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Potential of Lactic Acid Bacteria and *Bacillus* spp. in a Bio-Detoxification Strategy for Mycotoxin Contaminated Wheat Grains

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Abstract: Mycotoxins present in cereals are a worldwide problem and are a result of the presence of mycotoxin producing fungi. A strategy to reduce these fungi and mycotoxin levels in contaminated grains is with the use of lactic acid bacteria (LAB) or *Bacillus* spp., which can degrade or bind toxins. In this study, LAB and *Bacillus* spp. were isolated from mycotoxin contaminated wheat grains and, together with additional plant-derived strains, an antifungal screening against Fusarium graminearum was performed. Furthermore, these strains were screened for their ability to reduce zearalenone (ZEA) and deoxynivalenol (DON). Finally, the mode of action of the most promising microorganisms was investigated by analyzing toxin reduction with viable and dead cells, cell extracts and supernatants. Out of 212 tested strains, 70 showed high antifungal activity and 42 exhibited the ability to detoxify more than 90% ZEA, i.e., *Bacillus licheniformis* (19), *B. megaterium* (13), and *Levilactobacillus brevis* (10). None of the tested strains were able to decrease DON. The mode of action of ZEA reduction could not be fully elucidated. Neither dead cells (<20%), nor cell extracts nor supernatants could reduce ZEA in high amounts, which exclude high binding capacity and the involvement of extra- or intra-cellular enzymes.

Keywords: mycotoxins; zearalenone; deoxynivalenol; lactic acid bacteria; *Bacillus*; *Fusarium*; cereals; wheat; bio-detoxification

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1. Introduction

Mycotoxins are of major concern worldwide and are found in many raw materials such as cereals, dried fruits, and nuts, from which they enter the food chain [1,2]. According to the Food and Agriculture Organization and a study from Eskola et al. [2], it has been estimated that around 25% of worldwide crops are contaminated with mycotoxins above the EU and Codex limits, Eskola et al. revealed that 60–80% of all crops are contaminated with mycotoxins above detectable levels.

Mycotoxins are secondary metabolites produced by filamentous fungi, these fungi can easily grow on crops on the field but most notably when crops are stored under insufficient storage conditions [3]. The most common mycotoxin producing filamentous fungi related to food and feed are *Aspergillus*, *Penicillium*, and *Fusarium* [4–6]. The mycotoxins, which are most frequently found in food and feed are aflatoxins, ochratoxins, trichothecenes, (deoxynivalenol (DON), T-2 toxin, fumonisins and other trichothecenes), and zearalenone (ZEA) [7,8]. DON is a vomitoxin produced by *Fusarium* spp. (e.g., *F. poae*, *F. sporotrichoides*, *F. acuminatum*, and *F. equiseti*), often found in cereals such as maize, barley, wheat, and oat as well as in products made thereof [9,10]. Intoxication with DON often causes diarrhea, emesis, endotoxemia, and, in rare cases, can lead to death [11]. ZEA, also produced by *Fusarium* spp. (e.g., *F. graminearum*, *F. culmorum*, and *F. cerealis*) is often found in plants,

especially in maize, but also in wheat, barley, and oat [12–15]. Its macrolide structure has analogies with estrogen and possess immunotoxic properties [12–16].

Due to their toxicity and the amount of food waste generated by mycotoxin contaminated food and feed, different strategies have already been established to decrease or minimize the mycotoxin concentration in crops. These methods include maintaining good storage conditions, application of chemical and physical treatments [12], including acidic/basic solutions [13], ozonation [14], UV irradiation [15] and adsorption [16], or the application of antagonistic fungal strains, which inhibit unwanted moulds including mycotoxin producers [17]. Various studies showed that lactic acid bacteria (LAB) [18–20] and *Bacillus* spp. [21–23], express species- and strain-specific antifungal activity against different moulds. Antifungal mechanisms are described as being related to bioactive substances produced by the previous mentioned microorganisms, such as organic acids (especially phenyl lactic acid produced by LAB) [24–26], fatty acids [27], reuterin [28,29], peroxide [30,31], antifungal peptides [32,33] or cyclic dipeptides [34,35]. Furthermore, the production of exopolysaccharides is known to contribute to antifungal activity [36,37]. In addition, lipopeptides [38–40], enzymes [41,42], or polyketones [43] are involved in antifungal mechanisms, especially in relation to *Bacillus* spp.

Biological treatments to eliminate mycotoxins, or so-called bio-detoxification strategies, are favored because of their efficiency, specificity, and environmental safety [44]. Beyond suppression of the toxin producing organism, bio-detoxification is a targeted biological method to reduce mycotoxins. This approach includes the application of plant extracts, enzymes, or microorganisms which are able to degrade or bind the mycotoxins [12,44–46]. Of these two mechanisms, binding or adsorption of toxins to the cell wall of microorganisms might be problematic since the bound toxins could be released again in the gastrointestinal tract, which would lead to health problems in consumers [47,48]. Various studies described the bio-detoxification potential of LAB and propionic acid bacteria (PAB) for zearalenone [46,49–52], ochratoxin A [52,53], DON [46,54–56], aflatoxin [52,57,58], patulin [59,60], and fumonisins [61,62], although the strains tested were found to bind rather than degrade the mycotoxins [63]. In addition to LAB or PAB, selected strains of *Bacillus* spp. showed high efficiency in reduction of DON [64–66], ZEA [67–70], aflatoxin B1 [71–73], ochratoxin A [74,75], patulin [76], and fumonisins [77], however, in these cases, the toxins can be degraded or might be bound to the cell wall or to cell proteins. Furthermore, the metabolites that can be formed during degradation might even be more toxic than the primary substance. For example, ZEA can be degraded to α- or β-zearalenol (ZOL), whereas α-ZOL has a higher binding affinity to estrogen receptors than ZEA [78].

