

## ORIGINAL ARTICLE

**Control of *Zymoseptoria tritici* cause of septoria tritici blotch of wheat using antifungal *Lactobacillus* strains**K.M. Lynch<sup>1</sup>, E. Zannini<sup>1</sup>, J. Guo<sup>2</sup>, C. Axel<sup>1</sup>, E.K. Arendt<sup>1</sup>, S. Kildea<sup>3</sup> and A. Coffey<sup>2</sup><sup>1</sup> School of Food and Nutritional Sciences, University College Cork, Cork, Ireland<sup>2</sup> Department of Biological Sciences, Cork Institute of Technology, Bishopstown, Cork, Ireland<sup>3</sup> Crop Science Department, Teagasc Crops Environment and Land Use Programme, Carlow, Ireland**Keywords**

antifungal, cell-free supernatant, lactic acid bacteria, *Lactobacillus brevis*, septoria tritici blotch, *Zymoseptoria tritici*.

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**Abstract**

**Aims:** This study explored an effective biological control agent based on lactic acid bacteria culture or culture supernatant, which was effective against fungicide-resistant *Zymoseptoria tritici*, which causes septoria tritici blotch (STB). **Methods and Results:** Three lactic acid bacteria strains which exhibited broad antifungal activity were investigated for their potential to control *Z. tritici*. Plate assays, liquid culture growth inhibition assays and STB biocontrol seedling tests were employed. *Lactobacillus brevis* JJ2P and *Lactobacillus reuteri* R2 caused significant fungal inhibition as observed by large mycelium clearing on modified MRS agar. Cell-free culture supernatants of *Lact. brevis* JJ2P and *Lact. reuteri* R2 showed antifungal activity against *Z. tritici*, as observed by mycelial radial growth inhibition and liquid culture growth inhibition. Cell-free supernatants of these anti-*Z. tritici* LAB strains were assessed *in vivo* for their abilities to inhibit STB development in seedling tests. *Lact. brevis* JJ2P was capable of inhibiting disease development and significantly reduced the diseased leaf area covered with pycnidia.

**Conclusions:** Biological control accomplished by beneficial micro-organisms such as *Lact. brevis* JJ2P may represent an alternative control strategy for reducing STB.

**Significance and Impact of the Study:** Globally, STB is regarded as one of the most important diseases of wheat. Control of *Z. tritici* is heavily reliant on fungicide application. The recent emergence of resistance or reduced sensitivity to fungicides among *Z. tritici* populations has urgently called for the development of new control strategies.

**Introduction**

Septoria tritici blotch (STB) caused by the ascomycete fungus *Zymoseptoria tritici* (synonym *Mycosphaerella graminicola*) is the most economically damaging disease of wheat (*Triticum aestivum* L.) worldwide (Eyal 1999; Hardwick *et al.* 2001; Bearchell *et al.* 2005). Wheat is currently the third main human food crop after maize and rice (FAOSTAT 2015), and is particularly at risk from numerous diseases due to the level of intensification necessary for profitable production since the 1970s (Hardwick *et al.* 2001; Bearchell *et al.* 2005). By infecting leaves and causing necrosis, *Z. tritici* reduces the

grain-filling capacity of wheat and can, if left unchecked, result in significant yield losses with associated economic repercussions (Forrer and Zadoks 1983; Eyal *et al.* 1987; Polley and Thomas 1991; Eyal 1999). In addition, global expenditures on fungicides to manage STB reach hundreds of millions of dollars each year (Hardwick *et al.* 2001; McDougall 2006). As *Z. tritici* commonly undergoes sexual reproduction local populations have extremely high levels of variability which provide the pathogen the ability to rapidly adapt to changing environments such as host resistance or fungicides (Linde *et al.* 2002). The consequences of this have included the development of resistance among global populations to

the most commonly applied fungicides, such as the Quoine outside Inhibitors (QoI) and azole fungicides (Fraaije *et al.* 2005). To counter these developments in fungicide resistance there is an increased need to develop alternative control strategies, including biological control. Despite these issues, safe and environmentally friendly products for plant protection represent an insignificant portion of the pesticide market, which remains dominated by synthetic chemicals.

In addition to the need to find alternatives to fungicides due to the development of resistance, changes in the regulatory processes within the European Union are also expected to curtail the availability of effective fungicides (Jess *et al.* 2014). The development of biological control agents is viewed as an alternative control measure. Such biological control treatments can include living micro-organisms or abiotic products that have the ability to (i) provide plant protection through production of antibiotics or other molecules that are deleterious for pathogen development, (ii) compete with the pathogen for nutrients and space, or (iii) induce plant resistance. While there are relatively limited published studies on the use of micro-organisms or their metabolites as *Z. tritici* control agents, those published include the use of *Pseudomonas* (Levy *et al.* 1988, 1989, 1992; Flaishman *et al.* 1996), *Paecilomyces lilacinus*, *Nigrospora sphaeric*, *Cryptococcus* sp., *Bacillus* sp. (Perellò *et al.* 2002; Kildea *et al.* 2008) and *Trichoderma* spp. Some of these studies have demonstrated the potential for biological control of *Z. tritici*, but to the best of our knowledge none of these have been employed commercially.

