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Biochemical and Microbiological Soil Effects of a Biostimulant Based on *Bacillus licheniformis*-Fermented Sludge

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Abstract: Biostimulants are substances and/or microorganisms that are applied to plants or to the rhizosphere in order to enhance the natural process improving the absorption of nutrients and the quality of crops as well as the tolerance to abiotic stresses. A new biostimulant was developed from sewage sludge through its fermentation with *Bacillus licheniformis* as a plant growth-promoting bacteria (PGPB). The fermented product includes three classes of biostimulant components: the *B. licheniformis* biomass; the enzymatic secretion of said microorganism, which are mainly peptidases and amidases related to nitrogen metabolism and glucanases, related to carbohydrate metabolism; and finally, the hydrolyzed sludge organic matter, with a high content of protein hydrolysates. The biostimulant was evaluated in soil at the biochemical (enzymatic activities) and microbiological levels (metabarcoding analysis). Metabarcoding analysis revealed that the biostimulant complex, mainly the soluble fraction containing the *Bacillus* multienzyme complex and protein hydrolysate, induced PGPB soil bacteria, and it was detected that the inoculation in the soil of *B. licheniformis* remained active throughout the study. These results show the fermentation process with *B. licheniformis* as an interesting option for the total valorization of activated sewage sludge aimed at obtaining products of agronomic/environmental interest.

Keywords: sludge; biostimulants; *Bacillus*; enzymes; biodiversity

Citation: Caballero, P.; Macías-Benítez, S.; Moya, A.; Rodríguez-Morgado, B.; Martín, L.; Tejada, M.; Castaño, A.; Parrado Rubio, J. Biochemical and Microbiological Soil Effects of a Biostimulant Based on *Bacillus licheniformis*-Fermented Sludge. *Agronomy* **2022**, *12*, 1743. https://doi.org/10.3390/agronomy12081743

Academic Editor: José L. S. Pereira

Received: 15 June 2022
Accepted: 19 July 2022
Published: 23 July 2022

1. Introduction

Biostimulants are natural substances that, in small doses, promote plant development and growth by improving nutrient intake and bioavailability and conferring resistance to such abiotic stresses that may affect crops [1]. The soil application of these biostimulants has shown a positive effect over the soil biological fraction, which have a direct implication over the soil fertility [2–4]. Biostimulants induce microbial stimulation and the enhancement of specific enzymes involved in the nutrient recycling in soil, which have been established as indicators of the quality and state of the fertility of soil [5,6]. Moreover, biostimulants have shown an enhancing effect of the soil microbiota involved in the bioremediation of polluting compounds [7,8].

The use of biostimulants, particularly the natural ones, can play an important role in the sustainable development of cropping systems [9,10]. The development of new biostimulants occurs through the development of economically viable bioprocesses [2]. In this way, it leads to the choice of low-priced organic by-products with the absence of toxins, their economically viable collection and storage, their production in large quantities and on a non-seasonal basis, and the absence of competition with other uses for them [11].

Considering these factors, sewage sludge, which is the inevitable organic by-product resulting from the treatment of wastewater, is an ideal raw material for the formulation of biostimulants as long as it does not exceed the limit values for organic pollutants, nor heavy metals, and is sanitized in order to eliminate the pathogenic microorganisms that comprise it.

In recent years, our group has made advances in this field by applying enzymatic and/or fermentative technology. We obtained biostimulants composed of low molecular weight peptides, free amino acids, and microbial metabolites such as phytohormone analogues, polysaccharides, humic substances, etc. as well as in the case of fermentations, the microorganisms of agronomic interest that are used to perform such processes [12,13]. These biostimulants show positive effects not only on the stimulation of the soil microbiota, having implications over the soil fertility [13,14] and enhancing the degradation of polluting compounds in soil [7].

Recent results in our group have revealed that the exogenous application of naturally-produced-in-soil microbial enzymes yielded interesting results not only at the biochemical level, stimulating soil microbial enzymes, but also over the microbial biodiversity [15]. Thus, by applying subtilisin from *Bacillus licheniformis*, we found an interesting stimulation of PGPB. Knowing such changes in depth is something that is gaining increasing interest nowadays thanks to the advances in metabarcoding techniques using 16S rRNA sequencing, which allows for the detection of variations that occur in the microbial biodiversity in the soil [16].

In this work, we describe a fermentative technology applied to a sludge from slaughterhouse wastewater for conversion into a biostimulant and to evaluate its biostimulating capacity both at the biochemical level and over bacterial biodiversity.

2. Materials and Methods

2.1. Obtaining the Biostimulant

Sludge was supplied by the wastewater treatment plant of the slaughterhouse *Ntra. Señora del Carmen* located in Morón de la Frontera (Sevilla, Spain).

Biostimulant products were obtained through a physical-fermentative process as described by Rodriguez-Morgado [13]. Sludge was first physically conditioned by concentration (up to 63.1 $\pm$ 0.17 g L^{-1}) and by autoclaving (121 °C, 30 min) in order to sanitize it. Next, it was subjected to a fermentation process carried out by *Bacillus licheniformis* (ATCC 21415). The fermentation took place in a 1 L fermenter for 6 days under constant conditions of temperature and stirring (45 °C and 150 rpm, respectively). Fermented sludge (the first biostimulant product, FS) was then separated by centrifugation (12,000$\times$ *g*, 30 min, 4 °C) into its insoluble and soluble fractions (second and third biostimulant products, IFS and SFS, respectively).

Before applying to soil, the humidity of the three biostimulants was matched at 70 g L^{-1} by concentration using a rotary evaporator (45 °C, vacuum pressure). The scheme of the process is detailed in Figure 1 and the products obtained are listed below:

Fermented sludge (FS): This is the biologically modified sludge after fermentation with Bacillus. Two fundamental changes have occurred in this process: a large part of the C and N has been converted into bacterial biomass, and an enzymatic solubilization of part of the organic components present in the sludge, specifically proteins, has occurred.

Insoluble Fermented Sludge (IFS): This is an insoluble product, composed of the *Bacillus* biomass together with all of the insoluble matter of fermented sludge that has not been metabolized or solubilized by the hydrolytic enzymes of *Bacillus*.

Soluble Fermented sludge (SFS): This is a soluble product, composed of the enzymatic secretion of *Bacillus* and by highly bioavailable soluble hydrolyzed organic matter, mainly composed of peptides and free amino acids.

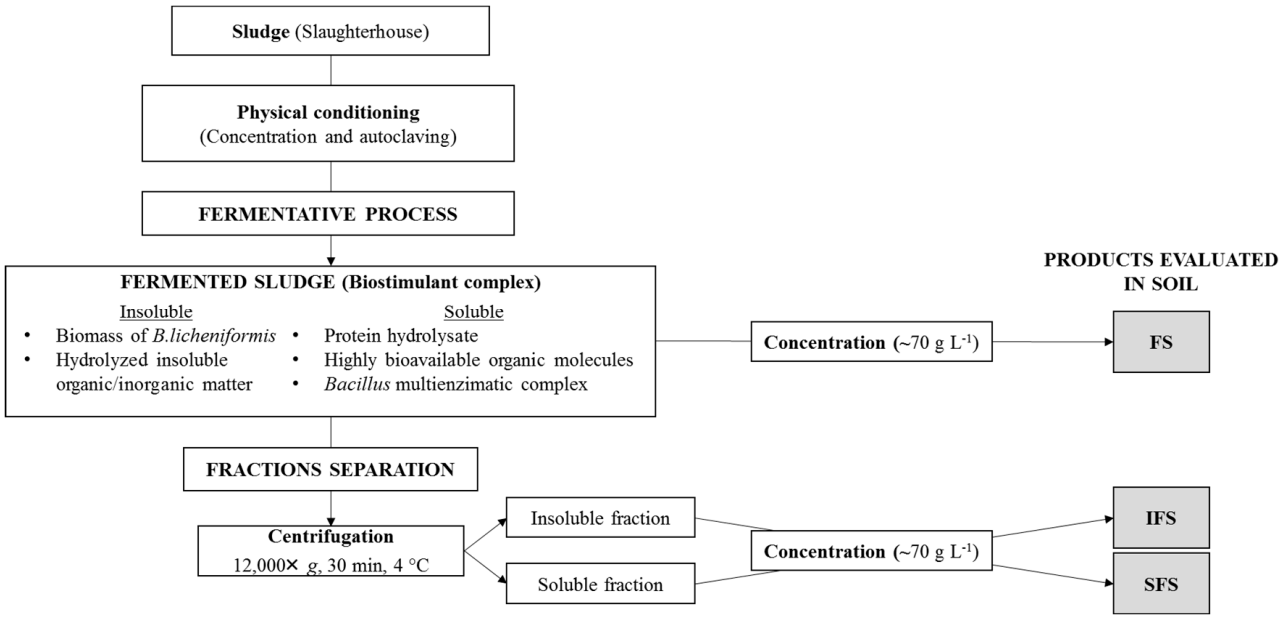


Figure 1. A diagram of the process to obtain the experimental products.