The aim of this study was to evaluate plant-derived strains of LAB and *Bacillus* spp. with qualified presumption of safety (QPS) status, according to the European Food Safety Authority (EFSA) [79], regarding their potential to reduce ZEA and/or DON in a bio-detoxification strategy for wheat grains. Firstly, 109 LAB and *Bacillus* spp. strains were isolated from mycotoxin contaminated wheat grains. These strains were complemented by plant-derived strains isolated in previous studies [80,81] from products such as malt, spent grain, sourdough, and others. An antifungal screening was performed using a total of 212 strains, additionally these strains were tested for their ability to reduce ZEA and/or DON. In a second part, investigations were conducted to understand the bio-detoxification mechanism of the most promising ZEA reducing strains. This study underlines the potential for the use of LAB and *Bacillus* spp. in biodetoxification strategies aiming at preventing food waste and improving food safety of cereal-based products.

2. Materials and Methods

2.1. Standards and Chemicals

Pure standards of zearalenone (ZEA) and deoxynivalenol (DON) were obtained from Sigma-Aldrich (Art. Nr. 32939 and CRM46911, respectively; Merck AG, Zug, Switzerland). All solvents and mobile phase modifiers were of LC-MS grade. Methanol, ammonium formate, and acetic acid were supplied by Sigma-Aldrich (Merck AG, Zug, Switzerland),

water by Carl Roth AG (Arlesheim, Switzerland), formic acid by VWR International GmbH (Dietikon, Switzerland), and ethanol supplied by Thermo Fisher Scientific AG (Reinach, Switzerland).

2.2. Isolation and Identification of Lactic Acid Bacteria and Bacillus spp. Strains from Mycotoxin Contaminated Grains

Mycotoxin contaminated wheat grains (DON 3300 μg/kg, ZEA 100 μg/kg, HT-2 toxin 11 μg/kg, enniatin B 100 μg/kg, enniatin B1 32 μg/kg (determined by Eurofins (Schönenwerd, Switzerland)) and wheat from Romania, (October 2019) were enriched in a minimal nutrition media containing a trace element solution (MM1 and TS2). MM1 consisted of 0.8 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 1 mg/L $CaCl_2$, 1.5 g/L NH_4Cl, 1 mg/L $FeCl_3$ [82] and 2 mL of trace elements (TS2 [83]), with 100 mg/L $ZnSO_4$, 30 mg/L $MnCl_2$, 300 mg/L H_3BO_3, 200 mg/L $CoCl_2$, 10 mg/L $CuCl_2$, 20 mg/L $NiCl_2$, 900 mg/L Na_2MoO_4, and 20 mg/L Na_2SeO_3. Prior to use, the pH of the minimal media including trace elements was set to 6.0. The contaminated grains were mixed in a ratio of 1:1 (10 g grains and 10 g MM1) and 1:4 (8 g grains and 32 g MM1) and incubated for 5 days at 30 °C. Each enrichment was performed in triplicates. After incubation, serial dilutions were plated on MRS agar (VWR), PC agar (Carl Roth), and DRBC agar (BD) aiming at isolating lactic acid bacteria, *Bacillus* spp., and yeast strains, respectively. All plates were incubated for 3 days at 30 °C, MRS plates were incubated under anaerobic conditions. Randomly selected colonies were purified by streaking them 3 times consecutively on the corresponding agar. The purified colonies were identified by MALDI-TOF MS [84] and stored at −80 °C in the culture collection of the Food Biotechnology Research Group of ZHAW, Wädenswil, Switzerland.

2.3. Microorganisms Used in This Study

A total of 212 strains of LAB and *Bacillus* spp. were selected according their QPS status and used for antifungal screenings against *Fusarium graminearum* strains and screenings for mycotoxin reduction (DON and ZEA). The strains included the isolates described in Section 2.2 as well as plant-derived bacterial strains from the culture collection of the Food Biotechnology Research Group of ZHAW (Wädenswil, Switzerland) previously isolated from malt, spent grain, sourdough, and others ([80,81] and unpublished data). Tested species were: *Bacillus flexus* (1), *Bacillus licheniformis* (39), *Bacillus megaterium* (13), *Bacillus pumilus* (2), *Bacillus subtilis* (2), *Levilactobacillus brevis* (10), *Lapidilactobacillus concavus* (3), *Loigolactobacillus coryniformis* (33), *Latilactobacillus curvatus* (12), *Limosilactobacillus fermentum* (1), *Lentilactobacillus kefiri* (1), *Lentilactobacillus parabuchneri* (4), *Lactiplantibacillus plantarum* (6), *Fructilactobacillus sanfranciscensis* (2), *Lactococcus lactis* (2), *Leuconostoc citreum* (19), *Leuconostoc lactis* (14), *Leuconostoc mesenteroides* (1), *Leuconostoc palmae* (2), *Leuconostoc pseudomesenteroides* (4), *Pediococcus acidilactici* (15), *Pediococcus pentosaceus* (22), *Weissella cibaria* (1), *Weissella confusa* (2) and not identified (1).

2.4. Antifungal Screening against Fusarium graminearum DSM 1095 and DSM 4527

The antifungal screening was performed in 6 well plates (TPP) that were filled with 4 mL of wheat flour hydrolysate agar medium (WFH) according to Müller et al. [80]. For the wheat hydrolysate, 200 g of wheat flour type 550 (Meyerhans Mühlen AG, Weinfelden, Switzerland) was mixed with 800 mL tap water and incubated at 30 °C and 90 rpm for 4 h. The mixture was stored in the fridge (4 °C) overnight (18 h) and after decanting, the obtained supernatant was used as WFH. WFH agar was prepared by supplementation of 1 L of WFH with 15 g glucose, 15 g maltose, 15 g sucrose, 15 g fructose, 10 g yeast extract, and 15 g agar. The pH was adjusted to 5.6 and the medium was sterilized at 121 °C for 15 min. 4 mL of WFH agar was distributed into each well of the 6-well plates. LAB strains were cultured on MRS agar anaerobically at 30 °C for 3 days, whereas *Bacillus* spp. were grown on PC agar at 30 °C for 1 day. Each well was inoculated using sterile toothpicks with fresh colonies of LAB or *Bacillus* spp. and the plates were incubated at 30 °C for

3 days anaerobically or at 30 °C aerobically for 1 day, respectively. Spore solutions of *Fusarium graminearum* DSM 1095 as well as DSM 4527, isolated from maize, were prepared in buffered peptone water (0.15%; Carl Roth). Each spore solution was inoculated separately into malt soft agar (18 g malt extract, 9 g agar) with concentrations of ~2 log or 3 log spores per mL soft agar, respectively. The wells were overlayed with 900 µL soft agar and the plates were incubated for 4 days at 25 °C. The inhibition of fungal growth was analyzed by categorization of the inhibition zone into no inhibition, weak- (small inhibition zone), moderate- (clear inhibition zone), and strong inhibition (no mould growth). The experiment was performed in triplicate.

