IJIE Int. J. Indust. Entomol. 37(1) 9-14 (2018)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2018.37.1.9

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Analysis of the Inhibitory Effect of two Bacterial Strains on *Metarhizium anisopliae* Induced Fatality Rates in *Protaetia Brevitarsis*

Kyu-Won Kwak[1, 2], Sung-Hee Nam[1], Kwan-Ho Park[1], Heuisam Lee[1] and Myung-Sae Han[2*]

[1]*National Institute of Agricultural Science, Department of Agricultural Biology, 55365, Wanju, Republic of Korea*
[2]*Kyungpook National University, Department of Bio-fibers and Materials Science, 41566, Daegu, Republic of Korea*

Abstract

Bacterial species, *Bacillus amyloliquefaciens* and Lactobacillus species (*L.* sp.5-1), are known to inhibit the growth of pathogenic bacteria and fungi. *Metarhizium anisopliae* is a pathogenic fungal species which causes fatal damage to *P. brevitarsis* populations. Therefore, we investigated the inhibitory effect of *B. amyloliquefaciens* and *L.* sp. 5-1 on *M. anisopliae* induced fatality rates in *P. brevitarsis.* Samples of *M. anisopliae*-infected sawdust were treated with strain *B. amyloliquefaciens* KACC10116, strain *L*. sp. 5-1 KACC19351, and a combination of the two. *P. brevitarsis* were fed treated sawdust samples, and their subsequent fatality rate was monitored. The fatality rate fell below 1.5% after 10 days and decreased by approximately 40% after 15 days. On average, the fatality rate decreased by 20%, compared to the control. The difference in the decrease in fatality rate between *B. amyloliquefaciens* treatment and *L*. sp. 5-1 treatment was not significant. Results indicate that both strains exhibit high anti-fungal activity, which may be useful in environmental purification efforts. These strains may be used for effective prevention of fungal infection in *P. brevitarsis.*

Int. J. Indust. Entomol. 37(1), 9-14 (2018)

Received : 3 Jul 2018
Revised : 3 Sep 2018
Accepted : 4 Sep 2018

Keywords:
Bacillus amyloliquefaciens, *Lactobacillus* sp. 5-1, *Protaetia brevitarsis*, *Metarhizium anisopliae*, effective prevention

Introduction

Bacillus species are known to produce various antibiotic substances such as bacteriocin and lipopeptides (Arnison *et al*., 2013; Ongena and Jacques., 2008). These lipopeptides are characterized by strong anti-bacterial, anti-virus, and anti-cancer properties as well as low toxicity (Meena and Kanwar, 2015). *B. amyloliquefaciens* is a gram-positive bacterium of the genus *Bacillus*. This species is known to stimulate plant growth while inhibiting the growth of pathogenic fungi. It reportedly produces antibiotic lipopeptides such as intrurin, fengycin, bacilysin, and surfactin (Koumoutsi *et al*., 2004; Yu *et al*., 2002). Further, *B. amyloliquefaciens* secretes extracellular enzymes that exhibit immune effects such as a-amylase, cellulase, metalloprotease, and protease (Ahmed *et al*., 2014). A series of studies investigated certain properties of *B. amyloliquefaciens*, such as extracellular secretion of enzymes including amylase, phytase, and protease, stimulation of plant growth and antibiotic action against plant pathogens (Borgiat and Campbell, 1978; Idriss *et al*., 2002; Liu *et al*., 2011). *B. amyloliquefaciens* is known to reduce fecal noxious gas emission and maintain intestinal microbiota equilibrium (Ahmed *et al*., 2014). *Lactobacillus* species are useful in fermented food production, and perform anti-fungal

***Corresponding author.**
Myung-Sae Han
Department of Bio-fiber and Materials Science, Kyungpook National Universtiy, Daegu 41566, Republic of Korea
Tel: +82-53-950-5738 / FAX: +82-53-950-6744
E-mail: mshan@knu.ac.kr

and anti-mycotoxin functions. While Lactic acid bacteria (LAB) which display anti-fungal properties mainly produce lactic acid, these acids are also able to generate formic, propionic, butyric, phenylllactic, hydroxyphenyllactic, and indole-3-lactic aicds (Guimaraes *et al*., 2018). These organic acids penetrate the fungal cell membrane to disrupt fungal metabolism and intracellular pH homeostasis, thus inhibiting the growth of fungi (Brul and Coote, 1999).