2.2. Chemical Characterization of the Biostimulant Products

The total dry matter content of the products was determined according to the methods standardized by the APHA (*American Public Health Association*) [17].

The total C and N contents were analyzed using an elemental analyzer (LECO TruSpec CHNS Micro, Leco Instrumentos SL, Madrid, Spain).

Macro- and microelements in raw sludge and the different products obtained after fermentation (FS, IFS, SFS) were analyzed in combusted samples by inductively coupled plasma and atomic emission spectrometry (ICP-AES) by using multi-element sequential equipment (Fisons-ARL 3410) with a data acquisition and control system.

The molecular size distribution profile of the soluble organic component of the fermented product was determined by HPLC size exclusion chromatography, measuring the absorbance at 215 nm following the operational parameters described in a previous work [18].

The soluble content was determined by relating the soluble dry matter to the total dry matter using the following formula:

$$\text{Soluble content } (\%) = \frac{\text{Soluble dry matter} \cdot 100}{\text{Total dry matter}}$$

2.3. Microbial and Enzymatic Characterization of Fermented Product

The *Bacillus* concentration and proteomic *Bacillus* secretion were analyzed in the fermented product.

2.3.1. *B. licheniformis* Concentration

This was determined by counting colonies in Petri dishes with LB agar medium. Units of bacterial concentration are expressed as colony-forming units per gram of product (CFU g^{-1}). Logarithmic dilutions of the products were made in sterile saline until countable concentrations of CFU were reached on the plates.

2.3.2. Proteomic Study

Given that the microbial community that conforms sludge includes the genus *Bacillus*, the basal expression of *Bacillus'* proteins in sludge was compared by mass spectrometry with the after fermentation.

Samples were centrifuged (14,000× *g*, 4 °C, 20 min) and the pellet was discarded in order to remove cellular debris and other insoluble particles. The soluble fraction was concentrated by ultrafiltration (*Vivaspin 20 filters*, 10,000 MWCO PES, *Sartorius Biolab Products*, Germany). Sample preparation and LC-MS analysis were carried out following the procedure described by Parrado et al. [19].

LC–MS analysis was performed in a Surveyor HPLC system in tandem with a Finnigan LTQ mass spectrometer (Thermo Electron, Bremen, Germany). A total of 5 µL of sample was injected into a C18 PepMap100 µ-Precolumn Cartridge (Dionex, Amsterdam, The Netherlands) for preconcentration and washing, then resolved in a Biobasic C18 75 µm × 10 cm column (ThermoFisher Scientific, Waltham, MA, USA). Peptides were eluted with a 120-min gradient of 5% acetonitrile with 0.1% formic acid to 40% acetonitrile with 0.1% formic acid, at a nominal post-split flow rate of 250 µL min^{-1}. The LTQ was run in positive ion mode using the nanospray source. The spray voltage was set at 2 kV, and the capillary temperature was set at 170 °C. The samples were scanned in the range of 400–1500 m/z using the full scan mode, and data dependent MS/MS on the top five ions with CID was carried out with the dynamic exclusion set to on.

The data were converted to SEQUEST format (DTA) and searched using a Sequest search engine with Proteome Discover 1.4 software, matching it to the UniProt-Bacillaceae and UniProt-*Bacillus licheniformis* databases.

2.4. Design of the Soil Biostimulation Study

The experimental design was stablished according to previous studies [15]. Thus, microcosms of 250 g of soil were preincubated at 30–40% of their water holding capacity for 7 days. After this phase, each product was added to the soil under the following experimental conditions:

C: Soil without addition of any product was used as the control.

S_{FS}: Soil with addition of fermented sludge.

S_{IFS}: Soil with addition of insoluble fraction of fermented sludge.

S_{SFS}: Soil with addition of soluble fraction of fermented sludge.

Each product was evaluated at two different concentrations, 0.1 and 0.5% w/w (dry matter).

2.5. Soil Analysis

2.5.1. Determination of Soil Enzymatic Activities

Soil enzymes were monitored during 28 days after application of the products in order to obtain a global vision of how the sludge-based biostimulants were acting in the soil at the biochemical level. By responding immediately to the changes in the soil fertility status, soil enzymes such as those involved in the nutrient turnover (phosphatases, β-glucosidases) and key enzymes in cellular energy metabolism (dehydrogenases) are considered as good soil quality indicators [5,6].

Dehydrogenase activity was measured as the reduction of 2-p iodophenyl-3-p nitrophenyl 5-phenyltetrazolium chloride (INT) to iodonitrotetrazolium formazan (INTF), as described by García et al. [20].

Phosphatase activity was determined using p-nitrophenyl phosphate as the enzyme substrate, which was hydrolyzed to produce p-nitrophenol (p-NF), a phosphate molecule and a proton. The determination of the activity was carried out as described by Tabatabai and Bremner [21].

β-glucosidase activity was determined using p-nitrophenyl-β-d-glucopyranoside as the substrate of the enzyme, which upon hydrolyzing releases the p-nitrophenol (p-NF) molecule that is quantifiable by spectrophotometry. The determination of the activity was carried out as described by Masciandaro et al. [22].

2.5.2. Metabarcoding Analysis

Changes produced in the soil bacterial biodiversity were studied through a metabarcoding analysis performed using the 16S rRNA marker.

Soil DNA extraction was performed using the DNeasy Power-Soil DNA Isolation Kit (Qiagen) according to the manufacturer's instructions. Illumina MiSeq sequencing and the analysis of the microbial community composition were performed as described previously [23].

Soil DNA Extraction and Illumina MiSeq Sequencing: Total genomic DNA was extracted from the soil samples using the DNeasy Power-Soil DNA Isolation Kit (Qiagen) according to the manufacturer's instructions.

For library preparation, the V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primer pair Bakt 341F (5′ CCTACG GGN GGC WGC AG 3′)/ Bakt 805R (5′ GAC TAC HVG GGTATC TAA TCC 3′) [24] as the forward and reverse primers with the Illumina-specific sequencing sequences attached to their 5′ ends.

The barcoding sequences required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions but with only five cycles and with 60 °C as the annealing temperature. A negative control containing no DNA was included in order to check for contamination during the library preparation.

Analysis of Microbial Community Composition: Sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0) as described previously [25]. Raw FASTQ files were demultiplexed, trimmed by CUTADAPT 1.3, merged by FLASH, and quality–filtered and labeled by QIIME 1.9.0 with the following criteria: (i) sequences whose overlap exceeded 30 bp were merged according to their overlap sequence; (ii) primers were matched allowing two nucleotide mismatches, (iii) reads shorter than 300 nucleotides were removed; and (iv) merged reads were quality-filtered at a minimum Phred quality score of 20. All chimeric sequences were identified and removed by the UCHIME algorithm implemented in VSEARCH by using the Greengenes reference database. The sequences were then clustered into operational taxonomic units (OTUs) using the de novo approach at the 100% identity threshold. Singleton OTUs were filtered out, and the representative sequence for each OTU was assigned to a microbial taxon using the RDP classifier with a confidence threshold of 97%.