2.5. Screening for Zearalenone and Deoxynivalenol Reduction

2.5.1. Screening Method

The screening for mycotoxin reduction was performed using WFH medium prepared as described in 2.4 with the following modifications: Yeast extract was mixed with WFH and autoclaved at 121 °C for 15 min. Afterwards, 15 g of each sugar (glucose, maltose, sucrose, and fructose) was added, pH was set to 5.6, and the obtained WFH medium was sterile filtered. For the screening, overnight cultures were prepared in MRS broth at 30 °C or BHI broth at 30 °C for LAB or *Bacillus* spp. strains, respectively. The cultures were washed twice by centrifugation (8000× *g*, 5 min) and resuspension using diluent (8.5 g/L peptone, 1 g/L sodium chloride). 1.5 mL WFH medium supplemented with either 0.1 µg/mL ZEA or 5 µg/mL DON was inoculated with 1% of the bacterial culture. A negative control was prepared using 1% diluent as inoculum. The samples were incubated at 30 °C for 72 h. After incubation, the samples were centrifuged (~30 s) and filtered (0.2 µm RC filters; Phenomenex, Torrance, CA, USA).

ZEA and DON were quantified by LC-MS/MS consisting of an Agilent 1290 Infinity II chromatographic system coupled to an Agilent 6530 Q-TOF mass spectrometry according to André et al. [85]. The column used was an Agilent Poroshell 120 EC-C18 (2.1 × 100 mm, 2.7 µm) protected by a guard column (Agilent EC-18, 2.1 × 5 mm, 2.7 µm). For ZEA, the flow rate was set to 0.28 mL/min, while the temperature was at 35 °C. The mobile phases consisted of water with 0.1% acetic acid (mobile phase A) and methanol with 0.1% acetic acid (mobile phase B). The gradient used was as follows: 0–0.5 min 10% B; 6–15 min 98% B; 15–17 min 10% B. For DON, the flow rate was set to 0.25 mL/min, and the column temperature at 35 °C. The two elution mobile phases were made up of water with 0.1% formic acid and 5 mmol ammonium formate (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). Gradient elution was as follows: 0–4.5 min, 10% B, 5–8 min, 100% B; 8.5 min, 10% B. For both mycotoxins the injection volume was 10 µL. The MS analyses were conducted for both mycotoxins in negative ionisation mode (ESI–) in the spectral range of 100–1000 Da. Nitrogen served as the nebuliser and collision gas. For ZEA the parameters of the mass spectrometer were as follows: gas temperature: 350 °C; drying gas: 10 L/min; nebulizer: 40 psi; sheath gas: 350 °C; sheath gas flow: 11 L/min; capillary voltage: 3500 V; fragmentor voltage: 100 V. For DON analysis, the parameters were as follows: gas temperature: 325 °C; drying gas: 6 L/min; nebulizer: 45 psi; sheath gas temperature: 350 °C; sheath gas flow: 11 L/min; capillary voltage: 2000 V; fragmentor voltage: 90 V. The screening of all strains was performed once. After which the cultivation and analyses were performed in triplicates for the strains showing a reduction of the mycotoxin content of more than 70%.

2.5.2. Determination of Zearalenone Detoxification Mechanisms

A selection of strains exhibiting a high reduction of ZEA (reduction of >90% of ZEA) were tested for their detoxification mechanism according to a method adapted from Franco et al. [25] and Gao et al. [86]. Five *B. licheniformis* strains (MA572, MA695, TR086, TR212, and TR374), three *B. megaterium* strains (Myk106, Myk145, and TR362), and five *L. brevis* strains (JR1, JR11, JR187, JR98, and MA278b) were inoculated in 20 mL of WFH medium and were incubated overnight (16 h) at 30 °C. Afterwards, each culture was split into 3 × 5 mL

samples. A first 5 mL sample was used for testing viable cells, whereas the cell pellet was washed twice (8000× *g* for 10 min) with sterile phosphate buffer (50 mM; pH 7), and was resuspended in WFH medium. A second 5 mL sample was used for testing dead cells and was therefore autoclaved for 15 min at 121 °C, followed by washing the inactivated cells twice with sterile phosphate buffer (50 mM; pH 7) by centrifugation (8000× *g* for 10 min), and resuspending them in WFH medium. A third 5 mL sample was centrifuged at 8000× *g* for 10 min and the supernatant was sterile filtered (0.2 µm), resulting in a cell-free supernatant. The remaining cell pellet was washed twice with sterile phosphate buffer (50 mM; pH 7) by centrifugation (8000× *g* for 10 min), resuspended in WFH medium and treated with ultrasonication (Bandelin Sonopuls with ultrasonic sonotrode TS 103; 10 kJ; 10 min, sonication 30 s, cool down 30 s; samples placed on ice; Bandelin, Berlin, Germany) for cell rupture. After centrifugation at 12000× *g* for 20 min, the supernatant was sterile filtered and used as cell extract. From the four different samples (viable cells, dead cells, cell-free supernatant, and cell extract), 1.5 mL was taken and supplemented with 1.5 µL ZEA (100 µg/mL in ethanol). After brief homogenization (vortex) the samples were incubated at 30 °C for 72 h. The samples were filtered (0.2 µm; RC filters; Phenomenex, Torrance, CA, USA) and ZEA was quantified by LC-MS/MS as described in Section 2.5.1. The experiment was performed in triplicate.