Lactic acid bacteria (LAB) function as natural food-grade biocontrol agents in a wide range of food and feed products (Stiles 1996; Carr *et al.* 2002; Schnurer and Magnusson 2005; Broberg *et al.* 2007). They are also naturally associated with plant material. LAB are known to have antifungal activity against a broad spectrum of fungi including *Alternaria* spp., *Aspergillus* spp., *Botrytis* spp., *Candida* spp., *Endomyces* spp., *Fusarium* spp., *Monilinia* spp., *Penicillium* spp., *Phytophthora* spp., *Rhizopus* spp., *Sclerotium* spp., *Trichophyton* spp., *Microsporum* spp. and *Epidermophyton* spp. (Corsetti *et al.* 1998; Okkers *et al.* 1999; Stiles *et al.* 2002; Dal Bello *et al.* 2007; Mandal *et al.* 2007; Sathe *et al.* 2007; Valerio *et al.* 2009; Falguni *et al.* 2010; Guo *et al.* 2011, 2012; Wang *et al.* 2012). This antifungal nature of LAB suggests a potential for the use of such strains in terms of restricting the fungal proliferation of STB. The mechanism by which LAB are antifungal is believed to be due to the production of several low molecular weight compounds linked to their metabolism, such as organic acids, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds (Dalić *et al.* 2010).

This study explored the capacity of three selected LAB to inhibit *Z. tritici*. Previous work conducted in our laboratories isolated a large number of LAB from a variety of environments and tested their antifungal performance (Guo *et al.* 2011, 2012). A number of strains were active against different fungi responsible for food spoilage and human mycoses such as *Penicillium*, *Aspergillus*, *Trichophyton*, *Microsporum* and *Epidermophyton*. Thus, three LAB isolates which exhibited strong antifungal activity were selected, and their potential to control fungicide-resistant *Z. tritici* was evaluated using plate assays, liquid culture growth inhibition assays and *in vivo* STB biocontrol tests. To the best of our knowledge this study is the first report of the use of food-grade LAB, which have QPS (Qualified Presumption of Safety) status, as biocontrol agents against STB, and to employ *Z. tritici* as an indicator fungus.

## Materials and methods

### Lactic acid bacterial cultures and growth conditions

Three strains of LAB, namely *Lactobacillus brevis* JJ2P, *Lactobacillus arizonensis* R13 and *Lactobacillus reuteri* R2 were isolated from porcine gut, cheese and porcine gut respectively (Guo *et al.* 2011). These LAB strains have previously shown strong inhibitory activity against a wide range of food spoilage and human pathogenic fungi. An additional three strains of LAB, namely *Lact. brevis* L1105, *Lact. arizonensis* R14 and *Lact. reuteri* M13 with low or no antifungal activity were chosen as negative control strains. All LAB were grown routinely on MRS medium (Fluka Chemie AG, Buchs, Switzerland) for 48 h at 30°C. Long-term storage was done in 40 % glycerol at -80°C. The growth rate of the LAB strains was monitored by following optical density in MRS broth at 620 nm (OD620).

### Fungal cultures and preparation of the spore-mycelia suspension

*Zymoseptoria tritici* strains 46·10, 552·11, 560·11 and 563·11 were obtained from the Teagasc phytopathological collection. Strain 46·10 and strains 552·11, 560·11 and 563·11 were isolated from STB-infected winter wheat leaves collected from Irish crops in both 2010 and 2011 respectively. Isolate 560·11 exhibits high levels of resistance to the azole fungicides and has the CYP51 mutations L50S, V136C, S188N, A379G, I381V, Δ459/460 and S524T. All isolates were grown on potato dextrose agar (PDA) (Oxoid Ltd., Basingstoke, UK) plates at 20°C in the dark for 7–10 days and subsequently stored at 4°C until required. For *in vitro* tests, spore suspensions were

created by transferring yeast-like spores from the PDA plate to 500 ml of synthetic-nutrient-poor bouillon (SNB) (Nirenberg 1976). These were then incubated at 20°C in the dark for 10–15 days with stirring (120 rev min<sup>-1</sup>) after which spore concentrations were determined by plating serial dilutions on PDA plates, followed by enumeration. Typically the spore suspension was adjusted to the required concentration (1.0 × 10<sup>6</sup> ml<sup>-1</sup>) prior to use. For seedling tests, yeast-like spores were scraped from the surface of 4-day-old cultures into a 0.2% (v/v) Tween 20 sterile distilled water (SDW) solution and, where required, filtered through sterile cheesecloth. Spore concentration was adjusted to 1.0 × 10<sup>6</sup> ml<sup>-1</sup> using 0.2% Tween 20-SDW. For all *in vitro* tests, *Z. tritici* strain 46.10 was used as the target organism, while strains 552.11, 560.11 and 563.11 were used for the *in vivo* wheat seedling trials.