Alpha diversity indices Chao, Good's coverage, Simpson, Shannon, and phylogenetic diversity were calculated to analyze the complexity of species diversity in each sample. Operational taxonomic unit data files generated by QIIME were imported into R version 3.5.1 to further process and visualize the results using the phyloseq, Vegan, and ggplot2 packages [26].

2.6. Statistical Analysis

Soil enzymatic activities resulting from the application of different treatments were compared using a one-way analysis of variance (ANOVA), followed by a Tukey test. The level of significance was established at $p < 0.05$.

3. Results

3.1. Characterization of Biostimulant Products

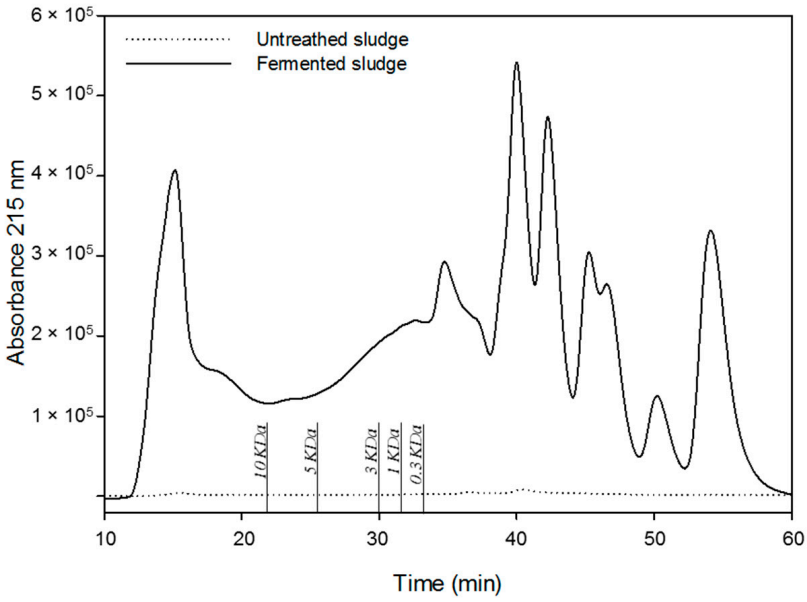
The initial sludge was totally insoluble and it did not present any soluble fraction, while the soluble content of the fermented sludge, FS, reached 14.6% of the total dry matter.

The bacterial concentration (*B. licheniformis*) in the FS was $2.01 \times 10^8 \pm 1.11 \times 10^8$ CFU g^{-1}.

The chemical characterization and molecular size distribution profile of the soluble organic fraction of the biostimulant products are shown in Table 1 and Figure 2 respectively.

Table 1. The chemical characterization of the soluble organic component of the different fermented sludge-based biostimulants (media ± SD, n = 3).

	Fermented Sludge	Insoluble Fraction of Fermented Sludge	Soluble Fraction of Fermented Sludge
pH	8.82 ± 0.09	8.82 ± 0.09	8.82 ± 0.09
Organic matter % *w*/*w*	71.26 ± 0.31	68.88 ± 0.47	79.66 ± 0.22
C (% *w*/*w*)	36.20 ± 0.03	34.73 ± 1.21	40.23 ± 1.07
N (% *w*/*w*)	5.63 ± 0.01	4.56 ± 0.40	7.94 ± 0.36
P (mg Kg^{-1})	17,663.19 ± 0.51	18,896.71 ± 0.15	6338.29 ± 0.03
K (mg Kg^{-1})	4452.18 ± 1.38	3521.13 ± 0.79	8289.96 ± 1.97
S (mg Kg^{-1})	17,219.07 ± 0.42	18,853.05 ± 0.15	6303.72 ± 0.21
Si (mg Kg^{-1})	10,313.57 ± 0.26	11,623.00 ± 0.09	≤3.72
Sn (mg Kg^{-1})	≤0.24	≤0.24	≤0.24
Al (mg Kg^{-1})	5690.70 ± 0.05	6570.89 ± 0.04	215.99 ± 0.01
Ca (mg Kg^{-1})	35,608.16 ± 4.07	40,610.33 ± 4.67	7955.39 ± 1.84
Cd (mg Kg^{-1})	≤0.24	≤0.24	≤0.24
Cr (mg Kg^{-1})	9.06 ± 0.00	21.13 ± 0.00	2.23 ± 0.00
Cu (mg Kg^{-1})	161.13 ± 0.02	166.67 ± 0.05	134.94 ± 0.04
Fe (mg Kg^{-1})	9388.99 ± 0.02	10,848.83 ± 0.06	1208.18 ± 0.40
Mg (mg Kg^{-1})	5014.23 ± 0.02	5314.55 ± 0.09	2814.13 ± 0.08
Mn (mg Kg^{-1})	167.97 ± 0.01	192.49 ± 0.01	6.69 ± 0.00
Na (mg Kg^{-1})	3548.29 ± 0.47	2499.06 ± 0.36	9209.29 ± 1.57
Ni (mg Kg^{-1})	22.57 ± 0.00	23.47 ± 0.00	7.99 ± 0.00
Pb (mg Kg^{-1})	56.96 ± 0.00	61.03 ± 0.01	30.86 ± 0.00
Zn (mg Kg^{-1})	1080.36 ± 0.01	1251.17 ± 0.04	208.18 ± 0.00
Mo (mg Kg^{-1})	≤0.24	≤0.24	≤0.24
Se (mg Kg^{-1})	≤0.47	≤0.47	≤0.47
Hg (mg Kg^{-1})	0.04 ± 0.00	0.05 ± 0.00	0.01 ± 0.00



	Untreated sludge (%)	Fermented sludge (%)
> 5 KDa	ND	25.62 ± 0.06
5 – 3 KDa	ND	2.33 ± 0.01
3 – 1 KDa	ND	6.1 ± 0.02
< 1 KDa	ND	65.95 ± 0.09
1 – 0.3 KDa	ND	8.76 ± 0.03
< 0.3	ND	57.19 ± 0.06

Figure 2. The molecular size distribution profile of the organic fraction of the untreated sludge and after fermentation with *B. licheniformis*.

Proteomic characterization: Fermentation induced a high diversity of secreted proteins, mainly comprised of proteins with hydrolytic and transport functions (Table 2). Secreted hydrolases, which are mainly produced during the stationary growth phase [27], were 50% peptidases and amidases, related to N metabolism, and 33.3% glucanases, related to carbohydrate metabolism.

Table 2. The identification of *Bacillus* extracellular proteins in both the untreated and fermented sludge with *B. licheniformis*, using the Sequest search engine pitted against the UniProt database. ([a]) Common proteins in untreated and fermented sludge.