3. Results

3.1. Microbiota of Mycotoxin Contaminated Wheat Grains

Microorganisms which were isolated from mycotoxin contaminated wheat grains are listed in Table 1.

Table 1. Microorganisms isolated from mycotoxin contaminated wheat grains enriched in the minimal nutrition medium MM1 in ratios 1:1 and 1:4 (n = 3) and identification by MALDI-TOF MS.

Microorganisms	1:1	1:4	Total
Acinetobacter baumannii	2	0	2
Bacillus cereus	2	1	3
Bacillus megaterium	3	8	11
Bacillus thuringiensis	0	1	1
Citrobacter amalonaticus	1	0	1
Cronobacter sakazakii	1	0	1
Enterobacter cloacae	3	1	4
Enterococcus durans	0	3	3
Enterobacter ludwigii	1	0	1
Enterococcus faecium	8	2	10
Enterococcus gallinarum	1	0	1
Enterococcus hermanniensis	0	1	1
Enterococcus hirae	1	0	1
Enterococcus mundtii	1	0	1
Escherichia coli	0	1	1
Escherichia hermannii	3	5	8
Klebsiella pneumoniae	0	1	1
Kosakonia cowanii	1	0	1
Lapidilactobacillus concavus	1	2	3
Loigolactobacillus coryniformis	23	15	38
Latilactobacillus curvatus	11	1	12
Lentilactobacillus kefiri	0	1	1
Lactococcus lactis	1	1	2
Leuconostoc pseudomesenteroides	1	0	1
Pediococcus acidilactici	6	9	15
Pediococcus pentosaceus	16	6	22
Weissella cibaria	0	1	1
not identified	19	23	42
Total	106	83	189

In total, 189 strains were isolated, whereas 42 could not be identified with MALDI-TOF MS. The identified lactic acid bacteria strains belonged mainly to the group of lactobacilli with *L. concavus* (3), *L. coryniformis* (38), *L. curvatus* (12) and *L. kefiri* (1). Additionally, *P. acidilactici* (15), *P. pentosaceus* (22), *Lc. lactis* (2), *Ln. pseudomesenteroides* (1), and *W. cibaria* (1) were identified of the LAB group. The isolated *Bacillus* spp. strains consisted of *B. cereus* (3), *B. megaterium* (11), *B. thuringiensis* (1). No yeasts were isolated from contaminated grains.

3.2. Inhibition of Growth of Fusarium graminearum DSM 1095 and DSM 4527 by Lactic Acid Bacteria and Bacillus spp.

Table 2 represents the *Bacillus* spp. and LAB strains tested for antifungal activity with no, weak, moderate, or strong inhibition against *Fusarium graminearum* DSM 1095 and DSM 4527.

Table 2. *Bacillus* spp. and LAB strains with antifungal activity against *Fusarium graminearum* DSM 1095 and DSM 4527 (n = 3). No = no inhibition of mold growth; weak = small inhibition zone; moderate = clear inhibition zone and strong = complete inhibition of mould growth.

Strains	**DSM 1095**				**DSM 4527**			
Inhibition	**No**	**Weak**	**Moderate**	**Strong**	**No**	**Weak**	**Moderate**	**Strong**
B. flexus	1	0	0	0	1	0	0	0
B. licheniformis	5	11	10	13	18	7	10	4
B. megaterium	11	1	1	0	6	6	1	0
B. pumilus	0	1	1	0	0	0	2	0
B. subtilis	0	0	0	2	0	0	0	2
L. brevis	0	0	0	10	0	0	2	8
L. concavus	3	0	0	0	3	0	0	0
L. coryniformis	23	10	0	0	31	2	0	0
L. curvatus	11	1	0	0	12	0	0	0
L. fermentum	0	1	0	0	1	0	0	0
L. kefiri	1	0	0	0	1	0	0	0
L. parabuchneri	0	0	0	4	0	0	3	1
L. plantarum	1	2	3	0	5	1	0	0
F. sanfranciscensis	1	0	0	1	1	0	1	0
Lc. lactis	2	0	0	0	2	0	0	0
Ln. citreum	0	0	1	18	0	0	4	15
Ln. lactis	0	2	10	2	5	4	5	0
Ln. mesenteroides	0	0	0	1	0	0	1	0
Ln. palmae	0	0	0	2	0	0	2	0
Ln. pseudomesenteroides	0	0	1	3	0	0	2	2
P. acidilactici	9	5	1	0	14	1	0	0
P. pentosaceus	7	12	3	0	18	2	2	0
W. cibaria	1	0	0	0	0	0	1	0
W. confusa	0	0	0	2	0	0	0	2
not identified	0	0	1	0	1	0	0	0
Total	76	46	32	58	119	23	36	34

A total of 58 strains showed strong inhibition and 32 moderate inhibition of *F. graminearum* DSM 1095, whereas *F. graminearum* DSM 4527 was strongly and moderately inhibited by 34 strains and 36 strains, respectively. Furthermore, 46 and 23 strains showed weak inhibition of *F. graminearum* DSM 1095 and DSM 4527, respectively. The remaining strains showed no inhibition of *F. graminearum* DSM 1095 and DSM 4527. Strong inhibition was observed for *B. licheniformis* (13 and 4; *F. graminearum* DSM 1095 and DSM 4527), *B. subtilis* (2 and 2), *L. brevis* (10 and 8), *L. parabuchneri* (4 and 1), *F. sanfranciscensis* (1 and 0), *Ln. citreum* (18 and 15), *Ln. lactis* (2 and 0), *Ln. mesenteroides* (1 and 0), *Ln. palmae* (2 and 0), *Ln. pseudomesenteroides* (3 and 2), and *W. confusa* (2 and 2) against *F. graminearum* DSM 1095 and DSM 4527, respectively. *B. licheniformis* (10 and 10), *B. megaterium* (1 and 1), *B. pumilus* (1 and 2), *L. brevis* (0 and 2), *L. parabuchneri* (0 and 3), *L. plantarum* (3 and 0), *F. sanfranciscensis*