#### Tolerance testing of *Zymoseptoria tritici* to pH

The pH tolerance of *Z. tritici* 46.10 was evaluated by adjusting the pH of modified MRS (mMRS; both sodium acetate and potassium dihydrogen phosphate omitted) to 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using either HCl or NaOH. Ten microlitres of spore–mycelia suspension were inoculated as a spot in the centre of the mMRS plates after which they were incubated in the dark for 15 days at 20°C under aerobic conditions. Susceptibility was monitored by measuring the diameter of the fungal colonies at the different pH levels.

#### Antifungal activity of selected *Lactobacillus* strains

The selected LAB were tested for antifungal activity towards *Z. tritici* 46.10 using a dual-culture plate assay. Two millilitres of fungal spore–mycelia suspension was mixed into 18 ml of mMRS agar adjusted to pH 6.0. After 30 min, antifungal LAB and respective negative control LAB were inoculated as two parallel lines of 2 cm length; keeping a distance of 2 cm (approx.) between the lines and allowed to grow anaerobically at 30°C for 48 h. The plates were then incubated for 10–15 days under aerobic conditions at 20°C in the dark to promote fungal growth. The antifungal activity of each LAB was ascertained by measuring the zone of inhibition of *Z. tritici* surrounding the bacterial streaks.

#### Freeze-dried lactic acid bacterial supernatant powder preparation

Cell-free supernatant (CFS) powders of the antifungal strains (positive; CFS-P), and the negative control strains (CFS-N) belonging to the same species were produced to

serve as base material for the experiments describing the antifungal nature of the strains. Briefly, overnight cultures of bacteria were inoculated in 500 ml of mMRS broth to reach an initial concentration of 10<sup>4</sup> CFU ml<sup>-1</sup>. The bacteria were grown for 5 days at 37°C (temperature at which the antifungal strain showed its highest activity). Cells were separated from the supernatant by centrifugation and decanting twice at 3000 g for 15 min. The cell-free supernatant was carefully drawn off and freeze-dried; the powder was stored at 4°C. Powder prepared from freeze-dried mMRS broth (CFS-C) was used as a control. Typically, 500 ml of supernatant produced 25 g of lyophilized powder. Powders were routinely reconstituted in SDW.

#### Antifungal activity of bacterial supernatant powders

A microplate assay was used to assess the effect of different concentrations of *Lact. brevis* JJ2P and *Lact. reuteri* R2 CFS on the growth of *Z. tritici* 46.10 in liquid culture. To adjust the *Z. tritici* spore concentration to 1.0 × 10<sup>6</sup> ml<sup>-1</sup>, aliquots of 50-ml spore–mycelia suspension were centrifuged at 3000 g for 10 min and the supernatant was discarded. The spore pellets were resuspended in potato dextrose broth (PDB) to a final concentration of 1.0 × 10<sup>6</sup> ml<sup>-1</sup> of which 100 µl (1.0 × 10<sup>5</sup> spores) was added to the wells of a sterile 96-well microplate (Sarstedt AG & Co, Nuembrecht, Germany). One hundred microlitres of different *Lact. brevis* JJ2P CFS dilutions were added to the wells, to achieve final CFS concentrations of 0, 0.4, 0.75, 1.5, 3.0, 6.0, 12.5, 25.0 or 50.0 (% w/v). The microplate was sealed with an optically clear seal (Thermo Scientific, Waltham, MA) and incubated for 240 h at 20°C in a Multiskan FC microplate-reader (Thermo Scientific). The OD620 was recorded at 5-h intervals. Changes in OD620 were used to evaluate *Z. tritici* growth and hence fungal inhibition. The experiment was performed in duplicate.

In addition, the antifungal activity of *Lact. brevis* CFS was also assessed on solid media (PDA plates) as follows: working solutions of CFS-P, CFS-N and CFS-C were made up to 50 % (w/w) by dissolving the CFS powder in distilled water, adjusted to pH 4.0 using D/L-lactic acid (Sigma-Aldrich, St. Louis, MO; and 4 mol l<sup>-1</sup> NaOH as required), and then filter-sterilized using a 0.45 µm MIN-ISART<sup>®</sup>-plus filter. PDA plates were prepared containing 0 (control), 0.5, 1, 2% (m/v) CFS-P and CFS-N. For each concentration, acidified control plates were prepared containing the same concentration of CFS-C. After cooling, 10 µl of *Z. tritici* 46.10 spore–mycelia suspension were inoculated as a spot in the centre of the PDA plates. The plates were incubated for 15 days at 20°C in the dark. The fungal growth was monitored by measuring the growth area of fungal colonies.