Access	Description	Score	Function
Basal proteins of *Bacillus* in the unfermented sludge			
A0A0M0KXG6	Chemical-damaging agent resistance protein C	3.32	Stress
A0A160M9Z6	Phage tail protein	2.35	Structural
A0A0K9M8G9	Formamidase	3.3	Hydrolase (Amidase)
A0A0J5VPC3	Peptidase S8	5.35	Hydrolase (Endopeptidase)
V6SXF8	Peptidase S8	10.16	Hydrolase (Endopeptidase)
A0A0Q3WA41	Elongation factor G	2.71	Protein synthesis
A0A0D6ZBQ0	Peptide-binding protein	6.77	Transport
A0A135L4D3	Peptide ABC transporter substrate-binding protein	2.17	Transport
A0A160M9B5	ABC transporter substrate-binding protein	7.53	Transport
N0ASW2	Bmp family lipoprotein	2.14	Transport
Fermented sludge with *B. licheniformis*			
A0A0M0KXG6 [a]	Chemical-damaging agent resistance protein C	3.21	Stress
A0A068NC77	Cell surface protein	2.11	Structural
A0A068NDT9	Collagen adhesion protein	3.08	Structural
A0A068NE02	Cell wall anchor domain-containing protein	3.25	Structural
C3FA89	Spore coat protein GerQ	3.04	Structural
F0PM11	Hydrolase, alpha/beta fold family protein	3.41	Hydrolase
A0A0C2Y2U4	Formamidase	2.55	Hydrolase (Amidase)
A0A0K9M8G9 [a]	Formamidase	8.73	Hydrolase (Amidase)
A0A0A8X646	Aminopeptidase Y (Arg, Lys, Leu preference)	7.42	Hydrolase (Aminopeptidase)
Q93EJ5	Leucine aminopeptidase	4.01	Hydrolase (Aminopeptidase)
T5HJ93	Aminopeptidase	4.01	Hydrolase (Aminopeptidase)
W7R6U5	Aminopeptidase	4.01	Hydrolase (Aminopeptidase)
A0A0A8XED3	Subtilisin	6.68	Hydrolase (Endopeptidase)
A0A0J5VPC3 [a]	Peptidase S8	8.93	Hydrolase (Endopeptidase)
A0A0U1NYI7	Subtilisin-like serine protease	2.06	Hydrolase (Endopeptidase)
P29599	Subtilisin BL	12.39	Hydrolase (Endopeptidase)
P29600	Subtilisin Savinase	5.1	Hydrolase (Endopeptidase)
V6SXF8 [a]	Peptidase S8	11.85	Hydrolase (Endopeptidase)
A0A0D1IL93	Beta-glucanase	3.1	Hydrolase (Glucanase)
A0A0W8K3R3	Beta-glucanase	3.1	Hydrolase (Glucanase)
D0EWD5	Beta-1,3-1,4-glucanase	3.1	Hydrolase (Glucanase)
D7GAY2	Licheninase	3.1	Hydrolase (Glucanase)
Q6UNS4	Beta-1,3-1,4-glucanase	3.1	Hydrolase (Glucanase)
Q84GK1	Beta-1,3-1,4-endoglucanase (Fragment)	3.1	Hydrolase (Glucanase)
Q8GMY0	Beta-1-3,1-4-endoglucanase	3.1	Hydrolase (Glucanase)
W7R9E9	Beta-glucanase	3.1	Hydrolase (Glucanase)
A0A0K9GB73	UPF0173 metal-dependent hydrolase AC622_04030	3.17	Hydrolase (Beta-lactamase)
A0A068NEN6	UPF0173 metal-dependent hydrolase BcrFT9_03657	2.64	Hydrolase (Beta-lactamase)
A0A164CK25	Metal-dependent hydrolase (Fragment)	2.47	Hydrolase (Beta-lactamase)
C3DIS4	NH(3)-dependent NAD(+) synthetase	2.37	Metabolism
A0A0C3LQW7	Uncharacterized protein	2.62	Oxidoreductase
A0A0Q9HD35	Uncharacterized protein	3.53	Pectin lyase
A0A0M2SFX9	Uncharacterized protein	2.75	Protease inhibitor
A0A0D1L4A8	Valine–tRNA ligase	2.69	Protein synthesis
W7RS29	Peptide synthetase	2.82	Protein synthesis
A0A098F6B3	Phosphatidylethanolamine N-methyltransferase	1.84	Transferase
A0A072NRD1	Potassium uptake protein, TrkH family	2.94	Transport
A0A084GY51	Peptide-binding protein	2.78	Transport
A0A098EWU5	Putative lipoprotein	3.11	Transport
A0A0A8X5D1	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA	18.95	Transport
A0A0D6ZBQ0 [a]	Peptide-binding protein	9.47	Transport
A0A0J1ILE4	Oligopeptide-binding protein AppA	2.58	Transport
A0A0J5JQF2	Peptide ABC transporter substrate-binding protein	3.44	Transport

Table 2. *Cont.*

Access	Description	Score	Function
A0A0M2SQW2	Peptide ABC transporter substrate-binding protein OS=*Bacillus* sp. SA2-6 GN=WQ57_16375 PE=4 SV=1 - [A0A0M2SQW2_9BACI]	2.36	Transport
A0A0M3RFD5	Peptide ABC transporter substrate-binding protein	3.85	Transport
A0A150MCH0	Uncharacterized protein	25.07	Transport
E5WE49	Oligopeptide ABC transporter	6.38	Transport
Q2B8Z3	Oligopeptide ABC transporter (Binding protein)	3.07	Transport
V6T2D8	Uncharacterized protein	20.84	Transport
W4RN39	Oligopeptide ABC transporter	6.3	Transport
A0A068N9Y0	Conserved repeat domain protein	6.34	Unknown
A0A0B5NMV1	Uncharacterized protein	4.35	Unknown
A0A0D6Z7I3	Uncharacterized protein	5.9	Unknown
A0A164D5Z4	Putative internalin	2.62	Unknown
J7WX50	Uncharacterized protein	3.32	Unknown

3.2. Evaluation of the Biostimulant Capacity of Sludge-Based Products in Soil

3.2.1. Soil Biochemical Properties

The treatments stimulated, to a greater or lesser extent, the enzymatic activities of dehydrogenase, phosphatase, and β-glucosidase in comparison to the control. In relation to the dehydrogenase activity, it was observed that the soluble fraction of the fermented sludge (SFS) produced the greatest stimulation in the soil biological activity at both concentrations evaluated (0.97 ± 0.06 mmol INTF g^{-1} h^{-1} at the concentration of 0.1% *w*/*w* and 2.89 ± 0.10 mmol INTF g^{-1} h^{-1} at the concentration of 0.5 % *w*/*w* (Figure 3). Although to a lesser extent, the complete product (FS) also produced stimulation (1.66 ± 0.23 mmol INTF g^{-1} h^{-1}) at the highest of the concentrations studied (0.5 % *w*/*w*, Figure 3B). In all cases, the maximum peaks of stimulation were reached on day 5. On the other hand, IFS, the insoluble fraction of fermented sludge, induced dehydrogenase activity lightly and was only found at 0.5% *w*/*w* (0.95 ± 0.01 mmol INTF g^{-1} h^{-1}).

Regarding the phosphatase activity (Figure 4), a low stimulation of phosphatase activity was found after treatments with fermented-sludge based biostimulants. SFS, mainly at 0.5% *w*/*w*, produced the highest stimulation of phosphatase activity at day 5 (0.459 ± 0.022 mmol PNF g^{-1} h^{-1}; Figure 4B), coinciding with the peak of dehydrogenase activity. Although following a similar pattern, the biostimulant products FS and IFS produced 40% less stimulation than SFS on trial day 5. Finally, a belated increase in the phosphatase activity in IFS treatment was observed at a dose of 0.5% *w*/*w* between days 12 and 21 (Figure 4B).