(0 and 1), *Ln. citreum* (1 and 4), *Ln. lactis* (10 and 5), *Ln. mesenteroides* (0 and 1), *Ln. palmae* (0 and 2), *Ln. pseudomesenteroides* (1 and 2), not identified strain (1 and 0), *P. acidilactici* (1 and 0), *P. pentosaceus* (3 and 2), and *W. cibaria* (0 and 1) were moderately antifungal against *F. graminearum* DSM 1095 and DSM 4527. *F. graminearum* was weakly inhibited by *B. licheniformis* (11 and 7), *B. megaterium* (1 and 6), *B. pumilus* (1 and 0), *L. coryniformis* (10 and 2), *L. curvatus* (1 and 0), *L. fermentum* (1 and 0), *L. plantarum* (2 and 1), *Ln. lactis* (2 and 4), *P. acidilactici* (5 and 1), and *P. pentosaceus* (12 and 2). No inhibition against *F. graminearum* DSM 1095 and DSM 4527 was shown by *B. flexus* (1 and 1), *B. licheniformis* (5 and 18), *B. megaterium* (11 and 6), *L. concavus* (3 and 3), *L. coryniformis* (23 and 31), *L. curvatus* (11 and 12), *L. fermentum* (0 and 1), *L. kefiri* (1 and 1), *L. plantarum* (1 and 5), *F. sanfranciscensis* (1 and 1), *Lc. lactis* (2 and 2), *Ln. lactis* (0 and 5), not identified strain (0 and 1), *P. acidilactici* (9 and 14), *P. pentosaceus* (7 and 18), and *W. cibaria* (1 and 0).

3.3. Reduction of Zearalenone and Deoxynivalenol by Strains of Lactic Acid Bacteria and Bacillus spp.

In Table 3 the reduction of ZEA by LAB and *Bacillus* spp. is summarized by species showing the number of strains with >90%, 70–90%, 50–70%, 20–50%, and less than 20% reduction after 72 h of incubation at 30 °C.

Table 3. Reduction of ZEA (0.1 µg/mL) by LAB and *Bacillus* spp. with the total number of screened strains and the number of these strains able to reduce ZEA after 72 h incubation at 30 °C. $n = 1$ for strains exhibiting 50–70%, 0–50%, and <20% reduction and $n = 3$ for strains exhibiting >90% and 70–90% reduction.

Species	Total Screened	Reduction of ZEA				
		>90%	**70–90%**	**50–70%**	**20–50%**	**<20%**
B. flexus	1	0	0	0	0	1
B. licheniformis	39	19	6	5	6	3
B. megaterium	13	13	0	0	0	0
B. pumilus	2	0	1	1	0	0
B. subtilis	2	0	1	1	0	0
F. sanfranciscensis	2	0	0	1	1	0
L. plantarum	6	0	0	0	1	5
L. concavus	3	0	0	0	0	3
L. curvatus	12	0	0	0	0	12
L. kefiri	1	0	0	0	0	1
L. parabuchneri	4	0	0	3	1	0
L. brevis	10	10	0	0	0	0
L. fermentum	1	0	0	0	1	0
L. coryniformis	33	0	0	0	0	33
Lc. lactis	2	0	0	0	0	2
Ln. citreum	19	0	0	0	2	17
Ln. lactis	14	0	0	0	1	13
Ln. mesenteroides	1	0	0	0	0	1
Ln. palmae	2	0	0	0	0	2
Ln. pseudomesenteroides	4	0	0	0	2	2
P. acidilactici	15	0	0	0	0	15
P. pentosaceus	22	0	0	0	0	22
W. cibaria	1	0	0	0	0	1
W. confusa	2	0	0	0	0	2
not identified	1	0	0	0	0	1
Total	212	42	8	11	15	136

A total of 42 strains of *Bacillus licheniformis* (19), *Bacillus megaterium* (13), and *L. brevis* (10) showed ZEA reduction of more than 90% of the initial 0.1 µg/mL ZEA. Furthermore, strains of *B. licheniformis* (6), *B. pumilus* (1), and *B. subtilis* (1) showed ZEA reduction of

70–90%. ZEA reduction of 50–70% was observed in strains of *B. licheniformis* (5), *B. pumilus* (1), *B. subtilis* (1), *F. sanfranciscensis* (1), and *L. parabuchneri* (3); and a reduction of 20–50% by *B. licheniformis* (6), *F. sanfranciscensis* (1), *L. plantarum* (1), *L. parabuchneri* (1), *L. fermentum* (1), *Ln. citreum* (2), *Ln. lactis* (1), and *Ln. pseudomesenteroides* (2). The remaining strains, i.e., *B. flexus* (1), *B. licheniformis* (3), *L. plantarum* (5), *L. concavus* (3), *L. curvatus* (12), *L. kefiri* (1), *L. coryniformis* (33), *Ln. lactis* (2), *Ln. citreum* (17), *Ln. lactis* (13), *Ln. mesenteroides* (1), *Ln. palmae* (2), *Ln. pseudomesenteroides* (2), *P. acidilactici* (15), *P. pentosaceus* (22), *W. cibaria* (1), *W. confusa* (2) and an unidentified strain (1), showed less than 20% ZEA reduction.

None of the tested strains showed DON reduction higher than 15% (n = 1; see Supplementary Table S1).

3.4. Mechanism of Zearalenone Detoxification

ZEA detoxification of various samples (viable and dead cells, cell extract, cell-free supernatant) by strains belonging to the group with higher than 90% ZEA reduction was observed for *B. licheniformis* strains MA572 (100%), MA695 (100%), TR086 (100%), TR212 (98%), and TR374 (99%); *B. megaterium* strains Myk106 (100%), Myk145 (100%), and TR362 (99%) and *L. brevis* strains JR1 (96%), JR11 (97%), JR187 (95%), JR98 (97%), and MA278b (98%) and are represented in Figure 1.