### Effect of LAB cell-free supernatant on the development of STB on wheat seedlings

The ability of cell-free supernatants of both *Lact. brevis* JJ2P and *Lact. reuteri* R2 to control STB *in vivo* was determined. Wheat seedlings of the STB-susceptible winter wheat variety, Cordiale, were grown in 9 × 9 × 9 cm pots (5 seedlings per pot) under controlled conditions (75% relative humidity under a 12-h day/night cycle at 18/12°C respectively). After 18 days (second leaf fully emerged) pots were removed and inoculated to run-off with the LAB CFS (prepared as above) or treated with varying doses (6.91–124.5 g l<sup>-1</sup>) of the azole fungicides epoxiconazole in the form of Opus Max (BASF). Plants were allowed to dry for 24 h after which they were individually inoculated to run-off with spore suspensions of *Z. tritici* strains 552-11, 560-11, 563-11 or 0.2% Tween 20-SDW. Immediately following inoculation all pots were sealed in clear polyethene bags to increase humidity and placed back into the controlled environment in a completely randomized block design, with three replicates. The bags were removed after 48 h and any subsequent growth (greater than the second emerged leaf) were excised from the plants at 4-day intervals. Levels of disease on the second emerged leaf were assessed 28 days post fungal inoculation and recorded as diseased leaf area bearing pycnidia. Differences between the treatments and potential interactions with the different isolates were determined using a two-way ANOVA in GENSTAT 14th Edition (VSN International Ltd., Hemel Hempstead, UK).

### Characterization of antifungal compounds produced by *Lactobacillus brevis* JJ2P and *Lactobacillus reuteri* R2

CFS of both strains grown in mMRS were analysed for the presence of 20 known antifungal compounds: azelaic acid, benzoic acid, caffeic acid, catechol, p-coumaric acid, ferulic acid, hydrocaffeic acid, hydrocinnamic acid, hydroferulic acid, 4-hydroxybenzoic acid, 2-hydroxydodecanoic acid, β-hydroxylauric acid, 2-hydroxyisocaproic acid, hydroxymyristic acid, 4-hydroxyphenyllactic acid, methylcinnamic acid, 3-phenyllactic acid, phloretic acid, salicylic acid and vanillic acid. The analysis was carried out using a Waters Quattro premier XE UPLC system as described by Axel *et al.* (2015). The LC-MS/MS method

was operated in negative electrospray ionization mode to detect the antifungal compounds. The QuEChERS method (quick, easy, cheap, effective, rugged and safe) was applied to extract *in situ* antifungal compounds from the mMRS broth (Brosnan *et al.* 2014). Lactate, acetate and ethanol were quantified as described by Wolter *et al.* (2014).

## Results

### pH tolerance testing of *Zymoseptoria tritici*

The growth of *Z. tritici* 46-10 was similar from pH 4.5 to 8.0, with a colony diameter of 3.5 cm. At pH 3.5, 4.0 and 9.0 fungal growth was slightly reduced to 3.0 cm. When the pH was decreased to 3.0, the fungal colony diameter reduced to 2.3 cm and at pH 2.5 or less, no fungal growth was recorded (Table 1).

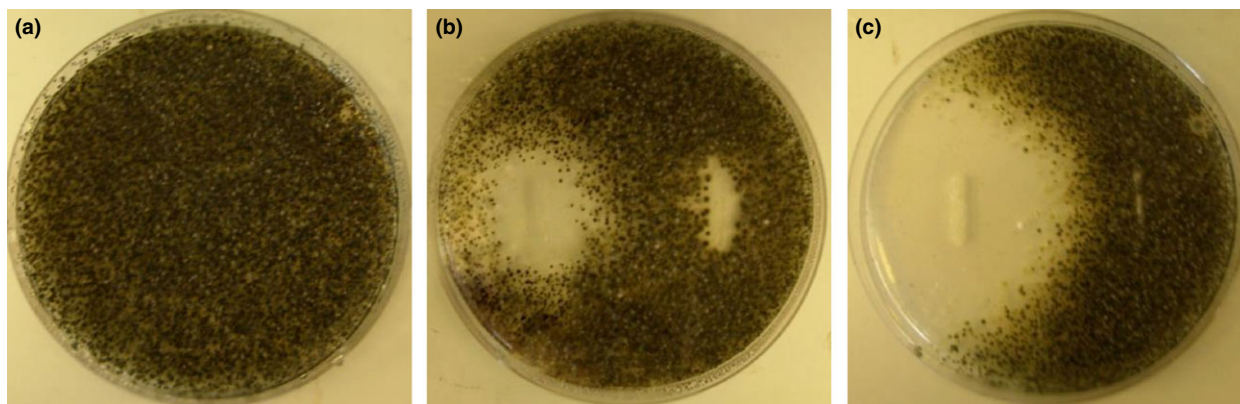
### Ability of *Lactobacillus* strains and LAB cell-free supernatants to inhibit *Z. tritici* growth *in vitro*

In preliminary dual-culture assays *Lact. brevis* JJ2P and *Lact. reuteri* R2 inhibited *Z. tritici* 46-10 growth on PDA, manifested by clear zones of inhibition surrounding the bacterial streaks (Fig. 1). The negative control LAB strains of the same species showed little or no activity against *Z. tritici* (Fig. 1). *Lactobacillus arizonensis* R13, selected based on previously identified broad range antifungal activity had very low inhibition against *Z. tritici* (data not shown) and thus was not included in any further analysis.