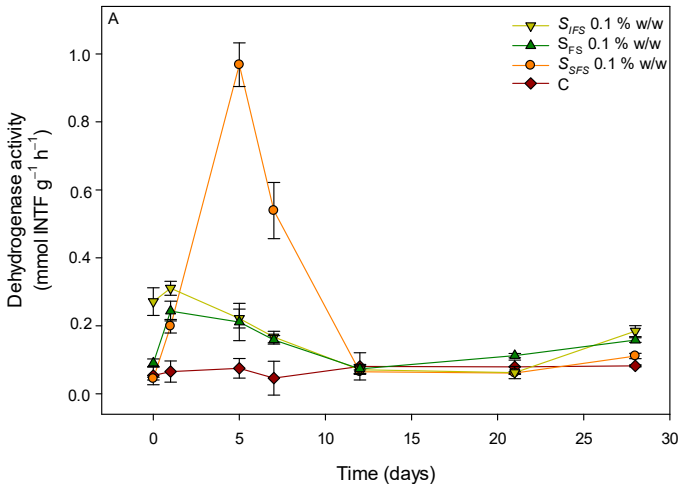


Figure 3. *Cont.*

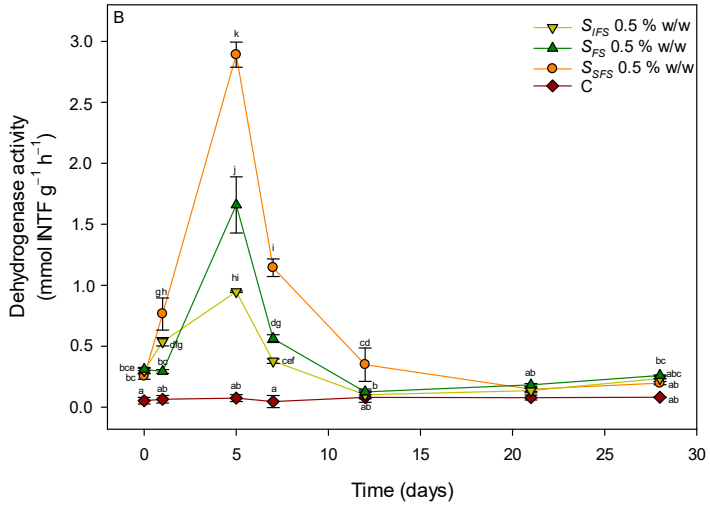


Figure 3. The dehydrogenase activity in the control soils and those treated with the different products at concentrations of 0.1% *w*/*w* (**A**) and 0.5% *w*/*w* (**B**). Points (mean ± SD) with the same letter(s) were not significantly different from each other ($p > 0.05$). INTF: 2-p-iodo-3-nitrophenyl formazan.

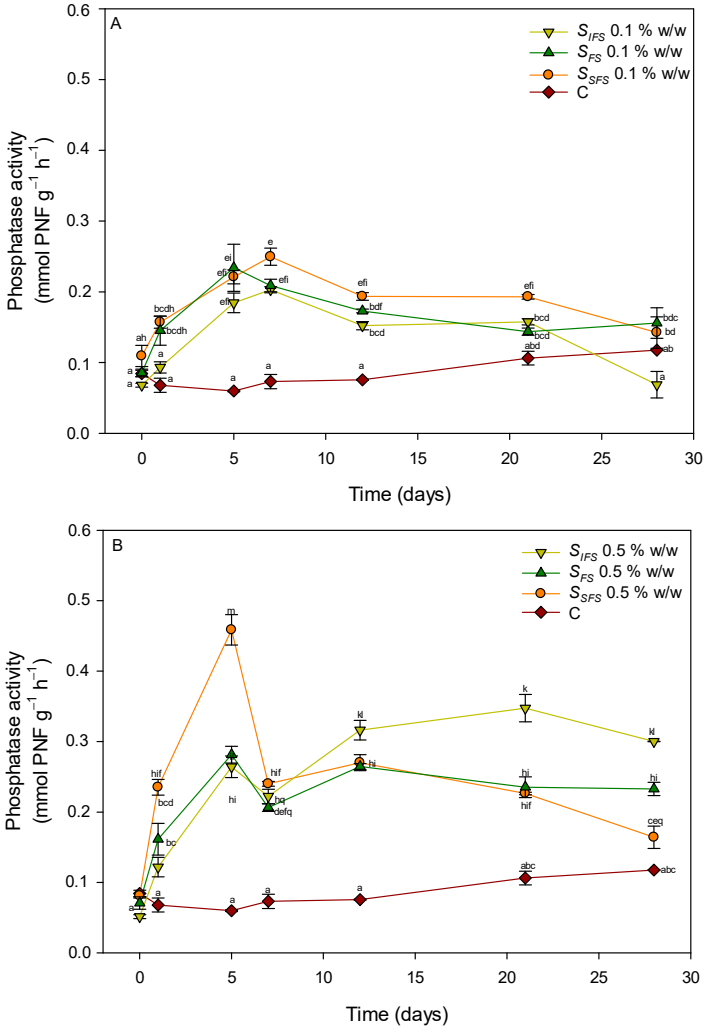


Figure 4. The phosphatase activity in the control soils and soils treated with the different products at concentrations of 0.1% *w*/*w* (**A**) and 0.5% *w*/*w* (**B**). Points (mean ± SD) with the same letter(s) were not significantly different from each other ($p > 0.05$). PNF: p-nitrophenol.

Regarding the glucosidase activity (Figure 5), an essential enzyme in the soil carbon cycle [28], only significant changes were observed compared with the control at the concentration of 0.5% *w*/*w* of the different treatments, thus not showing any relation to the bioavailability nor solubility degrees of the different biostimulants.

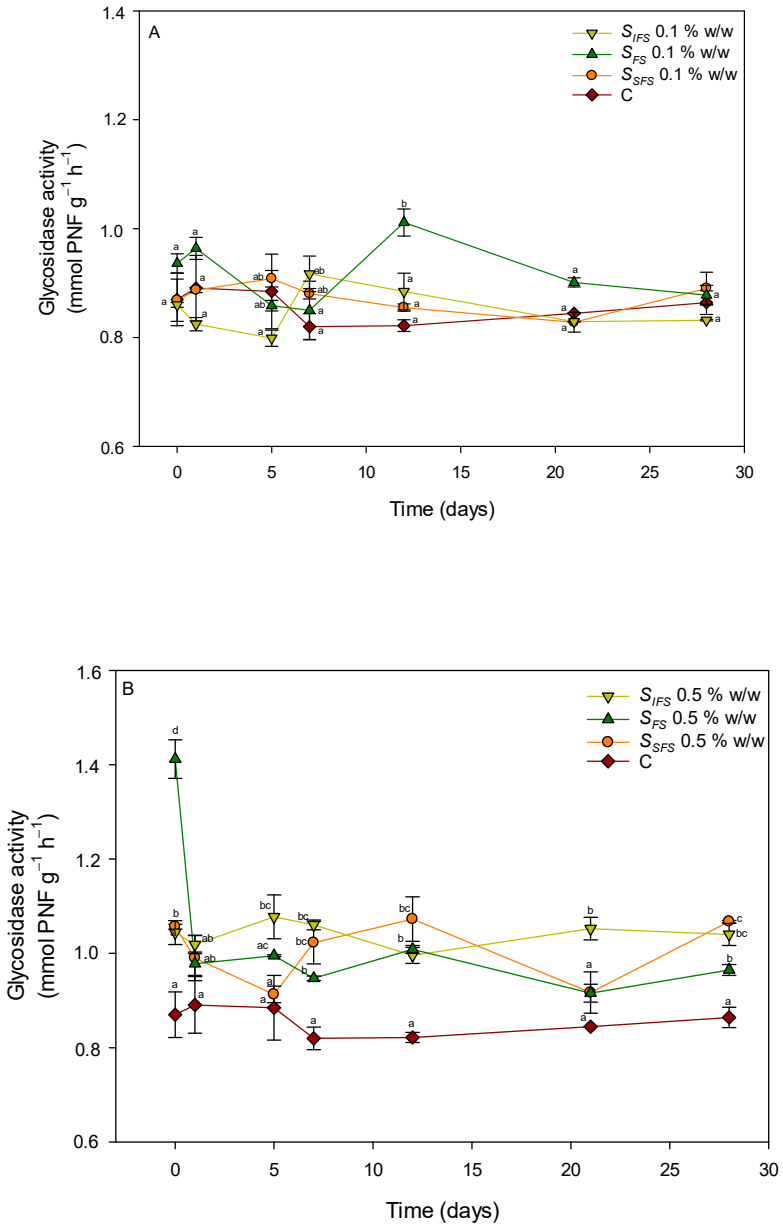


Figure 5. The glucosidase activity in the control soils and those treated with the different products at concentrations of 0.1% *w*/*w* (**A**) and 0.5% *w*/*w* (**B**). Points (mean ± SD) with the same letter(s) were not significantly different from each other ($p > 0.05$); only the points of each test time were compared with each other. PNF: p-nitrophenol.

3.2.2. Soil Microbiological Characterization

The bacterial biodiversity was analyzed in soil samples treated with the sludge-derived products at 0.5% *w*/*w*, which was the dose of biostimulant that produced the highest stimulation in soil.