There has been an increasing trend in *P. brevitarsis* farming in recent times, due to more people consuming edible insects. As the scale of farming is increases, so does the risk for a higher incidence of fungal infections in *P. brevitarsis*. The most prevalent infectious fungal species is *M. anisopliae,* which accounts for approximately 80% of infections (Kwak *et al*., 2018). During the course of this study, it was also observed that microorganisms such as *Bacillus subtilis*, *Lactobacillus plantarum* and yeast may all have an anti-fungal effect on *M. anisopliae*. Of these, *L*. sp. 5-1 and *B. amyloliquefaciens* were found to be safe for *P. brevitarsis* growth. Therefore, a strain each from these two species were selected for testing anti-fungal effects on *M. anisopliae* (Kwak *et al*., 2018).

Material and Methods

Fungi and Bacteria culture

Fungal strain, *M. anisopliae* KACC40969 was obtained from the Korean Agricultural Culture Collection, Rural Development Administration, Wanju, Korea. *M. anisopliae* KACC40969 was cultured on Sabouraud dextrose agar (SDA; BD, Franklin Lakes, NJ, USA) for 2 weeks in an incubator at 24°C. Bacterial strains, *B. amyloliquefaciens* KACC10116 and *L*. sp. 5-1 KACC19351 were obtained from the Korean Agricultural Culture Collection, Rural Development Administration, Wanju, Korea. The Bacillus strain was grown on Nutrient Agar (NA; BD, Franklin Lakes, NJ, USA), Reasoner's 2A agar (R2A; BD, Franklin Lakes, NJ, USA), and the Lactobacillus strain on de Man Rogosa and Sharpe (MRS; BD, Franklin Lakes, NJ, USA), respectively, for 8 hours in an incubator at 30°C. *L*. sp. 5-1 strain was diluted to concentrations of 2, 1.5, 1, 0.5, 0.25, and 0.125 measured at an optical density of 600 nm. *B. amyloliquefaciens* strain was diluted to concentrations of 1.5, 1, 0.5, 0.25, and 0.125.

Bioassay of *M. anisopliae* application to *P. brevit* arsis subsp. seluensis larvae

To investigate the mortality inhibition effect of *M. anisopliae* in the control group, *M. anisopliae*-inoculated sawdust was obtained from a sample in which the *P. brevitarsis* subsp. *seluensis* population had been wiped out due to *M. anisopliae* infection. This sawdust sample was mixed with water at a ratio of 3:1 (300 g of sawdust: 100 mL of water). Next, 10 g of *M. anisopliae* infected sawdust was placed in each petri dish (Diameter = 10 cm). Then, 5 ml of *L*. sp. 5-1. and *B. amyloliquefaciens* at varying concentrations were sprayed onto the *M. anisopliae* infected sawdust, ensuring even absorption into the sawdust. Stage 2 instars of *P. brevitarsis* subsp. *seluensis* were starved for 24 hours. One *P. brevitarsis* subsp. *seluensis* instar was placed per petri dish, and the fatality rate measured every 5 days, for 30 days.

Results and Discussion

M. anisopliae control by *B. amyloliquefaciens*

In testing *B. amyloliquefaciens* treatment, *M. anisopliae*-infected *P. brevitarsis* were treated with OD_{600}=1.5 culture medium. The fatality rate recorded after 15 days was 46.6%, showing a decrease of approximately 22%, compared to the 68% seen in the control. The fatality rate of *B. amyloliquefaciens* -treated *P. brevitarsis* remained below 1.5% after 10 days at all concentration levels (Fig. 1). Although the fatality rate increased 10 days following *B. amyloliquefaciens* treatment with OD_{600}=0.5, the overall fatality rate decreased by an average of 20% compared to the control (Data not shown). According to Lee's article (Lee *et al*., 2017), *Bacillus amyloliquefaciens* in culture medium with an OD_{600} of 1.5 was the most effective at removing zearalenone. Hence, this was defined as the optimal concentration of *B. amyloliquefaciens* (OD_{600} = 1.5). A concentration of OD_{600} = 1.0 of *B. amyloliquefaciens* is calculated as 1×10^8 cfu·ml^{-1} (Lee and Ryu, 2016), so it can be assumed that an OD_{600} of 1.5 contains more than 1×10^8 cfu·ml^{-1}. In this experiment, the highest concentration of *B. amyloliquefaciens* was at OD_{600} = 1.5.