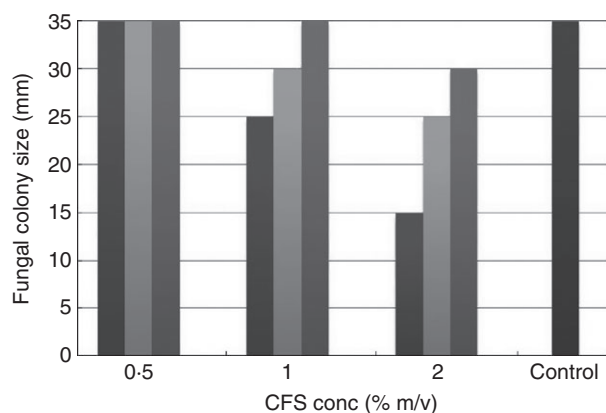
In the CFS agar-supplementation assay, using *Z. tritici* 46-10, the addition of CFS-P (*Lact. brevis* JJ2P) and CFS-N (*Lact. brevis* IL1105) at a concentration of 0.5 % did not affect fungal mycelial growth compared to negative control CFS-C (freeze-dried mMRS) or nonsupplemented control plate. Addition of CFS-P and CFS-N at a concentration of 1% slightly reduced *Z. tritici* growth compared to 1% CFS-C and the nonsupplemented control plate. At a concentration of 2%, strong inhibition of fungal growth was observed on the CFS-P plates, with lower levels of inhibition also observed on the CFS-N and slight inhibition on the CFS-C plates when compared to the nonsupplemented control (Fig. 2).

**Table 1** Effect of pH on fungal growth. Fungal colony diameter (cm) after inoculation of *Zymoseptoria tritici* 46-10 on potato dextrose agar adjusted to a pH ranging from 2.0 to 9.0

Genus	pH of agar											
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	
<i>Z. tritici</i> (colony diameter)	0.0	0.0	2.3	3.0	3.0	3.5	3.5	3.5	3.5	3.5	3.5	3.0



**Figure 1** Antifungal activity of different LAB against *Zymoseptoria tritici* 46-10 (a) *Z. tritici* grown 15 days at 20°C on mMRS agar plate with no LAB present, (b) *Z. tritici* grown with *Lactobacillus brevis* JJ2P (left, showing clear zone of fungal inhibition) and negative control *Lact. brevis* L1105 (right, with very low inhibition), (c) *Z. tritici* grown with *Lactobacillus reuteri* R2 (left, showing clear zone of fungal inhibition) and negative control *Lact. reuteri* M13 (right, with no inhibition).



**Figure 2** Diameter (mm) of *Zymoseptoria tritici* 46-10 colonies incubated for 15 days on PDA plates containing 2, 1 or 0.5% (m/v) of: freeze-dried cell-free supernatant (CFS) of *Lactobacillus brevis* JJ2P at pH 4 (CFS-P, ■); CFS of *Lact. brevis* L1105 at pH 4 (CFS-N, ■), freeze-dried mMRS at pH 4 (CFS-C, ■); PDA with no additives (control).

In the microtitre plate assay, when 12.5% (or greater) of CFS-P was added to PDB no growth of *Z. tritici* 46-10 was observed over the 240-h period. Decreasing the concentration of CFS-P to 6 or 3% inhibited fungal growth to some extent. Reducing the concentration of JJ2P CFS in PDB to 1.5% caused the loss of antifungal activity. Concentrations of JJ2P CFS lower than 1.5% did not affect the growth of *Z. tritici*. Similar results were detected for *Lact. reuteri* R2 (Fig. 3).

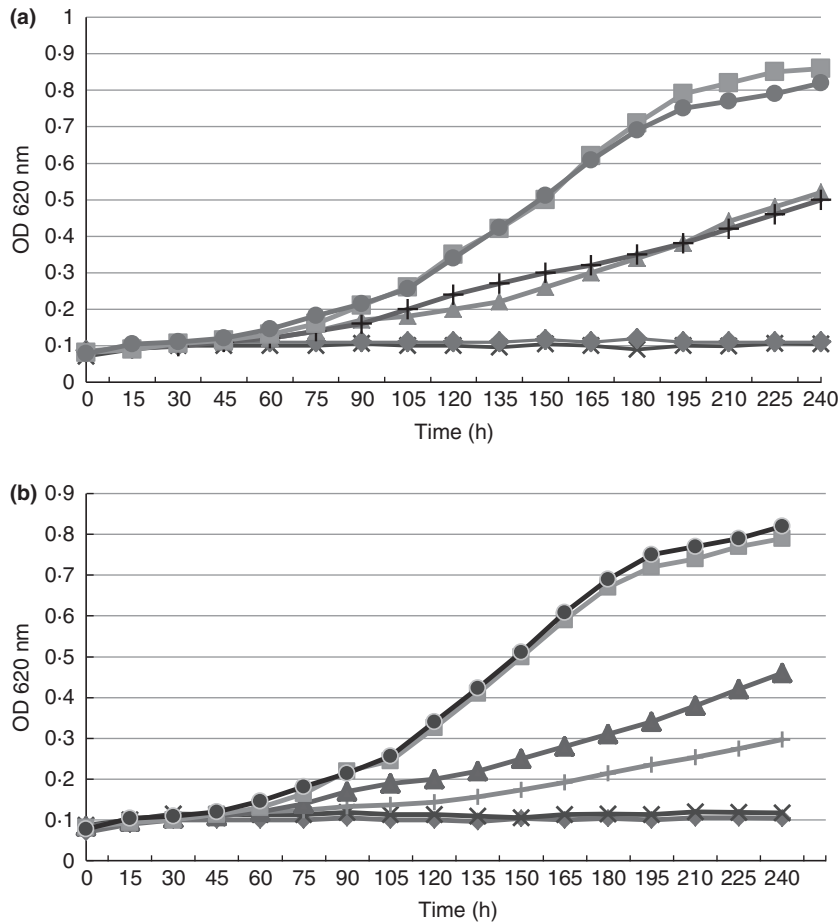
#### Effect of LAB cell-free supernatant on the development of STB disease on wheat seedlings