Effects on Soil Bacterial Community Diversity

A total of 185,193 quality bacterial sequences were obtained with a range of 3390–7006 sequences per sample after quality filtering processing, trimming the primers and barcodes, removing the chimeras and singletons, and low abundance OTU filtering. Before the downstream analyses, each sample was normalized to 3390, which was the minimum depth of the sequences.

As indicative of quality, it can be highlighted that the Good's coverage indices for all samples were 1.00 (Table 3).

Table 3. The alpha diversity index.

Treatment/Day		Goods_Coverage	Observed_Otus	Shannon	Simpson	PD_Whole_Tree	Chao1	Dominance
Control	0	1.000	165.667 ± 11.671	6.830 ± 0.074	0.984 ± 0.001	9.465 ± 0.593	165.667 ± 11.671	0.016 ± 0.001
	5	1.000	169.333 ± 11.672	6.558 ± 0.169	0.974 ± 0.002	11.166 ± 0.522	169.333 ± 11.672	0.026 ± 0.002
	28	1.000	186.667 ± 28.987	6.531 ± 0.228	0.966 ± 0.005	14.227 ± 0.705	186.667 ± 28.987	0.034 ± 0.005
FS	0	1.000	152.000 ± 15.578	6.272 ± 0.153	0.972 ± 0.003	9.592 ± 0.476	152.000 ± 15.578	0.028 ± 0.003
	5	1.000	163.333 ± 9.286	6.823 ± 0.040	0.987 ± 0.001	9.946 ± 0.339	163.333 ± 9.286	0.013 ± 0.001
	28	1.000	167.000 ± 7.000	6.602 ± 0.08	0.977 ± 0.000	11.982 ± 0.206	167.000 ± 7.000	0.023 ± 0.000
IFS	0	1.000	165.667 ± 15.326	6.301 ± 0.169	0.971 ± 0.004	9.585 ± 0.060	165.667 ± 15.326	0.029 ± 0.004
	5	1.000	139.667 ± 12.658	6.619 ± 0.101	0.984 ± 0.001	9.092 ± 0.469	139.667 ± 12.658	0.016 ± 0.001
	28	1.000	209.333 ± 11.557	7.031 ± 0.082	0.986 ± 0.001	13.850 ± 0.478	209.333 ± 11.557	0.014 ± 0.001
SFS	0	1.000	175.667 ± 11.671	6.922 ± 0.080	0.985 ± 0.001	11.045 ± 0.330	175.667 ± 11.671	0.015 ± 0.001
	5	1.000	148.333 ± 5.793	6.435 ± 0.072	0.975 ± 0.002	7.987 ± 0.119	148.333 ± 5.793	0.025 ± 0.002
	28	1.000	159.333 ± 21.061	6.540 ± 0.159	0.981 ± 0.002	9.866 ± 0.636	159.333 ± 21.061	0.019 ± 0.002

No major changes were found in the richness and diversity of the bacterial communities along the experiment and neither major changes were induced by any of the sludge-derived products applied as revealed values for the diversity indices of Shannon and Simpson (Table 3).

Bacterial Community Composition and Abundance in Soil

Although no relevant changes were found in the bacterial biodiversity indices, changes in the taxonomic composition were found after the application of the three sludge-fermented products, both in comparison with the control samples and over the time of the experiment. The most relevant changes affected five families, three of them belonging to Proteobacteria phylum (Oxalobacteriaceae, Comamonadaceae, and Moraxellaceae), one to Actinobacteria phylum (Rubrobacteraceae), and, as it would be expected, the Bacillaceae family (Firmicutes phylum), which includes *Bacillus genus* (Figure 6, Supplementary Materials).

Showing a low presence in the control samples (0.4%, 0.9%, and 0.7%, relative abundance for 0, 5, and 28 days, respectively), the relative abundance of the Oxalobacteriaceae family was induced in a similar way to the three treatments at 5 days (10.1%, 7.9%, and 8.9% for SF, SFS, and IFS, respectively). This induction lasted until the end of the trial (6.7%, 10.2%, and 7.2%, relative abundance at 28 days for TFS, SFS, and IFS, respectively, Figure 6; Supplementary Materials). Regarding the Comamonadaceae family, although it was present in the control samples, no changes were found along the experiment in this group (2.02%, 2.03, and 2.05%, relative abundance at 0, 5, and 28 days, respectively) (Figure 6; Supplementary Materials). However, there was a clear induction of this family after application of the sludge-based products. Therefore, at 5 days, the relative abundance increased more than 50% in the TFS and SFS treatments (0.3 and 15.9 for TFS; 0.3 and 13.8 for SFS) and the increase lasted until the end of the study (9.9 and 12.32, relative abundance at 28 days for TFS and SFS, respectively). The induction of this family is related mainly to the soluble fraction of the fermented sludge, as the changes induced for IFS were less pronounced (0.3, 6.33, and 5.12 for zero, 5 days, and 28 days, respectively) than those for TFS and SFS.