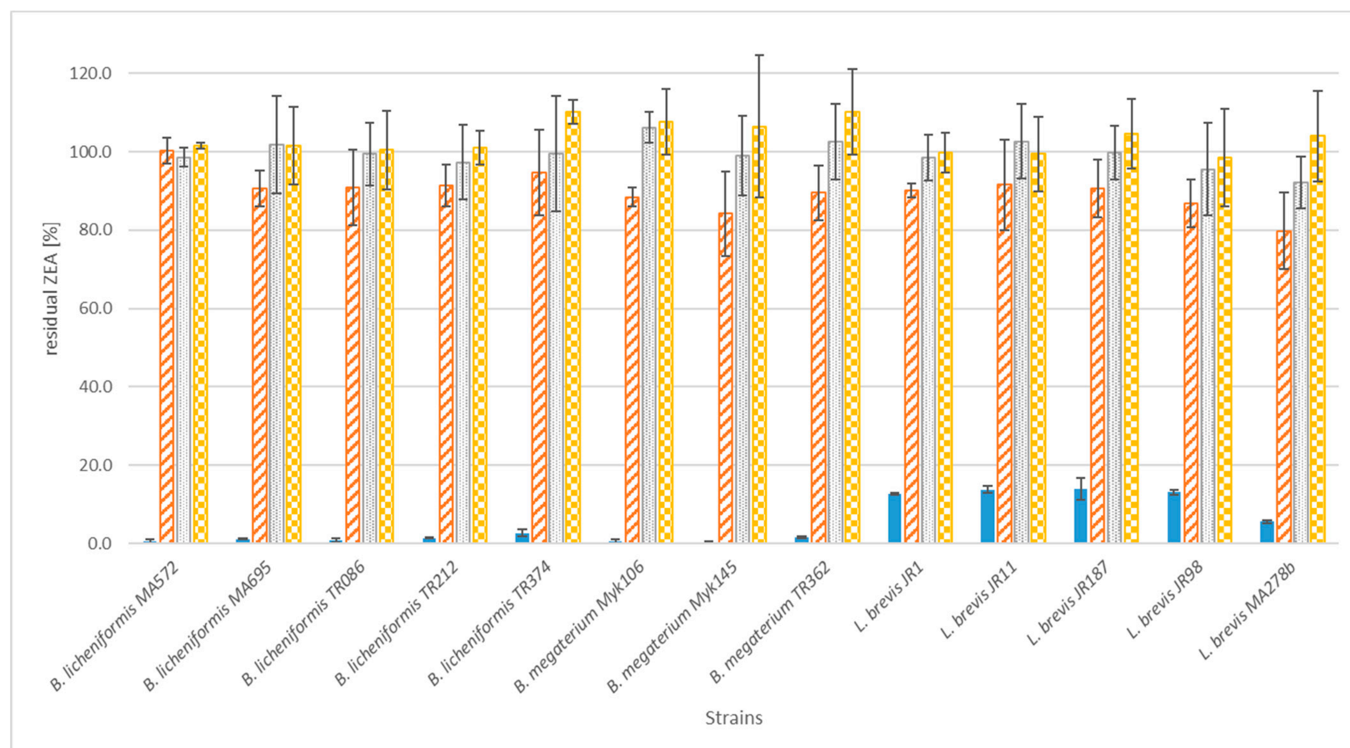

Figure 1. Residual zearalenone (ZEA) in % of the initial concentration (0.1 μg/mL) after incubation of cells (viable, blue bars), dead cells (sterilized at 121 °C for 15 min; orange striped bars), cell extract (ultrasonic; grey dotted bars), and cell-free supernatant (filtered; yellow squared bars) of *Bacillus licheniformis* MA572, MA695, TR086, TR212, and TR374, *Bacillus megaterium* Myk106, Myk145 and TR362 and *L. brevis* JR1, JR11, JR187, JR98 and MA278b at 30 °C 72 h; n = 3.

All tested viable cells showed a decrease of ZEA of >80% after 72 h of incubation, of which the five *B. licheniformis* and three *B. megaterium* strains showed between 97 and 100% reduction of ZEA and the five *L. brevis* strains showed between 85 and 95% reduction of ZEA. *L. brevis* MA278b revealed the highest ZEA reduction of all *L. brevis* strains tested, reducing ZEA content by an average of 95%. *B. licheniformis* MA572 showed an average reduction of 99.4%, and *B. megaterium* Myk145 an average reduction of 99.8% of ZEA content. The dead cells of all tested strains showed no or only little reduction of ZEA of

up to 20%. Dead cells of *L. brevis* MA278b showed a ZEA reduction of 20%, *B. megaterium* Myk145 and *L. brevis* JR98 reduced ZEA by 15%, whereas *B. licheniformis* MA695, TR086, and TR212, as well as *B. megaterium* Myk106 and TR362 and *L. brevis* JR1, JR11, and JR187 showed a ZEA decrease of 10% and *B. licheniformis* TR374 of less than 5%. Dead cells of *B. licheniformis* MA572 showed no reduction of ZEA. Cell-free supernatants of all tested strains showed no decrease in ZEA, as well as cell extracts, with exception to *L. brevis* JR98 (5% reduction) and MA278b (8% reduction). It has to be noted, that the standard deviations were rather high (up to 15%).

4. Discussion

The aim of this study was to find suitable microorganisms, which can inhibit the growth of *Fusarium graminearum* strains and bind or degrade ZEA and DON, two mycotoxins often found in contaminated cereal grains. The application of LAB has been previously shown to be a suitable strategy to detoxify ZEA, DON, T-2, HT-2 toxin, aflatoxin B1 and ochratoxin A [46,52,55]. Similarly, *Bacillus* spp. strains have already been described as showing a high capacity to degrade or bind mycotoxins such as DON and ZEA [66,68,87]. This study therefore focused on LAB and *Bacillus* spp. strains with QPS status allowing their later use in food and feed applications.

Since microorganisms have a better performance on substrates of which they had originally been isolated, as e.g., observed by Romanens et al. [88], microorganisms were isolated from mycotoxin contaminated wheat grains with focus on LAB and *Bacillus* spp. strains. Out of 189 isolated strains, *L. concavus* (3), *L. coryniformis* (38), *L. curvatus* (12), *L. kefiri* (1), *P. acidilactici* (15), *P. pentosaceus* (22), *Lactococcus lactis* (2), *Ln. pseudomesenteroides* (1), *W. cibaria* (1) and *B. megaterium* (11) were selected for further screenings due to their QPS status or their known uses as safe species. They were combined with strains of LAB and *Bacillus* spp. isolated in previous studies from various habitats and cultivated at the Culture Collection of Food Biotechnology Research Group of the Zurich University of Applied Sciences (ZHAW, Wädenswil, Switzerland).