High levels of disease were observed on the positive control plants (those treated with *Z. tritici* alone) for all

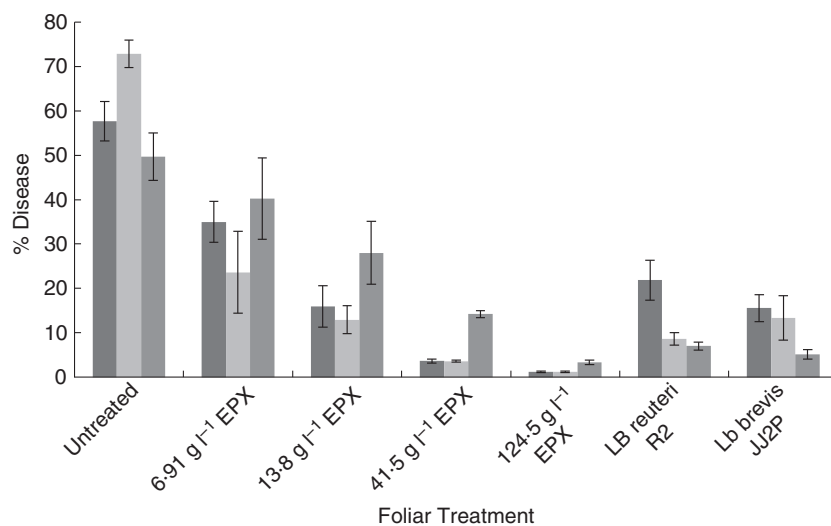
three isolates tested, and across the trial no significant differences were observed between the strains in the levels of disease observed ( $P = 0.073$ ). Foliar treatment had a significant effect on levels of disease ( $P < 0.001$ ), however, a significant interaction between foliar treatment and *Z. tritici* strain was observed ( $P < 0.001$ ). In the case of the epoxiconazole (EPX), higher levels of disease were observed for all four fungicide dosages following inoculation with strain 560-11, compared to 552-11 or 563-11, reflecting the higher levels of azole resistance detected in this strain. This was the opposite for *Lact. reuteri* R2, where seedlings treated with CFS from this strain and inoculated with 560-11 or 563-11 had lower levels of disease compared to those inoculated with 552-11; similarly, for *Lact. brevis* JJ2P CFS, seedlings inoculated with 560-11 again showed the lowest levels of disease (Fig. 4). Figure 5 clearly shows the reduction in diseased leaf area of seedlings when *Lact. brevis* JJ2P CFS was applied.

#### Antifungal compounds produced by *Lactobacillus brevis* JJ2P and *Lactobacillus reuteri* R2

Table 2 outlines the antifungal compounds produced by *Lact. brevis* JJ2P and *Lact. reuteri* R2 along with those produced by the negative control strains. Both *Lact. brevis* strains produced hydroxyphenyllactic acid, phenyllactic acid and leucic acid, while the negative control strain also produced benzoic acid. *Lactobacillus reuteri* strains produced a greater number of antifungal compounds, including hydroxyphenyllactic acid, phenyllactic acid, leucic acid, benzoic acid and hydroxylauric acid. Strain R2 additionally produced vanillic acid. Compared to the negative control strain, the antifungal strain, *Lact. brevis*