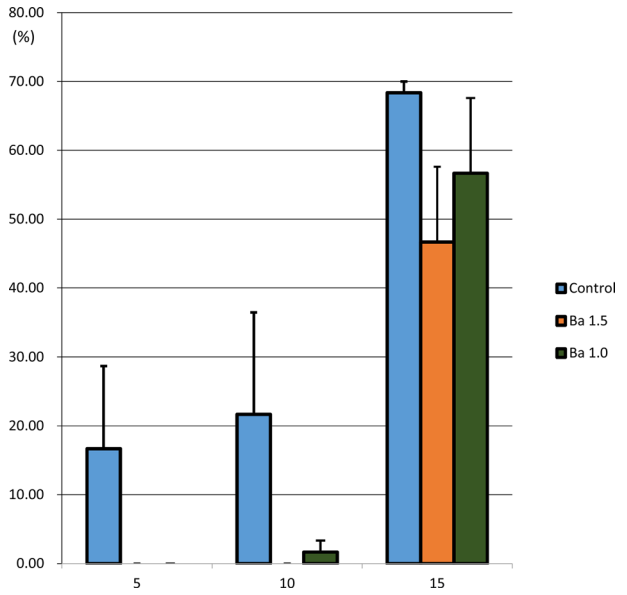

Fig. 1. *P. brevitarsis* mortality rate between *B. amyloliquefaciens* treatment and control groups in *M. anisopliae* sawdust. The mortality rate of control groups on third instar *P. brevitarsis* was about 68% after 15 days, but the mortality rate of the *B.a.* (OD_{600}= 1.5) treatment group was about 46%.

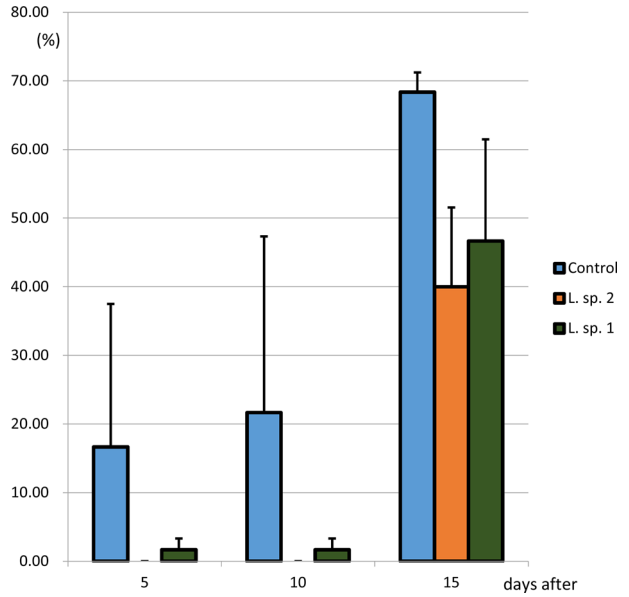

Fig. 2. *P. brevitarsis* mortality rate between *L.* sp.5-1 treatment and control groups in *M. anisopliae* sawdust. The mortality rate of control groups on third instar *P. brevitarsis* was about 68% after 15 days, but the mortality rate of the *L.* sp. 5-1 (OD_{600}=2) treatment group was about 40%.

M. anisopliae control by L. sp. 5-1

In this experiment, M*etarhizium anisopliae*e was as given a single application of *L.* sp. 5-1. According to studies by Huang *et al.* (2014) and Poppi *et al.* (2015), *L.* sp. 5-1 at concentrations of 10^7, 10^{8-9}, and 10^{9-10} cfu·ml^{-1} produced OD_{600} values of 1.0, 1.5, and 2.0 respectively (similar results for OD_{600} = 2.0 were observed in culture media. It is known that colony forming units are lowered depending on the pH of the culture medium and the incubation time (Huang *et al*., 2014; Poppi *et al*., 2015). The effective microorganism combination of *L.* sp. 5-1 at an OD_{600} of 1.0 (1×10^7 cfu·ml^{-1}) with *B. subtilis* and *Saccharomyces cerevisiae* were exposed to *M. anisopliae*, resulting in the inhibition of the growth of the entomopathogenic fungus (Kwak *et al*., 2018; Kwak *et al*., 2016). Therefore, this experiment was conducted by increasing or decreasing the concentration of *L.* sp. 5-1, based on the concentration of *L.* sp. 5-1 at OD_{600} = 1.0 (Kwak *et al*., 2018).