An antifungal screening with a total of 212 strains of LAB and *Bacillus* spp. revealed 58 LAB and 34 *Bacillus* spp. strains with strong antifungal activity and 32 and 36 strains, respectively, with moderate antifungal activity against *F. graminearum* DSM1095 and DSM4527. A higher resistance of *F. graminearum* DSM4527, compared to *F. graminearum* DSM1095, was observed. Strains of *B. licheniformis* (60% against DSM1095 and 36% against DSM4527), *L. brevis* (all tested strains), and *Ln. citreum* (all tested strains) showed moderate antifungal activity against *F. graminearum* DSM4527 and high antifungal activity against *F. graminearum* DSM1095. In the study of Wang et al. [89] comparable antifungal activities of *B. licheniformis* strains against different moulds such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinereapers*, *Gibberella zeae*, *Dothiorella gregaria*, and *Colletotrichum gossypii* were observed. In another study, Karthika and collaborators [90] showed that the combination of *B. tequilensis* and *B. licheniformis* was able to inhibit the growth of *F. oxysporum*. In the group of LAB, strains of *L. brevis* and *Ln. citreum* were described as having antifungal features. Abouloifa et al. [91] described *L. brevis* as being inhibitive towards *Aspergillus niger*, *Penicillium* sp., *Fusarium oxysporum*, and *Rhizopus* sp. Mauch et al. [92] determined *L. brevis* as having the best inhibition effect on *F. culmorum* growth. Furthermore, a *Ln. citreum* strain was found by Ogunremi et al. [93], which could inhibit the growth of *A. flavus* and *P. citrinum*, and similarly, Baek et al. [94] showed that a *Ln. citreum* strain had antifungal activity against *Cladosporium* sp., *Neurospora* sp., and *Penicillium crustosum*. The study from Müller et al. [80] confirms that *Ln. citreum* has strong antifungal activity against *Penicillium* sp., *Aspergillus* sp., and *Cladosporium* sp.

In the present study, the 212 LAB and *Bacillus* spp. strains were additionally tested for their bio-detoxification ability towards ZEA and DON. The most promising strains for ZEA detoxification belonged to *L. brevis* and *B. megaterium* as well as *B. licheniformis*. All tested *L. brevis* strains (10) were shown to decrease ZEA content by 92–100% within 3 days, whereas *L. brevis* MA278b was the strain with the highest activity (97–100%) in all triplicates.

Concurring with this study, Chlebicz and Śliżewska [95] found strains of *L. brevis* with high potential to detoxify ZEA (~50% reduction after 24 h), and Adunphatcharaphon et al. [96] revealed *L. brevis* strains with 18% of ZEA detoxification within 1 h of incubation.

All of the tested *B. megaterium* strains in this study (13) showed ZEA detoxification of 96–100% within 3 days of incubation. Eleven of those strains were isolated from mycotoxin contaminated wheat grains and 2 from spent grains, which are also susceptible to mycotoxin contaminations. The 2 strains originating from spent grain were slightly less efficient (96–99% ZEA reduction after 3 days) than the strains isolated from contaminated wheat grains, which all showed 100% decrease in ZEA content after 3 days of incubation. Likewise, Hassan et al. [97] determined strains of *B. megaterium* which could bio-detoxify ZEA to 100% within 44 h of incubation. Besides the strains of *B. megaterium*, 39 strains of *B. licheniformis* were tested in this study, and 20 of them showed an average decrease of more than 90% of ZEA after 3 days of incubation. In the study by Yi et al. [98] *B. licheniformis* isolates exhibited a bio-detoxification of 98% of ZEA within 36 h of incubation, and Hsu et al. [99] showed a reduction of 75% of ZEA by a *B. licheniformis* strain. All other LAB and *Bacillus* spp. strains tested in the current study did not show sufficient ZEA detoxification (<90% reduction after 3 days).

In general, biodetoxification of mycotoxins seems to be strain dependent, hypothesized to be dependent on the production of enzymes or cell wall compartments [52,100]. Also, the origin of the bio-detoxifying microorganism might be important, as suggested in this study, where most of the strains with detoxification ability were isolated from cereal products, which are highly susceptible to mycotoxin contaminations. There is a correlation between the antifungal activity of *L. brevis* and *B. licheniformis* and their ZEA reduction potential ($r = -0.71$ for DSM 1095 and $r = -0.5$ for DSM 4527; $p < 0.05$). Overall, 42 strains were found to have a high capacity for detoxifying ZEA within 3 days of incubation. In order to determine the efficiency of ZEA reduction by the tested strains, shorter incubation times should be tested.

For DON none of the 212 strains showed a detoxification higher than 15% after 3 days of incubation. Jia et al. [66] showed DON reduction of around 80% after 8 h by a strain of *B. subtilis*. In this study only 2 strains of *B. subtilis* strains were tested. In a future study, the number of *B. subtilis* strains should be increased aiming at selecting active strains able to reduce DON. Chlebicz and Slizewska [95] showed that all tested LAB (*L. brevis* (1), *L. casei* (2), *L. paracasei* (1), *L. pentosus* (1), *L. plantarum* (2), *L. reuteri* (2), *L. rhamnosus* (3)) decreased DON by 20 to 40% and ZEA by 40 to 70% after 24 h, and additionally strains of the yeast *S. cerevisiae* reduced DON levels by 20 to 40% and ZEA levels by 40 to 50% after 24 h. In their study, they also showed that a high amount of the toxin was already reduced after 6 h of incubation (30–60% for ZEA, 6–20% for DON). Further, Franco et al. [55] showed that all tested LAB (*L. plantarum* (6), *L. pentosus* (1), *L. paracasei* (1)) could reduce DON by 16–55% after 4 h of incubation, and Juodeikiene et al. [46] revealed mycotoxin reduction by *P. acidilactici* and *P. pentosaceus* of DON (20–50%), T-2 toxin (20–80%), HT-2 toxin (20–80%) and ZEA (10–40%) in malting wheat after 30 min of treatment. *L. rhamnosus* and *P. freudenreichii* showed DON reduction of 40% and 60%, respectively, whereas the mechanism of reduction was most probably binding of the toxin to the cell wall since the dead cells showed the same amount of DON reduction [54]. Wang et al. [87] showed that *B. licheniformis* could decrease DON by 80% after 48 h. All these studies indicate that DON contents are able to be reduced by microorganisms, however, this was not evident in the current study.