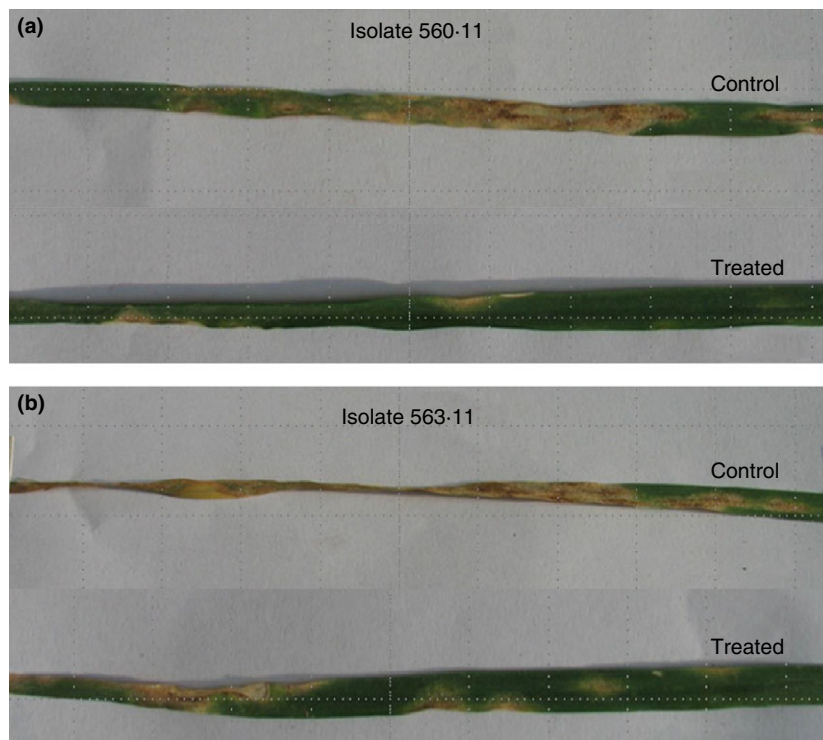
**Figure 3** Growth of *Zymoseptoria tritici* 46-10 in potato dextrose broth containing 25% (X), 12.5% (◆), 6% (+), 3% (▲), 1.5% (■) or 0% (●) of (a) *Lactobacillus brevis* JJ2P CFS and (b) *Lactobacillus reuteri* R2 CFS. Values are the mean of triplicate experiments.



**Figure 4** Comparison of epoxiconazole (EPX) fungicide and *Lactobacillus* CFS on disease levels of the winter wheat cultivar equinox (grown under contained environmental conditions), infected with different *Z. tritici* strains. Strains 552-11 (■), 560-11 (■) and 563-11 (■).

JJ2P, produced higher levels of leucic acid. Regarding *Lact. reuteri* R2, benzoic acid was produced at higher levels by this strain compared to the negative control. In addition, levels of organic acids and ethanol produced by

the strains were measured and found to be produced at higher levels by the antifungal strains, which likely contributed to their increased antifungal activity compared to the control strains.



**Figure 5** Effect of *Lactobacillus* CFS application on STB symptoms on winter wheat seedlings. The CFS of *Lactobacillus brevis* JJ2P significantly reduced the diseased leaf area caused by STB strain (a) *Zymoseptoria tritici* 560-11 and (b) *Z. tritici* 563-11 compared to the control plants (diseased wheat leaf without application of CFS).

**Table 2** Antifungal compounds produced by *Lactobacillus brevis* JJ2P and *Lactobacillus reuteri* R2 and respective negative control strains, *Lact. brevis* L1105 and *Lact. reuteri* M13

Compound	Strain							
	JJ2P		L1105		R2		M13	
	Conc.	RSD	Conc.	RSD	Conc.	RSD	Conc.	RSD
Hydroxyphenyllactic acid	0.3	3.3	0.4	7.8	1.9	0.6	0.2	8.3
Leucic acid	9.7	2.4	3.3	5.9	9.5	3.2	8.0	2.5
Phenyllactic acid	1.1	17.0	3.7	3.6	3.8	1.2	1.3	6.7
Benzoic acid	nd	–	2.1	10.0	5.4	8.4	3.4	2.8
Vanillic acid	nd	–	nd	–	0.3	6.7	nd	–
Hydroxylauric acid	nd	–	nd	–	0.2	17.3	0.3	11.5
Lactic acid	8229.7	–	7385.6	–	8221.6	–	7805.4	–
Acetic acid	1041.3	–	368.12	–	1032.3	–	414.3	–
Ethanol	4504.2	–	4122.8	–	4965.5	–	3645.6	–

RSD, relative standard deviation (%),  $n = 3$ ; Conc., concentration ( $\mu\text{g ml}^{-1}$ ); nd, not detected.

## Discussion

In this work the antifungal activity of *Lact. brevis* JJ2P and *Lact. reuteri* R2 were investigated against *Z. tritici* strains representative of current Irish populations. To ensure any antifungal activity observed was not dependant on the assay employed, a range of *in vitro* tests and an *in vivo* assessment were conducted. Whether applied as living cultures or cell-free-supernatants (CFS) both agents consistently inhibited *Z. tritici* growth,

demonstrating the antifungal activity of metabolites produced by the bacteria. As the LAB investigated and their CFS are slightly acidic in nature, the pH tolerance of the *Z. tritici* strains under test was initially determined. While pH 3.5, 4.0 and 9.0 did slightly reduce fungal growth, the growth was not affected at pHs from 4.5 to 8.0, indicating that while the lower pH of the cultures and CFS may have had a slight effect on fungal inhibition, other factor (s) were contributing to the observed inhibition of *Z. tritici*. The strength of this inhibition was

demonstrated in the liquid medium assay where the addition of *Lact. brevis* JJ2P and *Lact. reuteri* R2 CFS at 3 or 6% caused inhibition of fungal growth over a 10-day period. When 12.5% (or greater) CFS of either strain was added, no fungal growth was detected. This is in agreement with the work of Mauch *et al.* (2010) who reported a *Lact. brevis* strain showing strong antifungal activity against *Fusarium culmorum*. In that study, the addition of CFS of *Lact. brevis* PS1, under similar conditions as those described here, at concentrations of 2% (or greater) altered the growth morphology of *F. culmorum*, while the addition of 10% completely inhibited the outgrowth of fungal macroconidia.