At OD_{600}=2, the mortality rate was around 40% after 15 days, which was a 28% decrease compared to that of the control. At OD_{600}=0.5, the fatality rate recorded was 51.67%, showing an approximately 17% decrease. For *L.* sp. 5-1 single treatment, the fatality rate after 10 days was about 1.5% for most of the concentrations (Fig. 2). The control recorded a 20% fatality rate and thus, the fatality rate fell by approximately 19% in the treated group (Fig. 2).

M. anisopliae control by *B. amyloliquefaciens* and *L.* sp. 5-1 combined treatment

The purpose of this experiment was to test whether a combination of *B. amyloliquefaciens* and *L.* sp. 5-1 results in a synergistic effect compared to single treatments of each; however, the results did not indicate a significant difference. Infected sawdust was treated with a 1:1 combination of *B. amyloliquefaciens*: *L.* sp. 5-1, cultured in OD_{600} concentrations 1.5, 1.0, 0.5, and 0.25. The fatality rate recorded after 15 days was 40%, which was a 30% decrease compared to the 70% seen in the control (Fig. 3). After 10 days, the fatality rate in the combined treatment decreased to approximately 1%, compared to the 20% recorded in the control. In summary, the combination decreased the 10-day fatality rate by 19% (Fig. 3).

B. amyloliquefaciens and *L.* sp. 5-1 treatment of *M. anisopliae*-infected *P. brevitarsis* did not demonstrate a noticeable difference in fatality rates. However, the fatality rate fell to 40% when treated with high concentration *B. amyloliquefaciens* and *L.* sp. 5-1. In this case, the fatality rate was similar to that of 15 days

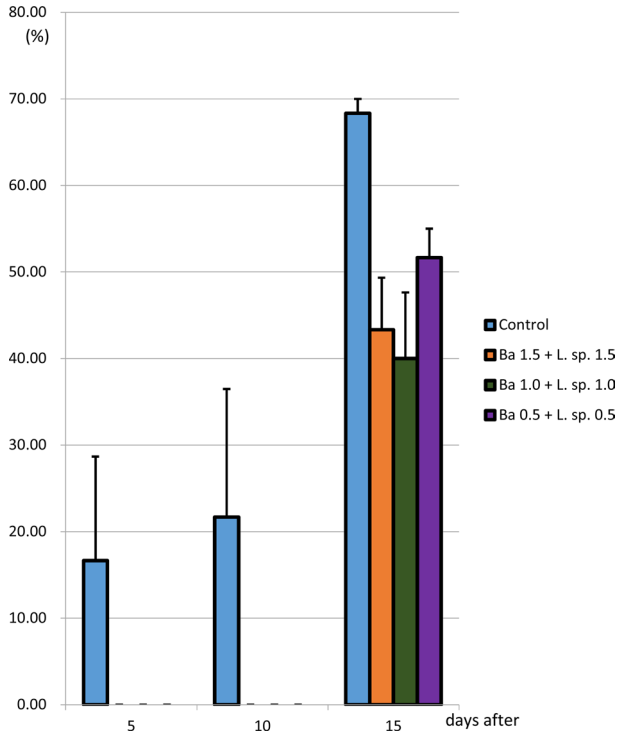

Fig. 3. *P. brevitarsis* mortality rate between *B. amyloliquefaciens* plus *L.* sp.5-1 treatment and control groups in *M. anisopliae* sawdust. The mortality rate of control groups on third instar *P. brevitarsis* was about 68% after 15 days, but the mortality rate of the *B.a* (OD_{600}=1.0) plus *L.* sp.5-1(OD_{600}=1) treatment group was about 40%.

after *B. amyloliquefaciens* and *L.* sp. 5-1 treatment. Therefore, combined treatment did not demonstrate a synergistic effect of any considerable magnitude. The fatality rate following *L.* sp. 5-1 treatment (OD_{600}=2) was 40%, whereas it was 46% following *B. amyloliquefaciens* treatment (OD_{600}=1.5). The fatality rate decreased by 28% and 22%, respectively, compared to 68% in the control. However, *L.* sp. 5-1 concentration was higher compared to that of *B. amyloliquefaciens*. Hence, the anti-fungal inhibition of *B. amyloliquefaciens* is more effective when concentration levels are taken into account. At OD_{600}, *B. amyloliquefaciens* was not cultured beyond the concentration level of 1.5.

