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Antifungal Activity and Mycotoxin Degradation Potential of Bioprotective Microorganisms to Application in Animal Food Production Chain

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Simple Summary: This study investigated the potential of bioprotective microorganisms to degrade mycotoxins and control fungal contamination in the animal food production chain. Twenty-three strains of bacteria and yeasts were tested for their ability to degrade zearalenone (ZEA) and fumonisin B1 (FB1), as well as inhibit mycotoxigenic fungi. Four bacterial strains exhibited a high capacity to degrade ZEA: *Bacillus amyloliquefaciens* MLB3, *Bacillus subtilis* MLB2, *Bacillus velezensis* CL197, and *Streptomyces griseus* CECT 3276. However, none of them achieved significant success in degrading FB1. In addition to mycotoxin degradation, these strains showed antifungal activity in co-culture assays with mycotoxin-producing fungi such as *Aspergillus* and *Fusarium*, inhibiting the growth of up to 100% of the tested fungal strains. In simulated swine and poultry digestion, the selected strains fully degraded ZEA, reinforcing their potential to prevent mycotoxin absorption during animal feeding. Metabolite analysis revealed that the main products of ZEA degradation were low-toxicity conjugates. These findings indicate that these bacteria could be promising for the development of biotechnological solutions to improve food safety and reduce mycotoxin contamination in animal feed, protecting both animal and human health.

Abstract: The global meat industry has grown substantially, producing 357.39 million tons in 2021, with poultry and pork comprising nearly 73% of this total. However, contamination by mycotoxins, such as zearalenone (ZEA) and fumonisin B1 (FB1), presents a major issue, as these toxins resist common preservation methods. This study explores the potential of bioprotective microorganisms in mycotoxin degradation and fungal control within the animal food production chain, a sector facing significant challenges due to fungal contamination. In this study, 23 bacterial and yeast strains were tested for their ability to degrade ZEA and FB1, and to inhibit mycotoxigenic fungi. Four bacterial strains were highly effective in degrading ZEA: *Bacillus amyloliquefaciens* MLB3, *Bacillus subtilis* MLB2, *Bacillus velezensis* CL197, and *Streptomyces griseus* CECT 3276. However, no strain achieved satisfactory FB1 degradation. The strains also displayed antifungal activity, inhibiting up to 100% of fungi growth in solid media co-culture tests. Simulated swine and poultry digestion demonstrated complete ZEA degradation after 2 hours of incubation. Metabolite analysis revealed that low-toxicity conjugates were formed. These findings suggest that these bacteria hold significant promise for biotechnological applications in animal production, helping to reduce mycotoxin contamination, improve food safety, and protect both animal and human health.

Keywords: animal production; *Bacillus*; *Streptomyces*; zearalenone

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1. Introduction

Animal food production has increased dramatically in the last decade. Considering only meat industry, in 2012 the world produced 245.73 million tons; in contrast, 2021 data resulted in a production of 357.39 million tons, which means an increase of 111.66 million tons in just nine years. Among all meat commodities, pork and poultry stand out, accounting for 33.92% and 38.89% of world production, respectively [1].

One of the major problems affecting the animal food production chain is contamination by mycotoxins. These mycotoxins can cause production losses, since there is a decrease in the zootechnical indexes of the animais, and also cause harm to consumers due to their residues in animal-source foods, once their chemical characteristics make them resistant to the main preservation methods, such as freezing and cooking [2]. Mycotoxins can reach the animal food production chain through the use of contaminated raw materials in the preparation of animal feed, or through fungal contamination in feed stored in an inadequate situation. Research indicates that between 30 and 100% of foods and feed intended for human and animal consumption have some degree of fungal and/or mycotoxin contamination [3].

Among the most studied mycotoxins, fumonisin B1 (FB1) and zearalenone (ZEA) present important impact on both human and animal health. FB1 is a human possibly carcinogenic mycotoxin – group 2B by the International Agency for Research on Cancer (IARC) –, related to esophageal cancer and neural tube defects during embryogenesis. ZEA is a toxin with strong estrogenic activity – group 3 by the IARC –, related to precocious puberty and development disorders, especially in women. Additionally, there are evidence of immunotoxic, hepatotoxic, nephrotoxic, genotoxic, and hematotoxic effects [4].

The search for strategies to control fungal contamination and the consequent production of mycotoxins is constant, and bacterial biocontrol has been highlighted in recent years, with several research being developed in the area [5–8]. Considering that fungal and mycotoxin control is essential throughout the entire food production chain, and that animal-foods can be vehicles of these contaminants for humans, the objective of this research was to evaluate the antifungal and antimycotoxigenic potential of different microorganisms, focusing on food-animals production.

2. Materials and Methods

2.1. Strains and Materials

Twenty-three potentially bioprotective microorganisms and thirteen filamentous fungi were used, obtained from different microbiological collections (Table 1). The bacteria and yeasts were maintained in culture media + 20% glycerol, and the fungi in culture media + 30% glycerol, both at -80 ºC until use, and were reactivated in the same culture media, incubated at 37 °C for 24 - 48h (bacteria and yeast) or at 25 °C for 5 - 7d (fungi), with at least two passages prior to the analyses.

Table 1. Microorganisms used in this study, sourced from diverse microbiological collections.

Microorganism	Identification	Source	Original isolation
Bacteria and yeasts			
Bacillus amyloliquefaciens	CECT 493	Colección Española de Cultivos Tipo	Bacterial amylase HT concentrate, United States
Bacillus amyloliquefaciens plantarum	MLB3	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Bacillus licheniformis	CECT 20	Colección Española de Cultivos Tipo	Unknown

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Bacillus megaterium	CECT 44	Colección Española de Cultivos Tipo	Unknown
Bacillus subtilis	MLB2	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Bacillus thuringiensis	CECT 197	Colección Española de Cultivos Tipo	Mediterranean flour moth, unknown country
Bacillus velezensis	CL197	AgriFood Research and Innovation Laboratory, Pontifícia Universidade Católica do Paraná, Brazil	Soil, Brazil
Candida sake	CECT 1044	Colección Española de Cultivos Tipo	Lambic beer, Belgium
Levilactobacillus (*Lactobacillus*) *brevis*	BN3	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Limosilactobacillus (*Lactobacillus*) *fermentum*	20_PG2_BHI ZZMK	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Lacticaseibacillus (*Lactobacillus*) *paracasei*	DSM 2649	German Collection of Microorganisms and Cell Cultures GmbH	Silage, unknown country
Lactiplantibacillus (*Lactobacillus*) *plantarum*	DSM 1055	German Collection of Microorganisms and Cell Cultures GmbH	Bread dough, United States
Lacticaseibacillus (*Lactobacillus*) *rhamnosus*	DSM 20711	German Collection of Microorganisms and Cell Cultures GmbH	Unknown
Liquorilactobacillus (*Lactobacillus*) *satsumensis*	DSM 16230	German Collection of Microorganisms and Cell Cultures GmbH	Shochu mash, Japan
Metschnikowia pulcherrima	CECT 1691	Colección Española de Cultivos Tipo	Fruit of *Phoenix dactylifera*, Egypt
Paenibacillus chibensis	CECT 375	Colección Española de Cultivos Tipo	Unknown
Paenibacillus polymyxa	CECT 153	Colección Española de Cultivos Tipo	Water, unknown country
Pediococcus acidilactici	146 RLT	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Pseudomonas putida	MCA	BioTech AgriFood Laboratory, Universitat de València, Spain	Unknown
Pseudomonas syringae	CECT 312	Colección Española de Cultivos Tipo	*Nicotiana tabacum*, Hungary

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Saccharomyces cerevisiae	DSM 70868	German Collection of Microorganisms and Cell Cultures GmbH	African palm wine, unknown country
Streptomyces calvus	CECT 3271	Colección Española de Cultivos Tipo	Soil, India
Streptomyces griseus	CECT 3276	Colección Española de Cultivos Tipo	Soil, United States
Filamentous fungi			
Aspergillus steiiny	CECT 20510	Colección Española de Cultivos Tipo	Pollen of bee, Spain
Fusarium graminearum	ITEM 126	ITEM Microbial Culture Collection of ISPA	*Triticum durum* kernel, Italy
Fusarium graminearum	CECT 20924	Colección Española de Cultivos Tipo	Rice carypses, Spain
Fusarium langsethiae	ITEM 11031	ITEM Microbial Culture Collection of ISPA	Unknown
Fusarium oxysporum	CECT 2719	Colección Española de Cultivos Tipo	Unknown
Fusarium oxysporum	ISPA 7067	ITEM Microbial Culture Collection of ISPA	Stalk of rice, Italy
Fusarium sporotrichioides[1]	CECT 20165	Colección Española de Cultivos Tipo	Emetic material, unknown country
Fusarium verticillioides	ITEM 12043	ITEM Microbial Culture Collection of ISPA	Unknown
Fusarium verticillioides	ISPA 12044	ITEM Microbial Culture Collection of ISPA	Unknown
Fusarium verticillioides	ITEM 12052	ITEM Microbial Culture Collection of ISPA	Unknown
Gibberella zeae	CECT 20492	Colección Española de Cultivos Tipo	Wheat, Spain
Gibberella zeae	CECT 2150	Colección Española de Cultivos Tipo	Grain of *Zea mays*, United States
Penicillium verrucosum	VTT D-01847	VTT Culture Collection, VTT Technical Research Centre of Finland	Grain, Finland

[1]Cataloged as *Fusarium poae* until December 2021.

