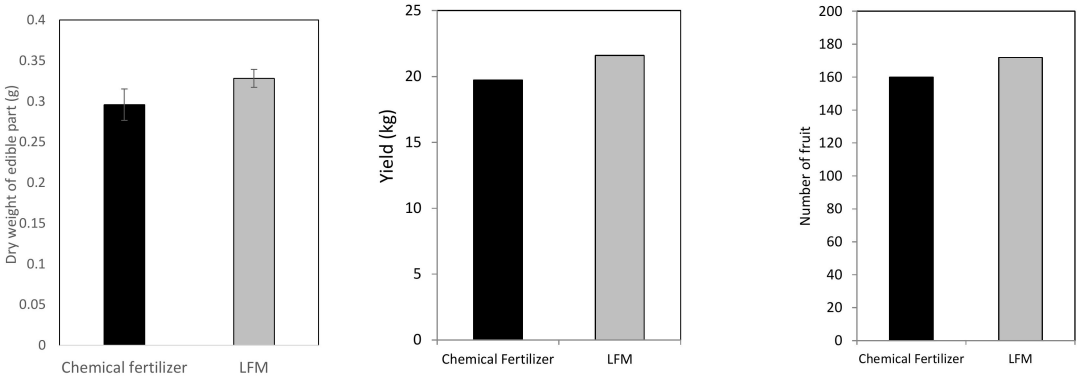


(**a**) (**b**)

Figure 3. Pot experiment testing lettuce (**a**) and *Brassica rapa* (**b**) growth using liquid food waste materials (LFM). Values presented are means and standard error ($n = 3$). Closed bar and Gray bar mean edible part and root, respectively. Treatments of the same crop with different letters are significantly different by Tukey's HSD ($p < 0.05$).

3.7. Field Experiments

Field experiments were conducted to assess the effect of LFM on the growth of *Brassica rapa*, lettuce and eggplant. The growth of *Brassica rapa* and lettuce in the field was similar to that in the pot experiment. The heights achieved by *Brassica rapa* were 33.0 ± 0.67 cm and 32.5 ± 0.78 cm, with LFM and chemical fertilizer, respectively. The lettuce grown with LFM amendment was slightly larger than that grown with chemical fertilizer, but not significantly so (Figure 4a). Eggplant also grew slightly better with LFM than with chemical fertilizer, but the differences were not significant (Figure 4b,c for eggplant).



(**a**) (**b**) (**c**)

Figure 4. Field experiment testing the effect of liquid food waste materials (LFM) on the growth of Lettuce (**a**) and eggplant (**b**,**c**).

4. Discussion

This study showed that useful microorganisms, such as PGPB, were present in LFM produced from the recycling of unused resources. This is an important finding that leads to the promotion of recycling, and also indicates that unused resources are useful as microbial resources. LFM can be used

as a fertilizer, and has other positive effects on vegetable growth. However, there are limitations to the plant growth-enhancement functions of LFM. If we apply isolated strains to LFM in order to promote a plant growth function more effective than that of the original LFM, some supplementary nutrients are needed to produce an effect. This point still remains an issue.

High-throughput sequencing analysis of the bacterial community composition in the LFM revealed substantial differences between the sampling months May and August. A similar bacterial community composition was observed for the samples from June, July and September. The May sample was still in the early stages of fermentation, and there was no school-provided lunch in August; therefore, LFM was stored in the tank, which might help to explain the differences in the community composition of these samples.

Most of the bacteria isolated from LFM were *Bacillus* spp., which is a spore-forming bacteria. This was because the LFM pH was reduced to 3.0 through the process of lactic acid fermentation, and the temperature exceeded 50 °C. However, this result was obtained because we used R2A media for isolation, and we detected the family of *Lactobacillaceae* in the LFM through high-throughput sequencing (Figure S3). The most frequently isolated *Bacillus* spp. strains were *Bacillus amyloliquefaciens* (56.7%) and *Bacillus vallismortis* (26.7%). *Bacillus* species are known to produce dormant spores [44], and enact an anti-pathogen activity through the assembly of non-ribosomal cyclic lipopeptides [45]. In addition, *Bacillus* species are considered PGPB because of their potential for antibiotic production, biofilm formation on the plant root surface, and production of plant hormones [46,47]. Furthermore, seed treatment with *Bacillus* species has been shown to significantly enhance shoot fresh and dry weight, as well as plant height, in various crops [48,49]. From the results of this study, >1000 bacterial OTUs were identified in LFM; therefore, it might be possible to isolate other useful strains, other than *Bacillus* spp., under a range of isolation conditions, including increased pH.