Anti-fungal activity of LAB strains is known to be activated by combinations of various organic acids such as acetic, caproic, popionic, formic, butryric, and n-valeric acid. Broad anti-fungal activity takes effect in broad molds (Laitila *et al*., 2002). LAB is thought to be associated with hydrogen peroxide and many inter-related factors apart from the generation of organic acid (Laitila *et al*., 2002). Therefore, it cannot be surmised that low pH and lactic acid alone may generate anti-fungal activity (Laitila *et al*., 2002).

LAB is reported to be highly useful for bio-preservation which is relevant to food safety with particular reference to cereal products. This may indicate a function for LAB as a food supplement, inhibiting potentially resistant molds (Russo *et al*., 2017)

On the other hand, the mycotoxin adsorption ability of gram-positive bacteria is associated with the cell membrane component peptidoglycan of *B. subtilis*. The thickness of *B. amyloliquefaciens* and *B. subtilis*-originated peptidoglycan wears thin, which causes the glycosidic linkage to break, and the pore size of peptidoglycan consequently increases (Lee *et al*., 2017)

B. amyloliquefaciens is known to produce inturin, a cyclic lipopeptide with antibiotic properties (Benitez *et al*., 2010). *B. amyloliquefaciens* and *B. subtilis* are closely associated, and the latter is also known to produce lipopeptide antibiotics including surfactins, inturins, bacillomycins, and mycosubtilis (Benitez *et al*., 2010).

In addition, lantibiotic subilin and mersacidin produced by *B. amyloliquefaciens* are peptides with anti-microbial properties. Lactic acids originating from *B. amyloliquefaciens* are known to reduce intestinal pH, and *B. amyloliquefaciens* is reported to be effective in reducing ammonia production (Ahmed *et al*., 2014)

Non-steroidal estrogenic mycotoxins generated by *Fusarium* species, such as Zearalenone, are known to, decontaminate food or feed. Compared to chemical methods such as the use of acids, bases, ozone, and hydrogen peroxide and physical methods such as irradiation, biological methods using effective microorganisms may be able to decontaminate food with minimal damage to nutrients in the food or feed and therefore offer a milder alternative to harmful decontaminants based on chemical methods (Lee *et al*., 2017). Further, *B. amyloliquefaciens* displays high tolerance to over 200 μg/ml ampicillin in honey (Li *et al*., 2009).

Another important function of Probiotics is resistance against potentially pathogenic microorganisms. For example, insect pathogens, *B. cereus,* and *Listeria monocytogenes*, associated with food poisoning and known to be harmful for humans and animals, are known to inhibit the growth of opportunistic fungi (Beloeil *et al*., 2003; Logan, 2012). *B. amyloliquefaciens* possesses probiotic functions but is unable to produce enterotoxin, and therefore considered as safe to be applied to feed (Lee *et al*., 2017)

In response to the growth of edible insect products for human consumption as well as for pet and animal feed, insect farming is on the rise. Therefore, application of useful microorganisms including LAB and *B. amyloliquefaciens* may be highly effective in the prevention and control of insect diseases.

Acknowledgements

This work was supported by the grant from the National Institute of Agricultural Science, Rural Development Administration, Republic of Korea (PJ012024012018). We express our sincere gratitude to Dr. An Jae-Hyung who provided us with *Lactobacillus* sp. 5-1 strain and made the experiment possible.

References

Ahmed ST, Islam MM, Mun H, Sim H, Kim Y, Yang C (2014) Effects of *Bacillus amyloliquefaciens* as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poult Sci 93, 1963-1971.

Arnison PG, Bibb MJ, Bierbaum G, Bowers AA, Bugni TS, Bulaj G, *et al.* (2013) Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. Nat Prod Rep 30(1), 108-160.