The bacteria and yeasts were chosen for the bioprotective potential presented by microorganisms of the same species, according to research available in scientific databases, and the fungi for their importance in food and feed contamination, and for their presumptive mycotoxin production. The taxonomy of *Lactobacillaceae* was actualized according to Zheng et al [9].

All culture media used were purchased from Oxoid (Hampshire, United Kingdom). All chemicals and reagents used were analytical or chromatographic grade, purchased from Fisher Scientific (Hudson, NH) and Sigma-Aldrich (St. Louis, MO). Ultra-pure water (~18.2 MΩ/cm resistivity) was obtained from a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

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2.2. Degradation of Mycotoxins in Culture Media

Initially, a mycotoxin degradation potential screening was conducted for the putatively bioprotective microorganisms. Bacteria and yeasts were acclimated for growth in a minimal medium (consisting of 5.0 g/L of tryptone, 1.0 g/L of glucose, and 2.5 g/L of yeast extract) for a minimum of two passages. Following adaptation, the medium was augmented with 1 µg/mL of ZEA or FB1, and was then inoculated with 1% of a fresh culture of each microorganism under examination, individually analyzed (adjusted to a 0.5 McFarland Standard, approximately ~1.5 × 10^8 CFU/mL). The tubes were incubated at 37 °C for 48h with agitation at 120 rpm. Subsequent to incubation, the content was filtered using a nylon syringe filter (0.22 µm pore size) and subjected to ultra-high performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC-MS/qTOF) for quantification of ZEA and FB1. Control tubes included minimal medium alone (negative control) and minimal medium supplemented with 1 µg/mL of ZEA or FB1 (positive control) [10].

Chromatographic analysis was carried out using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA), equipped with an auto-sampler, vacuum degasser, and binary pump. Analyte separation was achieved with a Gemini C18 column (50 mm × 2 mm, 110 Å, 3 µm particle size) sourced from Phenomenex (Palo Alto, CA). The mobile phases consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), with a flow rate of 0.3 mL/min in a gradient (0 min: 5% B; 30 min: 95% B; 35 min: 5% B), and a total analysis run time of 35 minutes. The injection volume was 5 µL [11].

For mass spectrometry analyses, an Agilent Ultra High-Definition Accurate Mass MS/qTOF system (6540, Agilent Technologies, Santa Clara, CA) was utilized, coupled with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI, Agilent Technologies, Santa Clara, CA) interface operating in positive ion mode. The optimized mass spectrometry parameters included a fragment voltage of 175 V, a capillary voltage of 3.5 kV, collision energies of 10, 20, and 40 eV, a nebulizer pressure of 30 psi, a drying gas flow of 8 L/min (using N2), and a temperature set at 350 °C. Data analysis was conducted using MassHunter Qualitative Analysis Software B.08.00 (Agilent Technologies, Santa Clara, CA) [11].

Following the initial screening of degradation activity, the microorganisms exhibiting a degradation rate of ≥90% were subjected to further assessments under the following conditions (analyses for FB1 were not conducted as the minimum degradation threshold was not met in the initial screening): i) the microorganisms were inoculated at a concentration of 10^4 CFU/mL in the presence of 1 µg/mL of ZEA, and incubated for 48h at 37 °C; ii) cell-free supernatants of each strain were obtained after a 48h-bacterial growth, followed by centrifugation (3500 × g, 10 min) and filtration using a nylon syringe filter (0.22 µm pore size). These supernatants were then incubated with 1 µg/mL of ZEA for 48h at 37 °C; iii) intracellular metabolites of each strain were obtained after rupturing the bacterial biomass's plasmatic membrane in a hypotonic solution (ultrapure water) and subjecting it to freezing cycles (-45 °C and 37 °C, 2h/cycle, repeated 4 times). The resulting solution was then subjected to centrifugation (3500 × g, 10 min), and the supernatant was collected. Subsequently, it was incubated with 1 µg/mL of ZEA for 48h at 37 °C; iv) autoclaved culture after a 48h-bacterial growth was incubated with 1 µg/mL of ZEA for 48h at 37 °C; and v) to evaluate the adsorptive potential, the recovery of ZEA from bacterial biomass after incubation for 48h at 37 °C in the presence of 1 µg/mL of ZEA was conducted. The degradation of ZEA, the adsorption potential of the strains, and the formation of degradation compounds, were evaluated using UHPLC-MS/qTOF, as described previously.

2.3. Inhibition of Fungi and Mycotoxins Production in Culture Media

The bioprotective microorganisms were cultivated in a liquid culture medium for 24h at 37 °C. Subsequently, 3 µL of the fresh culture were inoculated at the center of potato dextrose agar (PDA) plates. After drying, fungal explants with a diameter of 0.5 cm were placed at the edges of the plates. These explants were derived from recent fungal cultures that were initially prepared on PDA plates and incubated at 25 °C for 5 - 7d, until sporulation occurred. Plates containing both bioprotective microorganisms and fungal explants were incubated at 25 °C for 7d, and the extent of fungal growth

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was measured at 7d. Control plates contained only fungal explants (positive control) or only bioprotective microorganisms (negative control) [12].

Following the measurement, the agar was entirely removed from the plates and soaked in 15 mL of methanol to extract the mycotoxins. The flasks were agitated at 150 rpm for 24h, and then the methanol was filtered through a nylon filter with a porosity of 0.22 µm. Subsequently, the filtered methanol was subjected to UHPLC-MS/qTOF analysis, as described previously, for the quantification of mycotoxins and the detection of degradation compounds [13].

2.4. Characterization of Antifungal and Antimycotoxigenic Metabolites of Bioprotective Microorganisms

The selected bacteria (≥90% ZEA degradation in initial screening) were inoculated into 200 mL of tryptone soy broth (TSB) at a concentration of ~10^4 CFU/mL. The culture was maintained at 37 ^{o}C on an orbital shaker operating at 120 rpm. At specific time points (0, 24, 48, and 72h), aliquots were extracted, subjected to centrifugation at 3500 × g, diluted with Milli-Q water at a ratio of 1:5 (v/v), and then filtered using a nylon syringe filter with a 0.22 µm pore size. To prevent any potential interference with the growth process, separate samples were prepared for each of the specified time points [14]. These samples were subsequently analyzed using UHPLC-MS/qTOF as described previously.

Upon the identification of metabolites, additional samples were collected for lyophilization, performed using a Lab Freeze Dryer (OLT-FD-10N, Xiamen Ollital Technology Co., Ltd.) to antimicrobial activity analysis.

2.5. Antimicrobial Activity of the Lyophilized Metabolites

The antimicrobial activity of metabolites was conducted in two ways, both in solid and liquid media, involving the inhibition of fungal growth and mycotoxin production on solid media, and the determination of minimum inhibitory and fungicidal concentrations in liquid media.

For the analysis of growth inhibition zones, 100 µL of a spore suspension containing 10^4 spores/mL were inoculated onto the surface of PDA plates. After complete drying, spots were made on the plate, and were added the lyophilized metabolites reconstituted in ultrapure water at a concentration of 500 g/L. The plates were incubated at 25 ^{o}C for 3 - 7d, and the inhibition of fungal growth was measured. The negative control contained only ultrapure water, while the positive control consisted of PDA plates with fungal inoculum [15].

To determine the minimum inhibitory concentration, concentrations ranging from 0.98 to 500 g/L of lyophilized metabolites reconstituted in TSB were used, employing the microdilution technique. The wells contained 5 × 10^4 spores/mL with a final volume of 200 µL. The positive control contained only the spore suspension, and the negative control contained sterile TSB. Plates were incubated at 25 ^{o}C for 48h, and the lowest concentration exhibiting visible inhibition of fungal growth was determined as the minimum inhibitory concentration. After reading, the contents of wells with no visible growth were inoculated onto PDA plates. The lowest concentration required to kill the fungus was defined as the minimum fungicidal concentration [16].