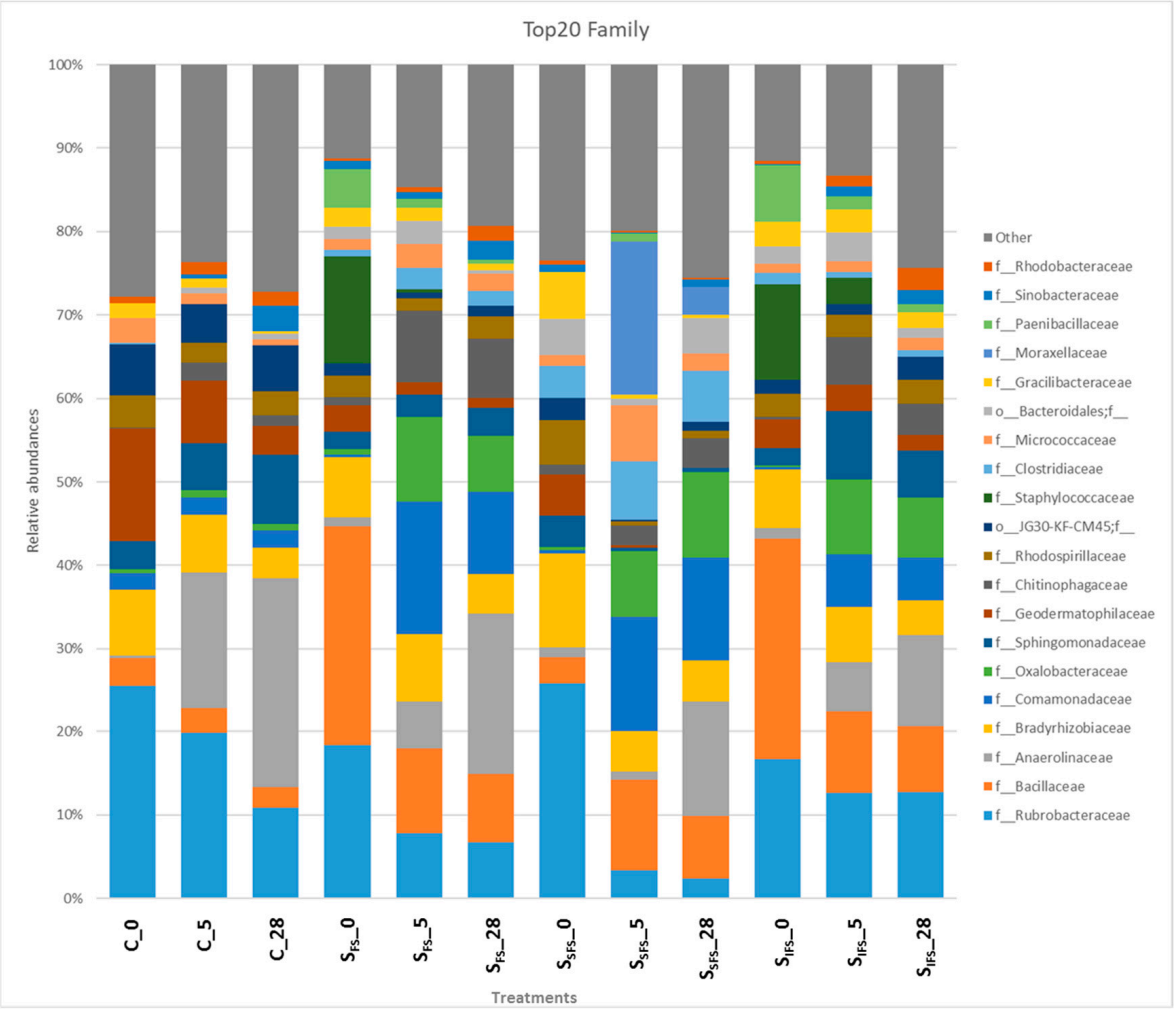


Figure 6. The top 20 most-abundant identified bacterial families. The remaining 120 families were collected into "Other". Figure shows the data of treatments with 0.5% *w*/*w* of the different sludge derived products. C_0: control soil at day 0; C_5: control soil at day 5; C_28: control soil at day 28; SFS_0: soil treated with the soluble total fermented sludge at day 0; SFS_5: soil treated with the total fermented sludge at day 5; SFS_28: soil treated with the total fermented sludge at day 28; SSFS_0: soil treated with the soluble fraction of fermented sludge at day 0; SSFS_5: soil treated with the soluble fraction of fermented sludge at day 5; SSFS_28: soil treated with the soluble fraction of fermented sludge at day 28; SIFS_0: soil treated with the insoluble fraction of fermented sludge at day 0; SIFS_5: soil treated with the insoluble fraction of fermented sludge at day 5; SIFS_28: soil treated with the insoluble fraction of fermented sludge at day 28.

Perhaps the most drastic change was found in the Moraxellaceae family, which was only detected in the SFS treatment, showing a relative abundance of 18.3% at five days and 3.4% at 28 days. The relative abundance of Moraxellaceae corresponded entirely to the genus *Acinetobacter* (see the Supplementary Materials).

Regarding the Bacillaceae family, as expected, it was represented by the genus *Bacillus*, (Supplementary Materials) and obviously, soils treated with TFS or IFS, which include the bacteria biomass, showed the highest relative abundance. At the initial times, the relative abundance in the TFS and IFS treatments were 8-fold higher than in the control soils (3.41%, 26.3%, and 26.5%, relative abundance at day 0 for the control, TFS, and IFS, respectively) (Figure 6; Table 3). However, after SFS treatment, the relative abundance of Bacillaceae was similar to the control soil due to the low *Bacillus* contribution with this (see Supplementary Materials). The relative abundance of Bacillaceae was decreased in

the TFS and IFS treatments along time (10.15% and 9.82% relative abundance at 5 days in TFS and IFS, respectively), however in the SFS treatment, with a relative abundance similar to the control at day 0 (3.26%), the relative abundance of this family increased at 5 days, reaching values similar to the TFS and IFS groups (10.84%). It should be highlighted that the *Bacillus* biomass was maintained throughout the course of treatment in all of the treatments (Supplementary Materials), which means that the initial microbial inoculum had established itself among the soil microbiome.

The Rubrobacteriaceae family was negatively affected throughout the experiment in all of the soil groups including the control. However, the decrease along the trait was mainly induced by SFS and IFS (91% and 64% decrease, respectively, at 28 days with respect to day 0). This family was represented by the *Rubrobacter* genus (Supplementary Materials).

4. Discussion

4.1. Characterization of Biostimulant Products

The fermented sludge was mainly an organic matter product (Table 1) with a high content in N and P (Table 1); the composition of heavy metals was below the limit values established by Spanish legislation for the use of this product in the agricultural sector (RD 1310/1990, of 29 October) (Table 1).

Proteomic characterization searching for the basal expression of *Bacillus'* proteins in the different products (FS, IFS, and SFS) found that fermentation induced a high diversity of secreted proteins, which means that the microbial metabolism was adapted to the substrates present in the environment. Secreted hydrolases were 50% peptidases and amidases, and 33.3% glucanases (Table 2). The difference in the level of induction of both groups of enzymes must be due to differences in the mechanism that regulates their expression, thus while glucanases are mainly inducible by the substrate, being secreted when potentially hydrolyzable carbon sources appear in the medium, peptidases and amidases are not only inducible by the substrate, but also in conditions of N, C, and P shortage [29].

4.2. Soil Biostimulant Capacity of Biostimulants

4.2.1. Soil Enzymatic Activities

All of the applied treatments stimulated the enzymatic activities of dehydrogenase, phosphatase, and β-glucosidase, all considered to be good soil quality indicators [5,6]. SFS, and to a lesser level, FSFS, produced a stimulation on the soil biological dehydrogenase activity at both of the concentrations evaluated. The main reason attributable to the stimulation produced by SFS and FS is the molecular size profile of the soluble organic component of the fermented sludge as a result of the enzymatic action of the *Bacillus* secretion. It presented a high content of small molecules constituted 65.95 ± 0.09% by organic molecules of a molecular size under 1 KDa (Figure 2), which implies that it was largely composed of peptides, free amino acids, and other highly bioavailable organic molecules, easily assimilated by the soil microbiota [13,14]. In this context, as revealed by the proteomic analysis (Table 2), the enzymatic composition of fermented products, rich in proteases enzymes, contributes to the stimulation of the microbiota. According to the present results, we previously reported that the soil application of subtilisin, one of the main proteolitic enzymes secreted by *Bacillus licheniformis*, induced the stimulation of the soil microbiota by making the organic matter more bioavailable, and interestingly, stimulated some possible PGPB [16].

The early stimulation profile of dehydrogenase activity (Figure 3) was also described by our group after applying biostimulant products based on enzymatically hydrolyzed sludge with subtilisin from *B. licheniformis* [3], which was comparable to SFS. However, in a later work [13] that evaluated a product obtained through a fermentation process similar to that in FS, a very slight stimulation of dehydrogenase activity was found, similar to that in S_{FS} and S_{IFS} at the doses of 0.1% w/w. It must be said that unlike in the present work, in the cited study, the sludge used as the raw material was obtained from urban wastewater treatment and it presented around 30% less organic matter content because

of a 4-month maturation-mineralization period to which it was subjected. Therefore, a discrepancy in the results may be due to the lower organic content of the mature urban sludge used and the low dehydrogenase activity was only attributable to the biological activity of the microbial biomass provided in the products, but the products themselves did not produce any biostimulation in the soil. The nitrogen content of our FS was double that of the product described in previous work [13]; moreover, the degree of hydrolysis of the products described in the present work was on average 30% higher, with a 65.95 ± 0.09% content of molecules lower than 1 KDa. Therefore, the protein hydrolysate contained in FS and SFS is more complex, presenting a higher content of peptides and free amino acids that give it a greater potential biostimulant capacity [30]. Therefore, we can assume that by increasing the number of lower molecular weight proteins, it would increase the stimulation of the soil dehydrogenase activity, so the degree of hydrolysis of the product is the determining factor in the stimulation of the soil microbiota. The decrease in molecular size of the protein means that the N is more readily available for soil microorganisms, which facilitates a greater proliferation of microorganisms in the soil [31].

The stimulation produced by IFS could be due both to the soluble fraction retained after centrifuging, and to the lytic enzymes produced by *B. licheniformis* that would have been established in the soil, promoting the hydrolysis of the insoluble organic matter provided in the treatment and indirectly stimulating the soil microbiota.