Beloeil PA, Chauvin C, Toquin MT, Fablet C, Le NoÃ tre Y, Salvat G, *et al*. (2003) *Listeria monocytogenes* contamination of finishing pigs: an exploratory epidemiological survey in France. Vet Res 34, 737-748.

Benitez LB, Velho RV, Lisboa MP, Medina LF, Brandelli A (2010) Isolation and Characterization of Antifungal Peptides Produced by *Bacillus amyloliquefaciens* LBM5006. Int J Microbiol 48(6), 791-7.

Borgiat PT, Campbell LL (1978) α-Amylase from five strains of *Bacillus amyloliquefaciens*:evidence for identical primary structures. J Bacteriol 134, 389-393.

Brul S, Coote P (1999) Preservative agents in foods. Mode of action and microbial resistance mechanisms. Int J Food Microbiol 50**,** 1-17.

Guimaraes A, Santiago A, Teixeira JA, Venancio A, Abrunhosa L (2018) Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. Int J Microbiol, 284, 31-38.

Huang R, Tao X, Wan C, Li S, Xu H, Xu F, *et al.* (2014) In vitro probiotic characteristics of *Lactobacillus plantarum* ZDY 2013 and its modulatory effect on gut microbiots of mice. J Diary Sci 98, 5850-5861.

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H*, et al*. (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiol 148, 2097-2109.

Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P*, et al.* (2004) Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186, 1084-1096.

Kwak KW, Kwon SW, Nam SH, Park KH, Kim ES, Lee HS, *et al.* (2018) Inhibition of *Metarhizium anisopliae* infection of *Protaetia brevitarsis seluensis* larvae using several effective microorganisms. Int J Indust Entomol 36, 1-9.

Kwak KW, Han MS, Nam SH, Park KH, Kim ES, Lee S, *et al.* (2016) Effect of *Saccharomyces cerevisiae* consumption on the pathogenicity of *Beauveria bassiana* in *Protaetia brevitarsis*. Int J Indust Entomol 33, 1-5.

Laitila A, Alakomi HL, Raaska L, Mattila-Sandholm T, Haikara A (2002) Antifungal activities of two *Lactobacillus plantarum* strains against *Fusarium* moulds in vitro and in malting of barley. J Appl Microbiol 93, 566-576.

Lee A, Cheng KC, Liu JR (2017) Isolation and characterization of a Bacillus amyloliquefaciens strain with zearalenone removal ability and its probiotic potential. PloS One 12(8), 1-21.

Lee GH, Ryu CM (2016) Spraying of Leaf-colonizing *Bacillus amyloliquefaciens* protects pepper from cucumber mosaic virus. Plant Disease 100, 2099-2105.

Li X, Quan CS, Yu HY, Wang JH, Fan S (2009) Assessment of antifungal effects of a novel compound from *Burkholderia cepacia* against *Fusarium solani* by fluorescent staining. World J Microbiol Biotechnol 25, 151-154.

Liu J, Zhou T, He D, Li XZ, Wu H, Liu W, *et al.* (2011) Functions of lipopeptides bacillomycin D and fengycin in antagonism of *Bacillus amyloliquefaciens* C06 towards Monilinia fructicola. J Mol Microbiol Biotechnol 20, 43-52.

Logan NA (2012) *Bacillus* and relatives in foodborne illness. J Appl Microbiol 112, 417-429.

Meena KR, Kanwar SS (2015) Lipopeptides as the Antifungal and Antibacterial Agents: Applications in Food Safety and Therapeutics. BioMed Res Intl 2015, 473050.

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16(3), 115-125.

Poppi LB, Rivaldi JD, Coutinho TS, Astolfi-Ferreira CS, Ferreira AJP, Mancilha IM (2015) Effect of *Lactobacillus* sp. isolates supernatant on *Escherichia coli* O157:H7 enhances the role of organic acids production as a factor for pathogen control. Pesquisa Veterinaria Brasileira 35, 353-359.

Russo P, Fares C, Longo A, Spano G, Capozzi V (2017) *Lactobacillus plantarum* with Broad Antifungal Activity as a Protective Starter Culture for Bread Production, Foods 176, 1-9.

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 34, 955-963