2.6. Zearalenone Degradation in Swine and Poultry In Vitro Digestion

For the simulated digestion analyses, encapsulated bacteria from the same batch produced for previous research conducted by our research group were used. Details regarding the encapsulation parameters, efficiency, and bacterial population of the resulting product can be found in the Supplementary Material. Before the digestions, the capsules were resuspended in sterile ultrapure water at a concentration of 50 g/L until complete homogenization and rehydration.

Swine digestion simulation was conducted in accordance with Evangelista et al [17] with modifications. One milliliter of the capsule suspension, contaminated with 1 µg/mL of ZEA, were utilized. In tubes containing the samples, 20 mL of phosphate buffer pH 6.0, 8 mL of 0.2 M HCl, and 50 U of pepsin were added. The pH was adjusted to 2.0±0.2, and the material was incubated for 2h at 39 ^{o}C with agitation at 250 rpm (stomach phase). After incubation, 7 mL of phosphate buffer pH 6.8,

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4 mL of 0.6 M NaOH, and 100 mg of pancreatin (4X USP) were added. The pH was adjusted to 6.8±0.2, and the material was incubated for 4h at 39 °C with agitation (intestinal phase). Samples were collected before and after the stomach phase, and after the intestinal phase, for the quantification of mycotoxins and degradation metabolites using UHPLC-MS/qTOF, as described previously.

Poultry digestion simulation was conducted as described by Evangelista et al [17] with modifications. One milliliter of the capsule suspension, contaminated with 1 µg/mL of ZEA, were used. In tubes containing the samples, 10 mL of sterile deionized water was added, and the pH was adjusted to 6.0±0.2. Incubation with agitation at 250 rpm at 41 °C for 30 minutes was performed (crop phase). After this period, 25 mL of sterile deionized water and 134,750 U of pepsin were added, with the pH adjusted to 2.5±0.2. The material was incubated at the same temperature for another 30 minutes with agitation (proventriculus phase). Glass beads with a diameter of 0.5 cm were added to simulate mechanical digestion in the gizzard, and the pH was adjusted to 3.5±0.2, with another incubation for 1h with agitation (gizzard phase). Then, 280 U of pancreatin and 135 mg of bile salts were added, and the pH was adjusted to 6.2±0.2, with another incubation for 2h with agitation (small intestine phase). Finally, the pH was adjusted to 7.0±0.2, and the material was incubated for 20 minutes with agitation (large intestine phase). Samples were collected before the crop phase, after the proventriculus phase, and after the large intestine phase, for the quantification of mycotoxins and degradation metabolites by UHPLC-MS/qTOF, as previously described.

2.7. Statistical Analyses

The results are presented as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 (San Diego, CA). Data were assessed for normality using the Shapiro-Wilk test, and an analysis of variance by ANOVA was conducted, followed by the Tukey test. The significance level was set at $p<0.05$.

3. Results

3.1. Screening of Degradation Potential and Effect of the Selected Strains

In the screening for ZEA degradation potential, only three strains showed no activity, and degradation rates ranged from 0.00 to 96.75%. In contrast, in the analysis with FB1, all strains exhibited some level of activity, ranging from 0.33 to 37.13%. Significant variability in degradation potential was observed, even among strains of the same species, as seen between *Bacillus amyloliquefaciens* CECT 493 and *Bacillus amyloliquefaciens plantarum* MLB3, which exhibited degradation of ZEA of 0.00% and 93.09%, respectively. For ZEA degradation, four strains demonstrated effectiveness within the established methodological criteria and were selected for further testing: *B. amyloliquefaciens plantarum* MLB3, *Bacillus subtilis* MLB2, *Bacillus velezensis* CL197, and *Streptomyces griseus* CECT 3276. Satisfactory FB1 degradation was not achieved with the applied methodology; therefore, further tests with this mycotoxin were not pursued (Table 2). Screening was also conducted for ochratoxin A degradation; however, none of the bacteria exhibited any degree of degradation (unpublished data).

Table 2. Screening for the degradation potential (%) of zearalenone (ZEA) and fumonisin B_1 (FB_1).

Microorganism	ZEA degradation	FB_1 degradation
Bacillus amyloliquefaciens CECT 493	0.00	21.10
Bacillus amyloliquefaciens plantarum MLB3	**93.09**	28.92
Bacillus licheniformis CECT 20	0.00	20.66
Bacillus megaterium CECT 44	40.65	18.71
Bacillus subtilis MLB2	**96.75**	11.63
Bacillus thuringiensis CECT 197	6.74	24.94

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Bacillus velezensis CL197	**94.27**	30.03
Candida sake CECT 1044	38.97	14.84
Levilactobacillus (*Lactobacillus*) *brevis* BN3	12.91	17.97
Limosilactobacillus (*Lactobacillus*) *fermentum* 20_PG2_BHI ZZMK	22.42	18.95
Lacticaseibacillus (*Lactobacillus*) *paracasei* DSM 2649	47.02	26.92
Lactiplantibacillus (*Lactobacillus*) *plantarum* DSM 1055	38.44	30.49
Lacticaseibacillus (*Lactobacillus*) *rhamnosus* DSM 20711	17.61	32.21
Liquorilactobacillus (*Lactobacillus*) *satsumensis* DSM 16230	4.45	36.45
Metschnikowia pulcherrima CECT 1691	36.20	18.22
Paenibacillus chibensis CECT 375	2.37	25.68
Paenibacillus polymyxa CECT 153	0.00	25.11
Pediococcus acidilactici 146 RLT	29.25	37.13
Pseudomonas putida MCA	42.55	0.33
Pseudomonas syringae CECT 312	48.41	30.45
Saccharomyces cerevisiae DSM 70868	42.15	24.11
Streptomyces calvus CECT 3271	53.30	24.81
Streptomyces griseus CECT 3276	**94.07**	14.66

The selected bacteria exhibited varying degradation rates when evaluated by the five established methodological parameters. The most effective degradation was achieved when using intracellular metabolites, resulting in a reduction of 89.34±0.72% to 92.93±2.17%. When assessing ZEA adsorption, low levels were observed, ranging from 10.10±0.68% to 18.59±4.80%. The combination of low adsorption with high degradation is a highly desirable characteristic since adsorption can be reversed along the gastrointestinal tract in potential *in vivo* applications, thereby restoring ZEA toxicity, whereas degradation is associated with a permanent modification in the toxin's structure (Table 3).

When assessing the metabolites produced after the inoculation of each bacterium with ZEA, the predominant presence was ZEA with conjugates, such as glucosides, acetyl, malonyl, or sulfate. Additionally, zeranol and zearalenol were observed in their original forms or as conjugates. The formation of conjugates can pose a concern, as they may potentially separate within the organism, allowing the toxin to exert its deleterious effects. Furthermore, zeranol and zearalenol are compounds that may exhibit increased toxic activity based on their chemical structure. However, it should be noted that the concentration of metabolites remained relatively low. While the analysis began with 1 µg/mL of ZEA, the concentration of metabolites ranged from 11.20±0.87 to 99.10±0.93 ng/mL (Table 4).

Table 3. Degradation of zearalenone (ZEA) under different conditions and recovery of the toxin through bacterial adsorption (%).

Bacteria	**T1**	**T2**	**T3**	**T4**	**T5**
Bacillus amyloliquefaciens plantarum MLB3	85.17±4.67	37.63±4.40	92.29±0.50	41.54±7.00	18.59±4.80
Bacillus subtilis MLB2	88.93±4.97	69.58±5.78	92.93±2.17	47.55±6.89	10.60±2.07
Bacillus velezensis CL197	83.92±1.83	60.04±1.64	91.61±5.17	44.39±1.61	10.10±0.68
Streptomyces griseus CECT 3276	84.71±2.98	24.82±6.16	89.34±0.72	11.01±4.08	12.72±3.30

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T1: 10^4 CFU/mL + 1 µg/mL of ZEA, 48h at 37 °C; **T2:** Cell-free supernatants + 1 µg/mL of ZEA, 48h at 37 °C; **T3:** Intracellular metabolites + 1 µg/mL of ZEA, 48h at 37 °C; **T4:** Autoclaved culture + 1 µg/mL of ZEA, 48h at 37 °C; **T5:** Recovery of ZEA from bacterial biomass adsorption after incubation for 48h at 37 °C in the presence of 1 µg/mL of ZEA.

Table 4. Metabolites of zearalenone (ZEA) produced by the inoculation of *Bacillus* and *S. griseus* at a concentration of 10^4 CFU/mL + 1 µg/mL of ZEA, with incubation for 48 hours at 37 ºC.