Organic materials slowly release nutrients, but they are still a promising alternative to chemical fertilizers, as their application can reduce nutrient leaching, volatilization and problems of toxicity [50]. In the present study, LFM was used as an alternative to chemical fertilizers in order to investigate the release of NO_3^--N and available phosphate (Figure S1). Low amounts of available phosphate were released from LFM during the incubation study because of the low total phosphate concentration in LFM. LFM released NO_3^--N from the third week of incubation, and released approximately 200 kg/ha^{-1} NO_3^--N during the 13 weeks of incubation. Moreover, the biomass richness of soil fertilized with LFM was higher than that treated with chemical fertilizer [51], and LFM did not change the soil pH after treatment through our study.

The growth-promoting effect of strain number 11, with an IAA-producing ability and an ACC deaminase activity, was confirmed in a pot experiment; growth was significantly promoted with inoculation by strain number 11, when compared with the control (Figure 2). The growth of lettuce in a pot was the same with both LFM and chemical fertilizer. For *Brassica rapa*, the growth of the edible (aboveground) part was the same with both LFM and chemical fertilizer, whereas the root biomass was significantly increased with LFM, when compared with chemical fertilizer (Figure 3b). These results indicate that the PGPB in LFM contributed to the increase in root biomass of *Brassica rapa*. A field experiment was conducted to assess the effect of LFM on the growth of *Brassica rapa*, lettuce and eggplant. Although the N input by LFM was less than half that of the chemical fertilizer, the growth of the eggplant with LFM was the same as that with chemical fertilizer. The yield with LFM was higher than, but not significantly different from, that achieved with chemical fertilizer. These results indicate the abundance and activity of PGPB in LFM, and their efficacy in supporting eggplant growth under the conditions tested. LFM could be a viable alternative to commercially available chemical fertilizers, without an adverse effect on soil and vegetable growth. A previous study showed the positive effect of PGPB inoculation on vegetable growth and yield [52].

All the selected strains that showed growth-enhancement in the pot experiment had an IAA-producing ability (Table 1). IAA is a type of plant hormone that promotes root elongation and enhances root growth. Many PGPBs with the IAA-production ability have been isolated in

previous studies [1,53]. Furthermore, all strains that were positive for IAA production also showed ACC deaminase activity (strain numbers 2, 4, 6 and 11). This suggests that IAA production and ACC deaminase activity contribute greatly to enhancing the plant growth in our isolated strains, while *Caulobacter* sp. had a negative impact on plant growth, even though it produced higher levels of IAA [54]. In addition, previous studies have shown that PGP microbes and PGPB can promote plant growth indirectly or directly, through the production of ACC deaminase and through reducing the ethylene level in the developing plants through the roots [52,55], by generating plant growth hormones like IAA [56]. It is likely that ACC deaminase and IAA production promote root growth in a similar fashion [57,58]. Of the selected strains, only strain number 11 showed phosphorus solubilizing potential and siderophore production (Table 1). Phosphate solubilization is effective in soils with low available phosphoric acid, and siderophore production chelates the iron in soils with high pH to help plant uptake [52,59]. However, the detailed mechanism of plant growth-enhancement is complex, and further investigation is needed [54,60].

The selection of PGPB strains from LFM was important to confirm the positive effect of the inoculants on plant growth, and to optimize their application for maximum impact on vegetable crops. The main aim of this study was to reduce the commercial use of chemical fertilizers, by utilizing LFM as an alternative fertilizer with the maximum impact on crop growth and soil, and minimal environmental impact. Further investigation into LFM use as an organic fertilizer should evaluate any adverse impact of its application to the soil environment.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/7/954/s1, Figure S1: Nitrate nitrogen (**a**) and available phosphate (**b**) release from organic materials, • rapeseed oil cake, ▲ Liquid food waste material (LFM). Figure S2: 31 strains isolated from liquid food waste material belong to genus *Bacillus*. Type A closely related to *Bacillus velezensis* strain FZB42, type B closely related to *Bacillus amyloliquefaciens* strain MPA 1034, type C closely related to *Bacillus vallismortis* strain NRRL B-14890, type D closely related to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, type E closely related to *Bacillus wiedmannii* strain FSL W8-0169, type F closely related to *Bacillus velezensis* strain NTGB-29, and type G closely related to *Bacillus vallismortis* strain DSM 11031. Figure S3: Top 30 bacterial compositions, in different taxonomic levels.

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