Regarding the phosphatase activity (Figure 4), significant induction was observed only with the SFS fraction at 0.5% *w*/*w*. Available phosphorus reduces the need for phosphatase secretion by the soil microbiota to make it accessible, so the low stimulation of phosphatase activity after treatments with the fermented-sludge based biostimulants could be due to a higher bioavailability of phosphorus in such products as a consequence of the physical-fermentative process increasing the soluble content (9.2 ± 0.14 g L^{-1}). Thus, the biostimulant products FS and IFS, presenting similar phosphorus content (17,663.19 ± 0.51 mg Kg^{-1} and 18,896.71 ± 0.15 mg Kg^{-1}, respectively; Table 1), showed similar phosphatase activities to each other for both the 0.1% *w*/*w* and 0.5% *w*/*w* concentrations, somewhat lower than the values obtained after SFS treatment, the soluble fraction that contains 6338.29 ± 0.03 mg Kg^{-1} (Table 1). These results agree with the biostimulant effect described by Rodriguez-Morgado and collaborators after the enzymatic hydrolysis of sewage sludge [3], and may be explained by the depletion of phosphorus as a consequence of the stimulation of the soil microbiota, which would promote the synthesis of microbial phosphatases to make it more bioavailable. Finally, the belated increase in the phosphatase activity in IFS treatment at 0.5% *w*/*w* may be due to the depletion of the initially slight amount of soluble phosphorous available that was assimilated by the soil microbiota, together with the new community of *B. licheniformis*, and the need to induce phosphatases in order to hydrolyze organic phosphorus to maintain the growth rate.

The results of the glucosidase activity (Figure 5) only showed changes at the concentration of 0.5% *w*/*w* for Fs, SFS, and IFS compared with the control. These results are not completely in agreement with those described by Rodríguez-Morgado et al. [3], who found an increase in the glucosidase activity during days 5–7, when the treated soil with biostimulants obtained from the sewage sludge by enzymatic process coincided with the stimulation peak of microbial activity.

However, when they used biostimulants obtained by the fermentative process, they also described no changes in the glucosidase activity [13]. As they discussed, during the fermentation process, B. licheniformis excretes a large number of enzymes in order to obtain energy and nutrients for its development, thus degrading practically all organic compounds in the media [21]. Thus, when this type of biostimulant is applied to the soil, the soil microorganisms do not need to excrete any extracellular enzymes to degrade the organic compounds that support their growth.

In summary, biochemical changes produced by the fermented sludge-based biostimulant fractions suggest that the soluble content is mainly responsible of the biostimulant power of the product. The soil biological stimulation was mainly due to its high content

of highly bioavailable soluble organic matter (65.95 ± 0.09% <1 KDa) and its content of Bacillus hydrolytic enzymes.

4.2.2. Changes in Soil at Microbiological Level

The most relevant changes in the taxonomic composition were affected by the Oxalobacteriaceae, Comamonadaceae, and Moraxellaceae families (Proteobacteria phylum); the Rubrobacteraceae family (Actinobacteria phylum); and the Bacillaceae family (Firmicutes phylum), which includes the *Bacillus genus* (Figure 6, Supplementary Materials).

The induction of abundance of Oxalobacteriaceae was observed at 5 days and lasted until the end of the trial for all treatments (Figure 6). Oxalobacteriaceae is a broad family that includes some mild plant pathogens, but also encompasses several endophytic bacteria classified as PGPB [32]. For example, the endophytic genus *Herbaspirillum* includes nitrogen-fixing species, producers of phytohormones such as gibberellin and auxin [33] and siderophores [34], and have the ability to solubilize inorganic phosphorus [35] among other PGPB capabilities. Furthermore, some species of this genus have been used as microbial inoculants in agronomic application, showing favorable results in the yield of crops such as sorghum [34].

The most differential shift in the bacteria composition was observed in the Moraxellaceae family, which was only detected after SFS treatment. Interestingly, the relative abundance of Moraxellaceae corresponded entirely to the genus *Acinetobacter* (see Supplementary Materials), which is a genus of high interest for agriculture, so certain strains of this genus are considered to be PGPB and are involved in the production of plant-growth-promoting hormones [36], the solubilization of phosphate [36], and the production of siderophores [37]. In addition, other *Acinetobacter* strains have exhibited potential biocontrol properties against the pathogenic bacteria [38].

The reason for the specific induction in the Moraxellaceae family after SFS application may perhaps be found in its chemical composition. SFS has a low Si content ($\leq$3.72 mg Kg^{-1}) compared to FS and IFS (10,313.57 ± 0.26 and 11,623.00 ± 0.09 mg Kg^{-1}, respectively, Table 1). It has been reported that Si application alters the soil physicochemical properties, which indirectly affect the soil microbial communities [39]. Moreover, Si application could change the soil microbial composition [40,41].

The Bacillaceae family was represented by the genus *Bacillus*, which was maintained throughout the course of treatment for all treatments (Supplementary Materials). Bacillus is a genus known to exert PGPR activity, thus Bacilli rhizobacteria species are known to protect plants from phytopathogen and simultaneously increase the yield in different crops [42,43]. Endospore forming *Bacillus* species exhibited physiological traits such as a multi-layered cell wall, endospore formation, and the synthesis of lipopeptides, antibiotics, and extra-cellular enzymes that make *Bacillus*, a potential PGPR, survive under adverse environmental conditions. These characteristics make *Bacillus*, a potential PGPR, survive under adverse environmental conditions [44]. Among the most studied Bacillus species for application in agriculture is *B. licheniformis*, which shows growth-promoting functions in plants such as the solubilization of phosphorus by the synthesis of phytases [45], the production of ammonium from phytohormones (auxins), and of siderophore compounds and compounds that inhibit the growth of pathogens [46].

The Rubrobacteriaceae family was negatively affected along the experiment in all of the soil groups including the control. We have previously described that after applying subtilisin, a soil extracellular endopeptodase from *Bacillus* sp., relative abundance of *Rubrobacter* decrease in soil and the effect was more pronounced when subtilisin was applied in combination with keratins [15]. In general, the *Rubrobacter* strains are associated with extreme environments (e.g., high temperature environments) such as the fumarole heated stream, soil adjacent to volcanic caldera, deteriorated monuments, or even halophilic species [47], and show low ecological value. Thus, we speculate that the abundance of *Rubrobacter*, a genus that grows preferentially in extreme conditions, decreases in enriched medias, probably due to the increase in the presence of bacteria with higher growth requirements.

In summary, all of the different biostimulants evaluated produced changes within the microbial structure of the soil compared to the control, causing the stimulation of some bacterial genera classified bibliographically as beneficial microbes, since they include strains of agronomic interest. The changes were mostly due to the SFS treatment, followed by the TFS treatment, so it can be concluded that it is the soluble fraction that is mainly responsible for the changes produced in soil diversity. This fraction is composed of hydrolyzed organic matter, mainly free peptides and amino acids, and the secretion of *B. licheniformis* during fermentation, mainly hydrolytic enzymes and other functional biomolecules.

5. Conclusions

These results position the fermentation process with *B. licheniformis* as an interesting option for the valorization of activated sewage sludge aimed at obtaining products of agronomic interest, and has been shown to be a viable alternative to the use of enzymatic catalysis technologies. Fermentative technologies also show certain advantages over enzymatic hydrolysis processes such as the greater complexity of the product as a result of the action of the wide variety of enzymes secreted by the fermentative microorganism, a cheaper process as it does not require expenditure on commercial enzymes, and the presence in the final product of the biomass of the fermentative microorganism, which, in this case, was a PGPR bacterium, enhances the agronomic interest of the product.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12081743/s1.

Author Contributions: Conceptualization, J.P.R. and M.T.; Methodology, S.M.-B. and P.C.; Software, S.M.-B.; Validation, P.C., M.T. and J.P.R.; Formal analysis, A.C. and J.P.R.; Investigation, S.M.-B., P.C., A.M., B.R.-M. and L.M.; Resources, J.P.R.; Data curation, P.C. and S.M.-B.; Writing—original draft preparation, A.C.; Writing—review and editing, P.C. and A.C.; Visualization, L.M.; Supervision, J.P.R. and M.T.; Project administration, L.M.; Funding acquisition, J.P.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministerio de Ciencia e Innovación (Spain), Proyectos I+D+i<<Retos Investigación>>Convocatoria 2018 RTI2018-097425-B100.

Data Availability Statement: Not applicable.

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

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