Molecular formula	*m/z*	Presumptive compound	Concentration*	Ref.
***Bacillus amyloliquefaciens plantarum* MLB3**				
C24 H34 O11	557.2210	hydroxy-zearalenol-Glc	98.92±0.28	[18]
C24 H32 O11	495.1890	hydroxy-zearalenone-Glc	94.07±0.28	[18]
C30 H44 O15	689.2625	zearalenol-di-Glc	26.54±0.30	[18]
C26 H36 O11	523.2167	zearalenol-Glc-Ac	29.46±0.81	[19]
C24 H32 O10	525.1971	zearalenone-4-beta-D-glucopyranoside	14.69±0.86	[20]
C36 H52 O20	863.3136	zearalenone-tri-Glc	99.10±0.93	[18]
***Bacillus subtilis* MLB2**				
C18 H20 O6	331.1174	hydroxy-dehydro-zearalenone	38.48±0.21	[18]
C32 H46 O16	745.2972	zearalenol-di-Glc-Ac	72.70±0.80	[19]
C26 H36 O11	523.2182	zearalenol-Glc-Ac	25.35±0.66	[19]
C36 H52 O20	849.3037	zearalenone-tri-Glc	26.71±0.79	[18]
C18 H26 O5	381.1930	zeranol	15.36±0.51	[21]
C18 H24 O8 S	445.1199	α- or β-zearalenol-Sulf	16.94±0.06	[18]
***Bacillus velezensis* CL197**				
C17 H24 O4	291.1597	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	15.50±0.91	[22]
C24 H34 O11	557.2226	hydroxy-zearalenol-Glc	86.98±0.52	[18]
C26 H36 O11	523.2164	zearalenol-Glc-Ac	45.66±0.41	[19]
C24 H32 O10	525.1981	zearalenone-4-beta-D-glucopyranoside	11.20±0.87	[20]
C36 H52 O20	849.3033	zearalenone-tri-Glc	15.49±0.87	[18]
***Streptomyces griseus* CECT 3276**				
C18 H20 O6	331.1174	hydroxy-dehydro-zearalenone	68.94±0.28	[18]
C24 H34 O11	557.2241	hydroxy-zearalenol-Glc	88.45±0.74	[18]
C32 H46 O16	731.2736	zearalenol-di-Glc-Ac	62.86±0.55	[19]
C24 H32 O10	525.1980	zearalenone-4-beta-D-glucopyranoside	16.73±0.12	[20]
C27 H34 O13	611.2001	zearalenone-Mal-Glc	59.44±0.02	[23]
C18 H26 O5	321.1704	Zeranol	27.50±0.95	[21]
C18 H24 O8 S	399.1137	α- or β-zearalenol-Sulf	17.84±0.96	[18]

Glc: glucoside; Ac: acetyl; Mal: malonyl; Sulf: sulfate. *Equivalent concentration in ng/mL of ZEA.

3.2. Antifungal and Antimycotoxigenic Activity of the Selected Bacteria in Solid Culture Media

When fungi and bacteria were co-inoculated in solid media, all bacteria were able to inhibit the growth of at least one fungal species. The best result was observed with *B. amyloliquefaciens plantarum* MLB3, which exhibited inhibitory activity against 100% of the fungi (13 strains), while the lowest result was observed with *S. griseus* CECT 3276, showing only 7.69% effectiveness (1 strain). This

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difference may be attributed to the considerably slower growth of *S. griseus* CECT 3276 in solid media compared to other bacteria, allowing the fungi more time to develop and occupy the available growth space. The strains *B. velezensis* CL197 and *B. subtilis* MLB2 exhibited 84.62% (11 strains) and 15.38% (2 strains) effectiveness, respectively (Table 5). Although the fungi were selected for their documented ability to produce mycotoxins, no production was detected in either the control or treatment groups. This may be due to routine cultivation in a nutrient-rich medium where there is no stimulus for the production of secondary metabolites responsible for the microorganism's defense and survival.

Table 5. Inhibition of fungal growth (mm) when in co-culture with the selected bacteria.

Microrganism	Control	Treatment
***Aspergillus steiiny* CECT 20510**		
Bacillus amyloliquefaciens plantarum MLB3	34.5±0.26	21.5±0.62*
Bacillus subtilis MLB2		31.0±0.14
Bacillus velezensis CL197		28.5±0.23*
Streptomyces griseus CECT 3276		34.0±0.44
***Fusarium graminearum* ITEM 126**		
Bacillus amyloliquefaciens plantarum MLB3	26.5±0.71	16.5±0.23*
Bacillus subtilis MLB2		26.5±0.15
Bacillus velezensis CL197		23.5±0.71
Streptomyces griseus CECT 3276		27.0±0.66
***Fusarium langsethiae* ITEM 11031**		
Bacillus amyloliquefaciens plantarum MLB3	36.5±0.71	23.5±0.32*
Bacillus subtilis MLB2		21.5±0.18*
Bacillus velezensis CL197		19.5±2.12*
Streptomyces griseus CECT 3276		35.5±0.49
***Fusarium oxysporum* ITEM 2719**		
Bacillus amyloliquefaciens plantarum MLB3	23.5±0.71	13.5±0.29*
Bacillus subtilis MLB2		21.5±0.73
Bacillus velezensis CL197		19.5±0.71*
Streptomyces griseus CECT 3276		23.5±0.51
***Fusarium oxysporum* ITEM 7067**		
Bacillus amyloliquefaciens plantarum MLB3	27.5±0.71	20.5±0.80*
Bacillus subtilis MLB2		22.0±0.27*
Bacillus velezensis CL197		23.0±1.41*
Streptomyces griseus CECT 3276		26.5±0.23
***Fusarium verticillioides* CECT 12043**		
Bacillus amyloliquefaciens plantarum MLB3	29.0±2.83	18.5±0.58*
Bacillus subtilis MLB2		27.5±0.51
Bacillus velezensis CL197		25.5±0.71
Streptomyces griseus CECT 3276		28.0±0.50
***Fusarium verticillioides* CECT 12044**		
Bacillus amyloliquefaciens plantarum MLB3	42.5±2.12	23.0±0.79*
Bacillus subtilis MLB2		42.5±0.28

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Bacillus velezensis CL197		25.5±0.71*
Streptomyces griseus CECT 3276		43.0±0.38
***Fusarium verticillioides* CECT 12052**		
Bacillus amyloliquefaciens plantarum MLB3	36.0±1.41	24.5±0.61*
Bacillus subtilis MLB2		32.0±0.64
Bacillus velezensis CL197		22.5±0.71*
Streptomyces griseus CECT 3276		31.5±0.24*
***Gibberella zeae* CECT 2150**		
Bacillus amyloliquefaciens plantarum MLB3	42.0±1.41	34.5±0.38*
Bacillus subtilis MLB2		40.5±0.47
Bacillus velezensis CL197		28.5±4.95*
Streptomyces griseus CECT 3276		39.5±0.88
***Fusarium graminearum* CECT 20924**		
Bacillus amyloliquefaciens plantarum MLB3	34.5±10.61	23.5±0.71*
Bacillus subtilis MLB2		34.0±0.59
Bacillus velezensis CL197		23.0±5.66*
Streptomyces griseus CECT 3276		33.5±0.63
***Fusarium sporotrichioides* CECT 20165**		
Bacillus amyloliquefaciens plantarum MLB3	34.5±0.71	15.5±0.31*
Bacillus subtilis MLB2		33.5±0.78
Bacillus velezensis CL197		24.5±0.71*
Streptomyces griseus CECT 3276		34.0±0.77
***Gibberella zeae* CECT 20492**		
Bacillus amyloliquefaciens plantarum MLB3	41.5±9.19	24.0±0.53*
Bacillus subtilis MLB2		42.0±0.49
Bacillus velezensis CL197		25.5±0.71*
Streptomyces griseus CECT 3276		41.0±0.49
***Penicillium verrucosum* VTT D-01847**		
Bacillus amyloliquefaciens plantarum MLB3	18.0±0.12	11.5±0.38*
Bacillus subtilis MLB2		15.5±0.79
Bacillus velezensis CL197		13.5±0.73*
Streptomyces griseus CECT 3276		17.0±0.78

*Significant difference (p<0.05) compared to the control group.

3.3. Bacterial Metabolites Characterization and Antifungal Effects

Among the evaluated bacteria, it was possible to provide a better characterization of the antifungal metabolites generated by *B. velezensis* CL197, with the detection of Fengycin C, Gageostatin B, Gageostatin C, Marihysin A, Plipastatin A2, and Plipastatin B1. For the bacterium *B. amyloliquefaciens plantarum* MLB3, the metabolites Gageostatin C and Marihysin A were identified and quantified (Table 6). No metabolites were detected in the cultures of *B. subtilis* MLB2 under the conditions used in this study. A total of 8 metabolites with potential antifungal activity were detected in the cultures of *S. griseus* CECT 3276. However, for their identification, a database common to metabolites produced by bacteria of the *Streptomyces* genus was employed. Given that this genus is widely recognized for its production of metabolites, either through natural pathways or by

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induction/bioengineering, the results presented here may be compromised. The compounds identified could potentially correspond to similar ones not specifically indicated, such as isomers (Table 7).