The mechanism of bio-detoxification is an important factor when considering the future use of microorganisms in food and feed since binding of the toxin to the cell wall could be problematic in a later application because of a possible release in the body during digestion. Therefore, different states of the bacterial cells such as viable cells, dead cells, cell extracts, or cell-free supernatants were tested for their efficiency in ZEA reduction. Viable cells should indicate if the cells must be active to reduce ZEA, dead cells if ZEA is bound to the cell wall, cell extract if intracellular proteins or enzymes are responsible for ZEA

decrease, and cell-free supernatant if extracellular proteins or enzymes are responsible for ZEA reduction. A selection of 13 ZEA degrading strains (>95% ZEA reduction after 3 days) of *B. licheniformis*, *B. megaterium*, and *L. brevis* were therefore tested in these 4 states for their ZEA reduction. Viable cells of all tested strains showed again high potential in ZEA detoxification (85–100%), whereas *L. brevis* strains revealed the lowest decrease (85–95%). Dead cells of all tested strains showed only low reduction in ZEA contents (0–20%), which indicated that part of the ZEA might be bound to the cell wall, this was observed for all strains except *B. licheniformis* MA572. Within the tested strains, *B. megaterium* Myk145 and *L. brevis* MA278b showed the strongest putative cell-wall binding capacity for ZEA (15 and 20% reduction of ZEA, respectively). Franco et al. [55] showed that dead cells of LAB decreased DON levels to a higher extent than viable cells. Tinyiro et al. [68] stated that cell binding capacity of toxins is dependent on cell concentration, which could be an explanation for the different results observed in the current study and the high standard deviations. El-Nezami et al. [54] recommended a LAB cell concentration of $>10^9$ cells per mL for trichothecene reduction, and Zhao et al. [101] even showed that *L. plantarum* strains with the ability to reduce ZEA content, showed that detoxification is cell concentration dependent with 10^{10} cells per mL in PBS showing the best results compared to 10^9 or 10^8 cells per mL. The cells could bind the toxin, whereas heat treatment increased the binding capacity. Cell binding was also observed as being strain dependent [100], and some studies indicated a pH dependency on cell wall binding of mycotoxins [102], while other findings were contradictory [103]. Topcu et al. [104] hypothesized that the pH dependency could also be strain dependent.

By using cell extracts, as well as cell-free supernatants, no reduction of ZEA contents was shown, indicating that neither intracellular nor extracellular proteins or enzymes were present to bind or degrade ZEA. In contrast to these findings, Tinyiro et al. [68] tested *B. natto* and *B. subtilis* strains, both with very good adsorption of ZEA if tested either as viable cells for 1 h incubation (90 and 60% binding, respectively) or as their cell extracts within 24 h of incubation (100 and 80% reduction). For the cell extracts, the degradation process was shown to be pH and temperature dependent with optimal conditions at pH 8 and 42 °C. Zou et al. [56] showed that viable cells and disintegrated cells (cell wall) of *L. plantarum* reduced DON concentration (20% after 4 h of incubation), whereas cell-free supernatants and cell extract did not reduce DON contents, therefore binding was hypothesized as mode of action. They showed, that heat-treated (121 °C for 20 min) and acid treated cells were better in binding (35% and 27% removal of DON, respectively) than viable cells or lysozyme treated cells whereas alkaline treated cells showed no reduction in DON amounts.

To summarize, the mechanism of ZEA bio-detoxification is still unclear and the method should be optimized. To obtain dead cells, a sterilization process was applied, which could also disturb the cell wall components that might be responsible for ZEA binding. Alternative methods for cell inactivation might therefore be treatments with ethanol, formalin, or sodium hydroxide [105], or a gentler heating process, e.g., in a 100 °C water bath for 1 h [106]. To obtain cell extracts, ultrasonication was applied without verifying sufficient cell lysis, which should be examined in a next study.

A possible way to find LAB which can degrade and not only bind ZEA would be to select esterase active strains. Cell-free supernatants of esterase active LAB showed ZEA reduction by up to 45% after 3 h incubation at 37 °C [107]. As there were no cells present, the enzymes produced by LAB most likely degraded ZEA. Likewise, Wang et al. [108] screened for esterase active microorganisms and identified a *B. pumilus* strain, which was capable of decreasing ZEA in its viable state but not as dead cells. The degradation rate was dependent on ZEA concentration, pH, and temperature. The higher the concentration the lower the degradation rate, whereas a pH of 8 combined with 37 °C were optimal.

5. Conclusions

In this study, antifungal and bio-detoxifying microorganisms were successfully isolated from mycotoxin contaminated wheat grains. Strains with high antifungal activity against *F. graminearum* and the potential to degrade or bind ZEA were found. The most promising species were *B. licheniformis*, *B. megaterium*, and *L. brevis*. In contrast, no microorganisms were found that showed significant DON reduction. Analysis of the detoxification mechanisms of ZEA revealed first insights into the respective mechanisms of the most promising species. In general, bio-detoxification using food grade (QPS) and safe strains is a promising strategy to increase food safety and reduce food waste. The elucidation of the exact mechanism of action is important for future application in grains for food or feed products, in order to ensure an irreversible inactivation of the mycotoxin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010007/s1, Table S1: Reduction of DON (5 µg/mL) by LAB and *Bacillus* spp. with the total number of screened strains and the number of strains showing DON reduction after 72 h incubation at 30 °C. $n = 1$.

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