Under controlled conditions, *Lact. brevis* JJ2P and *Lact. reuteri* R2 significantly reduced the development of STB on wheat seedlings resulting from infection with three different *Z. tritici* strains. The greatest inhibition was observed on seedlings inoculated with *Z. tritici* strain 560.11 which exhibits high levels of azole resistance *in vitro* and which showed the highest levels of disease on the azole-treated seedlings. This strain has accumulated six mutations and two amino acid deletions in the azole target site, 14 $\alpha$ -demethylase, in addition to an insert in the promoter region of the CYP51 gene, which codes for 14 $\alpha$ -demethylase. While the exact impact of these changes on the fitness of the pathogen remains to be determined, the differences observed in the seedling screen, while preliminary, do suggest such a fitness penalty in the presence of the LAB CFS. Further investigations into how both *Lact. reuteri* R2 and *Lact. brevis* JJ2P inhibit *Z. tritici* may, in addition to providing information on the antifungal activity of LAB, also provide information on how the various mutations affecting azole sensitivity are impacting the fitness of *Z. tritici*. The differences in efficacy of the LAB CFS against the different *Z. tritici* strains also highlights the need to screen biological agents against target pathogens that have been fully characterized and are representative of the field populations.

Regarding the mechanism of the antifungal nature of LAB, several fungicidal compounds have been isolated and characterized to date. The organic acids, lactic and acetic acid, are the main products of the fermentation of carbohydrates by LAB. In addition, further low molecular weight compounds are produced, which have previously been implicated in antifungal activity and these include carboxylic acids, carbon dioxide, diacetyl, hydrogen peroxide, 3-hydroxy fatty acids, cyclic dipeptides, reuterin, fungicins and proteinaceous compounds (Batish *et al.* 1997; Magnusson *et al.* 2003; Schnurer and Magnusson 2005; Mandal *et al.* 2007; Rouse and Van Sinderen 2008; Ryan *et al.* 2011; Guo *et al.* 2012).

In this study, a number of carboxylic acids were identified in the CFS of the *Lactobacillus* strains used. These

included phenyllactic acid and hydroxyphenyllactic acid which have previously been implicated in the antifungal activity of LAB strains (Dal Bello *et al.* 2007; Ryan *et al.* 2011). However, the measured levels of the detected carboxylic acids (Table 2) cannot fully explain the observed antifungal effect as the negative control strains produced similar levels of carboxylic acids to the antifungal strains. In contrast, the levels of both lactic and acetic acid were higher in the antifungal LAB strains compared to the negative control strains. The mechanisms by which organic acids inhibit fungal growth are still not fully understood. They are believed to diffuse through the membrane of the target organisms in their hydrophobic undissociated form, dissociating within the cell and thus reducing the cytoplasmic pH which interferes with metabolic activities (Dalié *et al.* 2010). The organic acids are believed to act in synergy, however, acetic acid is described as more potent due to its higher pKa value causing it to have a higher level of dissociation inside the cell (Crowley *et al.* 2013). It is possible that the identified carboxylic acids may act in synergy with the organic acids, and that other metabolites, not investigated in this study, may contribute to the observed anti-*Z. tritici* activity. For example Mauch *et al.* (2010) demonstrated that proteolytic treatment reduced the antifungal activity of *Lact. brevis* PS1 against *F. culmorum*, indicating that at least some of the antifungal compounds produced were proteinaceous in nature.

LAB have been successfully applied as starter cultures in a wide range of foodstuffs in the baking and brewing industries, in wine fermentation, and in dairy products to prevent food spoilage (Lavermicocca *et al.* 2000; De Muynck *et al.* 2004; Sathe *et al.* 2007; Mauch *et al.* 2010; Muhialdin *et al.* 2011; Ndagano *et al.* 2011; Ryan *et al.* 2011; Lynch *et al.* 2014). Considering the well-established antifungal spectrum of LAB, use of *Lact. brevis* JJ2P as a biocontrol agent may provide the industry with a natural and 'green' alternative to fungicides currently used to fight STB of wheat. In addition, other antifungal LAB strains could be examined for their potential to protect against pathogenic fungi of crops. Indeed, the use of these micro-organisms or their metabolites for such biocontrol strategies is bolstered when considering that soil and plants are the hypothetical first niche of ancestral LAB strains (and those strains that inhabit the gut of plant-eating animals) (Morelli *et al.* 2012). However, any novel biocontrol method must first be tested for its efficacy under outdoor conditions, a point of weakness of many biocontrol strategies. Kildea *et al.* (2008) previously described the difficulties associated with bringing such agents from the controlled environment to uncontrolled field conditions. Efficacy under these conditions can be influenced by many

factors, including soil type, host plant cultivar, pathogen strain and inoculation strategy. In addition, developments in processes for the large-scale production and isolation of antifungal compounds from LAB-fermented media are necessary for such methods to be widely accepted and economically viable.

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## Conflict of Interest

No conflict of interest declared.

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