Table 6. Antifungal metabolites produced by *Bacillus velezensis* CL197 and *Bacillus amyloliquefaciens plantarum* MLB3 (mg/L).

Incubation	Metabolite					
	Fengycin C	Gageostatin B	Gageostatin C	Marihysin A	Plipastatin A2	Plipastatin B1
***Bacillus amyloliquefaciens plantarum* MLB3**						
24h	nd	nd	nd[a]	nd[a]	nd	nd
48h	nd	nd	0.55±0.03[b]	nd[a]	nd	nd
72h	nd	nd	1.11±0.07[c]	2.44±0.19[b]	nd	nd
***Bacillus velezensis* CL197**						
24h	17.49±2.52[a]	2.55±0.24[a]	16.48±1.49[a]	1.34±0.10[a]	3.98±0.47[a]	1.33±0.21[a]
48h	7.88±0.94[b]	3.05±0.33[b]	12.01±1.59[b]	2.24±0.07[b]	2.41±0.04[b]	0.55±0.16[b]
72h	5.12±0.12[c]	2.12±0.19[a]	5.71±0.57[c]	2.97±0.33[c]	2.19±0.44[b]	0.40±0.03[b]

Different letters within the same column in the same group represent significant differences ($p < 0.05$). nd: not detected.

Table 7. Antifungal metabolites potenttialy produced by *Streptomyces griseus* CECT 3276.

Molecular formula	Presumptive compound	Ref.
C16 H23 N O4	Streptimidone	[24]
C29 H36 N2 O6	Frontalamide B	[25]
C29 H36 N2 O7	Frontalamide A	[25]
C33 H52 O10	Thailandin B	[26]
C35 H58 O12	Fungichromin	[27]
C36 H58 O10	Faeriefungin	[28]
C39 H61 N O14	Rimocidin	[29]
C47 H75 N O17	Nystatin A1	[30]

In the liquid culture medium inhibition test, the metabolites produced by *B. amyloliquefaciens plantarum* MLB3, *B. subtilis* MLB2, and *S. griseus* CECT 3276 for 24h did not exhibit fungistatic or fungicidal effects. However, the metabolites produced by *B. velezensis* CL197 demonstrated fungistatic effects at concentrations ranging from 250 to 500 g/L. On the other hand, when utilizing metabolites produced for 48 and 72h, all of them were effective, leading to fungal inhibition at concentrations of 250 and 500 g/L, and fungicidal activity at a concentration of 500 g/L (Table 8).

Table 8. Minimum inhibitory and fungicidal concentrations (g/L) of lyophilized metabolites from bioprotective bacteria against different mycotoxigenic fungal strains.

Microrganism	*Bacillus amyloliquefaciens plantarum* MLB3		*Bacillus subtilis* MLB2		*Bacillus velezensis* CL197		*Streptomyces griseus* CECT 3276	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	24h-incubation							

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Aspergillus steiiny CECT 20510	>500	>500	>500	>500	500	>500	>500	>500
Fusarium graminearum ITEM 126	>500	>500	>500	>500	250	>500	>500	>500
Fusarium langsethiae ITEM 11031	>500	>500	>500	>500	500	>500	>500	>500
Fusarium oxysporum ITEM 2719	>500	>500	>500	>500	500	>500	>500	>500
Fusarium oxysporum ITEM 7067	>500	>500	>500	>500	500	>500	>500	>500
Fusarium verticillioides CECT 12043	>500	>500	>500	>500	250	>500	>500	>500
Fusarium verticillioides CECT 12044	>500	>500	>500	>500	500	>500	>500	>500
Fusarium verticillioides CECT 12052	>500	>500	>500	>500	500	>500	>500	>500
Gibberella zeae CECT 2150	>500	>500	>500	>500	500	>500	>500	>500
Fusarium graminearum CECT 20924	>500	>500	>500	>500	500	>500	>500	>500
Fusarium sporotrichioides CECT 20165	>500	>500	>500	>500	500	>500	>500	>500
Gibberella zeae CECT 20492	>500	>500	>500	>500	500	>500	>500	>500
Penicillium verrucosum VTT D-01847	>500	>500	>500	>500	500	>500	>500	>500
	48h-incubation							
Aspergillus steiiny CECT 20510	500	500	500	500	500	500	500	500
Fusarium graminearum ITEM 126	500	500	500	500	250	500	500	500
Fusarium langsethiae ITEM 11031	500	500	500	500	500	500	500	500
Fusarium oxysporum ITEM 2719	500	500	500	500	500	500	500	500
Fusarium oxysporum ITEM 7067	500	500	500	500	500	500	500	500
Fusarium verticillioides CECT 12043	500	500	500	500	250	500	500	500
Fusarium verticillioides CECT 12044	500	500	500	500	500	500	500	500
Fusarium verticillioides CECT 12052	500	500	500	500	500	500	500	500
Gibberella zeae CECT 2150	500	500	500	500	250	500	500	500

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Fusarium graminearum CECT 20924	500	500	500	500	500	500	500	500
Fusarium sporotrichioides CECT 20165	500	500	500	500	500	500	500	500
Gibberella zeae CECT 20492	500	500	500	500	500	500	500	500
Penicillium verrucosum VTT D-01847	500	500	500	500	500	500	500	500
	72h-incubation							
Aspergillus steiiny CECT 20510	500	500	500	500	500	500	250	500
Fusarium graminearum ITEM 126	500	500	500	500	250	500	500	500
Fusarium langsethiae ITEM 11031	500	500	500	500	250	500	500	500
Fusarium oxysporum ITEM 2719	500	500	500	500	250	500	500	500
Fusarium oxysporum ITEM 7067	500	500	250	500	500	500	500	500
Fusarium verticillioides CECT 12043	500	500	500	500	250	500	250	500
Fusarium verticillioides CECT 12044	500	500	250	500	500	500	500	500
Fusarium verticillioides CECT 12052	500	500	500	500	500	500	500	500
Gibberella zeae CECT 2150	500	500	500	500	250	500	250	500
Fusarium graminearum CECT 20924	500	500	500	500	500	500	250	500
Fusarium sporotrichioides CECT 20165	250	500	500	500	250	500	500	500
Gibberella zeae CECT 20492	500	500	500	500	250	500	500	500
Penicillium verrucosum VTT D-01847	500	500	500	500	500	500	500	500

When evaluated in solid culture medium, only the metabolites generated through 72h of incubation exhibited inhibitory activity at a dose of 500 g/L. Notably, the metabolites produced by *B. velezensis* CL197 and *S. griseus* CECT 3276 demonstrated inhibitions ranging from 5.5±0.1 to 6.2±0.2 mm and 5.2±0.2 to 6.2±0.6 mm, respectively. The inhibition generated by the metabolites of *B. amyloliquefaciens plantarum* MLB3 was slightly lower, ranging from 3.2±0.9 to 4.0±0.2 mm. On the other hand, the metabolites of *B. subtilis* MLB2 were effective in only 6 out of the 13 fungal strains evaluated, with inhibition ranging from 1.1±0.1 to 1.4±0.3 mm (Table 9).

Table 9. Inhibition halo of fungal growth (mm) when exposed to lyophilized metabolites generated after 72h of incubation of bioprotective bacteria, at a concentration of 500 g/L.

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Microrganism	***Bacillus amyloliquefaciens plantarum* MLB3**	***Bacillus subtilis* MLB2**	***Bacillus velezensis* CL197**	***Streptomyces griseus* CECT 3276**
Aspergillus steiiny CECT 20510	3.9±0.3	nd	5.9±0.8	5.2±0.2
Fusarium graminearum ITEM 126	3.7±0.3	nd	5.6±0.9	5.9±0.1
Fusarium langsethiae ITEM 11031	3.2±0.9	1.3±0.3	5.5±0.1	5.6±0.4
Fusarium oxysporum ITEM 2719	3.5±0.8	1.4±0.3	5.5±0.1	5.6±0.6
Fusarium oxysporum ITEM 7067	3.4±0.8	1.1±0.2	6.1±0.5	5.5±0.6
Fusarium verticillioides CECT 12043	4.0±0.2	nd	6.0±0.1	5.5±0.5
Fusarium verticillioides CECT 12044	3.9±0.3	1.1±0.3	6.2±0.2	5.8±0.1
Fusarium verticillioides CECT 12052	3.4±0.1	nd	6.0±0.9	5.5±0.5
Gibberella zeae CECT 2150	3.5±0.5	1.2±0.2	5.9±0.8	5.3±0.2
Fusarium graminearum CECT 20924	3.7±0.5	nd	5.6±0.1	6.2±0.4
Fusarium sporotrichioides CECT 20165	3.4±0.1	1.1±0.1	5.8±0.3	5.8±0.9
Gibberella zeae CECT 20492	3.5±0.4	nd	5.6±0.3	6.2±0.6
Penicillium verrucosum VTT D-01847	3.3±0.5	nd	5.5±0.6	5.7±0.1

3.4. Mycotoxin Degradation in Simulated Digestion

All bacteria exhibited effective ZEA degradation throughout the simulated digestive process. In the swine digestion simulation, *B. subtilis* MLB2 demonstrated remarkable efficacy, achieving complete toxin degradation after a 2h-incubation. In the poultry simulation, notable performances were observed for *B. velezensis* CL197, *B. subtilis* MLB2, and *B. amyloliquefaciens plantarum* MLB3, with each achieving 100% degradation, or statistically equivalent levels, within a 2h-digestion period (Table 10).

Table 10. Degradation of zearalenone (%) in simulated swine and poultry digestion.

Bacteria	**Swine simulation**		**Poultry simulation**	
	2h	**6h**	**2h**	**4h20min**
Bacillus amyloliquefaciens plantarum MLB3	80.14±5.71^{a}	100^{a}	97.12±4.36^{a}	100^{a}
Bacillus subtilis MLB2	100^{b}	100^{a}	100^{a}	100^{a}
Bacillus velezensis CL197	60.41±7.23^{c}	96.74±2.13^{a}	100^{a}	100^{a}
Streptomyces griseus CECT 3276	76.18±2.28^{a}	100^{a}	92.44±6.68^{b}	100^{a}

Different letters within the same column represent significant differences ($p < 0.05$).

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When evaluating the production of metabolites in simulated digestion, the same profile as observed in the culture media degradation was obtained. The metabolites mainly consisted of conjugates, all with concentrations at least 10 times lower than the initial ZEA concentration. In poultry digestion, the concentrations of metabolites ranged from 2.17±0.84 to 68.74±0.44 (Table 11), and in swine digestion, they ranged from 3.21±0.29 to 258.32±0.36 (Table 12). During *in vitro* simulations, the reversion of conjugates to their original condition was not observed. However, *in vivo* tests are required for a more in-depth evaluation, as there are various factors in the animal organism that may facilitate the reconversion of conjugates into ZEA.

Table 11. Presumptive metabolites of zearalenone obtained in poultry digestion simulation supplemented with different bioprotective bacteria.

	Molecular formula	**m/z**	**Presumptive compound**	**Concentration***	**Ref.**
	***Bacillus amyloliquefaciens plantarum* MLB3**				
2h	C32 H46 O16	731.2735	zearalenol-di-Glc-Ac	33.09±0.73	[19]
	C18 H24 O5	379.1760	α or β-zearalenol	17.10±0.46	[31]
	C24 H34 O11	543.2046	hydroxy-zearalenol-Glc	46.20±0.14	[18]
	C24 H34 O10	541.2286	zearalenol-Glc	36.51±0.11	[32]
	C24 H32 O10	525.1960	zearalenone-4-beta-D-glucopyranoside	33.44±0.71	[20]
	C30 H42 O15	641.2442	zearalenone-di-Glc	9.09±0.67	[33]
4h20min	C26 H34 O11	567.2059	Ac-zearalenone-Glc or zearalenone-Ac-Glc	42.85±0.85	[19]
	C18 H20 O6	377.1243	hydroxy-dehydro-zearalenone	8.11±0.56	[18]
	C24 H34 O11	543.2085	hydroxy-zearalenol-Glc	51.29±0.07	[18]
	C24 H32 O11	495.1883	hydroxy-zearalenone-Glc	54.74±0.38	[18]
	C24 H32 O10	539.2158	zearalenone-4-beta-D-glucopyranoside	19.44±0.66	[20]
	***Bacillus subtilis* MLB2**				
2h	C17 H24 O4	291.1604	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	3.67±0.25	[22]
	C24 H32 O10	525.1978	zearalenone-4-beta-D-glucopyranoside	4.69±0.85	[20]
	C24 H34 O11	557.2230	hydroxy-zearalenol-Glc	8.49±0.66	[18]
	C32 H46 O16	731.2748	zearalenol-di-Glc-Ac	28.61±0.41	[19]

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	C29 H40 O14	611.2341	zearalenone-Hex-Pen	7.17±0.25	[18]
4h20min	C26 H34 O11	567.2056	Ac-zearalenone-Glc or zearalenone-Ac-Glc	37.85±0.22	[19]
	C17 H24 O4	291.1600	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	8.60±0.83	[22]
	C24 H32 O10	525.1968	zearalenone-4-beta-D-glucopyranoside	15.08±0.16	[20]
	C24 H32 O11	555.2093	hydroxy-zearalenone-Glc	15.04±0.24	[18]
	C32 H46 O16	745.2958	zearalenol-di-Glc-Ac	15.09±0.30	[19]
	C39 H56 O23	891.3163	zearalenol-di-Mal-tri-Glc	16.41±0.26	[18]
	C27 H34 O13	625.2141	zearalenone-Mal-Glc	17.44±0.95	[23]
	***Bacillus velezensis* CL197**				
2h	C17 H24 O4	291.1595	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	3.12±0.42	[22]
	C24 H32 O10	539.2128	zearalenone-4-beta-D-glucopyranoside	10.84±0.34	[20]
	C32 H46 O16	745.2892	zearalenol-di-Glc-Ac	12.37±0.20	[19]
	C24 H34 O10	481.2057	zearalenol-Glc	61.40±0.09	[32]
	C29 H40 O14	657.2419	zearalenone-Hex-Pen	28.40±0.72	[18]
	C18 H24 O5	319.1550	α or β-zearalenol	8.03±0.54	[31]
4h20min	C17 H24 O4	291.1593	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	4.15±0.31	[22]
	C24 H32 O10	539.2149	zearalenone-4-beta-D-glucopyranoside	24.57±0.39	[20]
	C26 H34 O11	567.2057	Ac-zearalenone-Glc or zearalenone-Ac-Glc	56.51±0.32	[19]
	C32 H46 O16	731.2767	zearalenol-di-Glc-Ac	42.54±0.71	[19]
	C24 H34 O10	527.2133	zearalenol-Glc	7.03±0.93	[32]
	C29 H40 O14	657.2428	zearalenone-Hex-Pen	36.99±0.54	[18]
	C33 H44 O18	773.2529	zearalenone-Mal-di-Glc	4.28±0.47	[18]

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	***Streptomyces griseus* CECT 3276**				
2h	C18 H20 O6	377.1233	hydroxy-dehydro-zearalenone	12.27±0.88	[18]
	C18 H24 O6	395.1701	hydroxy-zearalenol	2.84±0.85	[18]
	C30 H44 O15	689.2652	zearalenol-di-Glc	2.17±0.84	[18]
	C36 H48 O21	815.2596	zearalenol-di-Mal-di-Glc	26.74±0.47	[18]
	C24 H32 O10	525.1964	zearalenone-4-beta-D-glucopyranoside	12.57±0.56	[20]
	C18 H22 O8 S	397.0954	zearalenone-4-Sulf	28.16±0.14	[19]
	C30 H42 O15	641.2415	zearalenone-di-Glc	14.28±0.56	[33]
	C29 H40 O14	611.2298	zearalenone-Hex-Pen	20.73±0.50	[18]
	C18 H24 O8 S	399.1114	α- or β-zearalenol-Sulf	6.72±0.55	[18]
	C20 H24 O6	405.1547	Ac-zearalenone	60.81±0.14	[19]
	C24 H34 O10	527.2149	zearalenol-Glc	10.40±0.69	[32]
	C39 H54 O24	965.3121	zearalenone-di-Mal-tri-Glc	68.74±0.44	[18]
	C33 H44 O18	727.2451	zearalenone-Mal-di-Glc	49.83±0.55	[18]
4h20min	C17 H24 O4	291.1595	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	8.89±0.02	[22]
	C20 H24 O6	405.1549	Ac-zearalenone	20.20±0.66	[19]
	C36 H48 O21	815.2594	zearalenol-di-Mal-di-Glc	25.86±0.48	[18]
	C39 H56 O23	891.3155	zearalenol-di-Mal-tri-Glc	27.40±0.06	[18]
	C32 H44 O16	743.2722	Ac-zearalenone-di-Glc or zearalenone-Ac-di-Glc	23.47±0.58	[19]
	C18 H20 O6	391.1381	hydroxy-dehydro-zearalenone	20.53±0.33	[18]
	C32 H46 O16	731.2730	zearalenol-di-Glc-Ac	30.01±0.80	[19]
	C24 H32 O10	539.2151	zearalenone-4-beta-D-glucopyranoside	21.85±0.74	[20]

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	C30 H42 O15	641.2459	zearalenone-di-Glc	15.80±0.60	[33]

Glc: glucoside; Ac: acetyl; Mal: malonyl; Sulf: sulfate; Hex: hexose; Pen: pentose. *Equivalent concentration in ng/mL of ZEA.

Table 12. Presumptive metabolites of zearalenone obtained in swine digestion simulation supplemented with different bioprotective bacteria.

	Molecular formula	**m/z**	**Presumptive compound**	**Concentration***	**Ref.**
	***Bacillus amyloliquefaciens plantarum* MLB3**				
2 h	C32 H44 O16	729.2679	Ac-zearalenone-di-Glc or zearalenone-Ac-di-Glc	11.52±0.33	[19]
	C26 H34 O11	521.1995	Ac-zearalenone-Glc or zearalenone-Ac-Glc	58.87±0.06	[19]
	C17 H24 O4	291.1598	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	6.15±0.30	[22]
	C20 H24 O6	405.1555	Ac-zearalenone	46.39±0.78	[19]
	C24 H32 O11	541.1920	hydroxy-zearalenone-Glc	13.14±0.41	[18]
	C24 H34 O10	481.2062	zearalenol-Glc	134.27±0.09	[32]
	C24 H32 O10	539.2136	zearalenone-4-beta-D-glucopyranoside	14.01±0.35	[20]
	C18 H24 O5	379.1764	α or β-zearalenol	7.17±0.86	[31]
	C18 H20 O6	377.1230	hydroxy-dehydro-zearalenone	24.96±0.39	[18]
	C24 H34 O11	543.2048	hydroxy-zearalenol-Glc	78.07±0.31	[18]
	C36 H48 O21	815.2601	zearalenol-di-Mal-di-Glc	19.23±0.28	[18]
	C18 H22 O8 S	397.0954	zearalenone-4-sulfate	17.41±0.72	[34]
	C29 H40 O14	657.2431	zearalenone-Hex-Pen	29.32±0.31	[18]
6 h	C24 H34 O11	497.2016	hydroxy-zearalenol-Glc	75.16±0.74	[18]
	C24 H34 O10	527.2127	zearalenol-Glc	99.47±0.54	[32]
	C18 H22 O8 S	397.0958	zearalenone-4-sulfate	254.37±0.62	[34]

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	C30 H42 O15	641.2448	zearalenone-di-Glc	10.33±0.20	[33]
	C18 H24 O8 S	399.1117	α- or β-zearalenol-Sulf	72.55±0.01	[18]
	C24 H32 O11	495.1862	hydroxy-zearalenone-Glc	44.32±0.83	[18]
	C24 H32 O10	479.1944	zearalenone-4-beta-D-glucopyranoside	11.25±0.93	[20]
	***Bacillus subtilis* MLB2**				
2 h	C26 H34 O11	521.2000	Ac-zearalenone-Glc or zearalenone-Ac-Glc	17.17±0.58	[19]
	C17 H24 O4	291.1604	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	5.04±0.96	[22]
	C18 H20 O6	331.1182	hydroxy-dehydro-zearalenone	3.38±0.58	[18]
	C24 H32 O11	541.1915	hydroxy-zearalenone-Glc	15.65±0.17	[18]
	C26 H36 O11	523.2175	zearalenol-Glc-Ac	74.89±0.49	[19]
	C18 H20 O6	331.1191	hydroxy-dehydro-zearalenone	9.34±0.85	[18]
	C24 H34 O11	557.2229	hydroxy-zearalenol-Glc	107.05±0.25	[18]
	C24 H32 O10	525.1958	zearalenone-4-beta-D-glucopyranoside	24.38±0.04	[20]
	C18 H22 O8 S	397.0963	zearalenone-4-sulfate	18.91±0.69	[34]
	C29 H40 O14	671.2568	zearalenone-Hex-Pen	38.04±0.25	[18]
6 h	C32 H44 O16	743.2777	Ac-zearalenone-di-Glc or zearalenone-Ac-di-Glc	5.68±0.45	[19]
	C30 H44 O15	689.2665	zearalenol-di-Glc	135.44±0.32	[18]
	C33 H44 O18	787.2613	zearalenone-Mal-di-Glc	14.81±0.44	[18]
	C18 H20 O5	375.1471	dehydro-zearalenone	6.36±0.92	[35]
	C18 H20 O6	331.1186	hydroxy-dehydro-zearalenone	8.75±0.61	[18]
	C24 H34 O11	543.2080	hydroxy-zearalenol-Glc	85.54±0.85	[18]
	C24 H32 O11	495.1885	hydroxy-zearalenone-Glc	36.37±0.79	[18]

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	C32 H46 O16	685.2689	zearalenol-di-Glc-Ac	7.92±0.61	[19]
	C24 H34 O10	527.2140	zearalenol-Glc	151.77±0.40	[32]
	C24 H32 O10	525.1963	zearalenone-4-beta-D-glucopyranoside	24.05±0.61	[20]
	C18 H22 O8 S	397.0958	zearalenone-4-sulfate	251.24±0.82	[34]
	C30 H42 O15	641.2443	zearalenone-di-Glc	8.73±0.18	[33]
	C27 H34 O13	625.2133	zearalenone-Mal-Glc	5.79±0.18	[23]
	C18 H24 O8 S	399.1114	α- or β-zearalenol-Sulf	72.85±0.22	[18]
	***Bacillus velezensis* CL197**				
2 h	C32 H44 O16	729.2565	Ac-zearalenone-di-Glc or zearalenone-Ac-di-Glc	16.48±0.38	[19]
	C17 H24 O4	291.1598	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	5.30±0.47	[22]
	C32 H46 O16	731.2733	zearalenol-di-Glc-Ac	75.54±0.81	[19]
	C18 H24 O5	379.1762	α or β-zearalenol	11.79±0.38	[31]
	C18 H20 O6	391.1378	hydroxy-dehydro-zearalenone	59.25±0.65	[18]
	C24 H32 O11	495.1881	hydroxy-zearalenone-Glc	196.25±0.98	[18]
	C39 H56 O23	937.3181	zearalenol-di-Mal-tri-Glc	83.98±0.35	[18]
	C24 H34 O10	541.2234	zearalenol-Glc	23.63±0.25	[32]
	C24 H32 O10	525.1956	zearalenone-4-beta-D-glucopyranoside	19.38±0.34	[20]
	C18 H22 O8 S	397.0960	zearalenone-4-sulfate	36.49±0.57	[34]
	C29 H40 O14	611.2360	zearalenone-Hex-Pen	75.46±0.42	[18]
	C27 H34 O13	565.1914	zearalenone-Mal-Glc	19.45±0.79	[23]
	C36 H52 O20	849.3013	zearalenone-tri-Glc	22.83±0.81	[18]
6 h	C17 H24 O4	291.1594	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	5.79±0.75	[22]

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	C39 H56 O23	937.3187	zearalenol-di-Mal-tri-Glc	17.54±0.63	[18]
	C24 H34 O10	481.2058	zearalenol-Glc	67.68±0.19	[32]
	C24 H32 O10	525.1969	zearalenone-4-beta-D-glucopyranoside	24.60±0.80	[20]
	C18 H22 O8 S	397.0958	zearalenone-4-sulfate	258.32±0.36	[34]
	C29 H40 O14	657.2412	zearalenone-Hex-Pen	17.90±0.43	[18]
	C18 H24 O5	379.1757	α or β-zearalenol	26.50±0.50	[31]
	C20 H24 O6	405.1573	Ac-zearalenone	5.32±0.86	[19]
	C24 H32 O11	541.1921	hydroxy-zearalenone-Glc	27.85±0.57	[18]
	C33 H46 O18	789.2807	zearalenol-Mal-di-Glc	10.88±0.19	[36]
	C33 H44 O18	773.2571	zearalenone-Mal-di-Glc	8.80±0.88	[18]
	C27 H34 O13	625.2141	zearalenone-Mal-Glc	8.81±0.85	[23]
	C18 H24 O8 S	399.1116	α- or β-zearalenol-Sulf	77.30±0.21	[18]
	***Streptomyces griseus* CECT 3276**				
2h	C20 H24 O6	405.1542	Ac-zearalenone	26.02±0.31	[19]
	C18 H20 O6	391.1390	hydroxy-dehydro-zearalenone	16.55±0.23	[18]
	C26 H34 O11	581.2232	Ac-zearalenone-Glc or zearalenone-Ac-Glc	3.39±0.75	[19]
	C17 H24 O4	351.1811	1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6-one	3.21±0.29	[22]
	C18 H24 O6	381.1547	hydroxy-zearalenol	4.70±0.14	[18]
	C32 H46 O16	731.2724	zearalenol-di-Glc-Ac	45.64±0.26	[19]
	C39 H56 O23	937.3252	zearalenol-di-Mal-tri-Glc	9.16±0.87	[18]
	C24 H34 O10	527.2116	zearalenol-Glc	41.92±0.39	[32]
	C26 H36 O11	523.2157	zearalenol-Glc-Ac	62.14±0.39	[19]

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	C24 H32 O10	525.1993	zearalenone-4-beta-D-glucopyranoside	31.71±0.78	[20]
	C18 H22 O8 S	397.0956	zearalenone-4-sulfate	23.88±0.92	[34]
	C29 H40 O14	611.2330	zearalenone-Hex-Pen	16.59±0.36	[18]
6 h	C26 H34 O11	521.2059	Ac-zearalenone-Glc or zearalenone-Ac-Glc	11.96±0.30	[19]
	C24 H32 O10	525.1964	zearalenone-4-beta-D-glucopyranoside	15.58±0.66	[20]
	C39 H54 O24	965.3100	zearalenone-di-Mal-tri-Glc	10.45±0.59	[18]
	C20 H24 O6	405.1545	Ac-zearalenone	20.06±0.73	[19]
	C24 H34 O10	541.2286	zearalenol-Glc	18.71±0.53	[32]
	C18 H22 O8 S	397.0958	zearalenone-4-sulfate	255.97±0.11	[34]
	C18 H24 O8 S	399.1117	α- or β-zearalenol-Sulf	75.65±0.94	[18]

Glc: glucoside; Ac: acetyl; Mal: malonyl; Sulf: sulfate; Hex: hexose; Pen: pentose. *Equivalent concentration in ng/mL of ZEA.

4. Discussion

Initially, during the screening for ZEA and FB1 degradation, significant variability in results was observed, even among strains of the same species. This variation is associated with the intrinsic characteristics of each strain, such as metabolite production, enzyme activity, etc. In the present study, none of the strains were capable of degrading FB1 satisfactorily. However, due to their mild performance, they may be subjects of future research, involving an in-depth assessment of their mechanisms of action. This would help determine the feasibility of enhancing their effectiveness through molecular and bioengineering techniques. During the screening for ZEA degradation, four bacteria stood out: *B. amyloliquefaciens plantarum* MLB3, *B. subtilis* MLB2, *B. velezensis* CL197, and *S. griseus* CECT 3276. Previous research had already demonstrated the potential of these species in ZEA control. Lee et al [37] showed that *B. amyloliquefaciens* completely degraded 3.5 μg/mL of ZEA in 24h of incubation. Lei et al [38] utilized a *B. subtilis* strain for ZEA degradation and achieved positive results, with approximately an 89%-reduction in the initial toxin concentration. Wang et al [39] used a *B. velezensis* strain and achieved 100% ZEA degradation with an initial concentration of 7.45 μg/mL after 72h of incubation. While recent research using *S. griseus* was not found, Harkai et al [40] demonstrated that *Streptomyces* species had the potential for ZEA degradation, with efficiency ranging from 87.85±6.68 to 99.64±0.23% at an initial concentration of 1 μg/mL of ZEA.

During the evaluation of different potential degradation mechanisms and the possibility of adsorption, it was observed that the most efficient form of utilization was the intracellular metabolites, followed by the use of the whole bacterial culture. A low adsorption rate, as mentioned earlier, is a desirable characteristic since adsorption can be reversed throughout the gastrointestinal tract. In this analysis, the most abundant ZEA metabolites were conjugates, albeit with a drastic reduction in their concentration compared to the initial ZEA concentration. This demonstrates that the mycotoxin was predominantly biotransformed, necessitating further in-depth tests to specifically determine the exact metabolization products. Notably, it is observed that compounds of higher

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toxicity, such as α-zearalenol, were either not formed or present in extremely low quantities relative to the initial ZEA concentration. This indicates that, even without a complete characterization of the degradation products, no compounds that could increase the risks associated with ZEA consumption were formed. The tests revealed that efficient degradation requires either an active bacterium or its metabolites, which exhibit a thermolabile nature, since when autoclaved culture was used (primarily with broken cells), the degradation rate decreased significantly. Considering the industrial process's stages, the most practical application involves using the complete culture without the need for further manipulation. Hence, this method was chosen for subsequent tests, as it still achieved acceptable degradation levels, facilitating the future development of biotechnological products for application in the productive sector.

The evaluated bacteria, besides showing potential for ZEA degradation in culture medium, also demonstrated the ability to inhibit fungal growth. This characteristic can be particularly interesting since, in addition to mitigating the effects of already-produced toxins, they can prevent fungal development, which leads to the subsequent production of mycotoxins. Their bioprotective activities were assessed against different types of fungi, producers of various mycotoxins, and at least one strain proved effective against each of the fungi. This demonstrates their wide applicability in this role, with the possibility of developing combinations between them to enhance results. No mycotoxin production was observed in the analyzed fungi, although their mycotoxin-producing ability has been reported in previous research, which hinders the assessment of bacteria in mycotoxigenic activities. Therefore, further tests are required in this area. Several studies support the effects observed here, where *Bacillus* and *Streptomyces* species exhibit antifungal and antimycotoxigenic properties, highlighting the biotechnological potential of the strains studied [41–44]. Among the evaluated bacteria, the most efficient were *B. amyloliquefaciens plantarum* MLB3 and *B. velezensis* CL197, which belong to closely related phylogenetic groups and have a significant history of antifungal use. They employ various mechanisms, including the production of antifungal lipopeptides and volatile organic compounds, as well as competition for nutrients. Furthermore, these bacteria are generally safe for use, posing minimal risks to both animals and humans and having minimal environmental impact [45].

The assessment of metabolite production revealed a wide array of compounds produced by *B. velezensis*, aligning with the literature, which highlights these as some of its primary mechanisms of action [45]. Although the literature also cites the high potential for antifungal metabolite production by *B. amyloliquefaciens* [45], only two compounds were detected. In the case of *S. griseus* CECT 3276, eight compounds were potentially identified; however, their confirmation is challenging due to the broad range of compounds produced by bacteria in this genus. This exacerbates the information available in the databases, making it difficult to conduct a detailed analysis using the techniques employed in this study. Further research specifically for this strain is required to complement the results obtained here. No compounds were detected to *B. subtilis* MLB2. However, since the metabolites exhibited antifungal activity in the subsequent tests, further research is necessary to determine the compounds produced. Among the compounds produced by *B. velezensis* CL197 and *B. amyloliquefaciens plantarum* MLB3 and identified in this study, lipopeptides stand out. This class of compounds, according to the available literature, is proven to be essential for bacteria to exert their antifungal and antimycotoxigenic activity. For instance, Chakraborty et al [43] demonstrated that lipopeptides from *Bacillus* have the potential to inhibit mycelial growth, conidiogenesis, and conidial germination. This effect was confirmed in this study, where the metabolites produced by the bacteria were able to inhibit fungal growth in both liquid and solid culture media.

To conclude this research, the potential of bacteria to degrade ZEA in simulated digestion was evaluated. In this analysis, the effects observed in liquid culture media were potentiated, with mainly complete degradation of the toxin by the end of the *in vitro* process and the production of metabolite products in the form of conjugates. It is worth noting that in the *in vitro* process, there was no reversion of the conjugates to their original form, but further research is needed to assess whether this will also be the observed result *in vivo*. The bacteria have demonstrated high effectiveness in

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fungal and mycotoxin control, with broad potential for use in the development of biotechnological products for animal production.

5. Conclusions

The evaluated bacteria demonstrated significant antifungal and antimycotoxin activities with diverse potential applications, such as preventing fungal contamination in stored materials, degrading mycotoxins present in animal feed due to raw material contamination, preventing the absorption of toxins ingested by animals, and more. For a comprehensive characterization of the strains used in this study, further research is required to evaluate the metabolites produced, particularly by *B. subtilis* MLB2 and *S. griseus* CECT 3276, and the metabolization products of ZEA generated by the four bioprotective bacteria.

Another unexplored possibility in this study is the establishment of combinations that complement each other and enhance the observed effects. Thus, the research field remains wide open, with significant potential for the development of a biotechnological product for application in animal production. This product could help maintain herd health, food quality and safety, and posing no risks to consumers, contributing to public health maintenance.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: A.G.E.: Conceptualization, Formal analysis, Investigation, Writing - Original Draft. T.dM.N.: Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Writing - Review & Editing. C.L.: Conceptualization, Methodology, Supervision, Writing - Review & Editing. V.D. and A.M.: Methodology. G.M. and F.B.L.: Conceptualization, Methodology, Supervision, Resources, Project administration, Writing - Review & Editing.

Data Availability Statement: All data supporting the findings of this study are available within the paper and its Supplementary Information.

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