

Weissella cibaria Fungistatic Activity Against *Fusarium* spp. Affecting Yellow Pitahaya

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Abstract: Fungistatic activity of a Lactic-Acid Bacteria (LAB) was assessed *in vitro* while isolated from its rumen fluid, against *Fusarium oxysporum* and *Fusarium fujikoroii*, basal-rot related species in yellow pitahaya. Fed batch was used to reproduce said LAB. Fungistatic activity kinetic tests were performed on the ferment (S1) and on two of the fractions obtained through centrifugation, supernatant (S2) and biomass (S3). The fungistatic activity was measured in two substrates, agar Man Rogosa Sharpe (MRS) and Potato Dextrose Agar (PDA). On S1 it was also performed lactic acid and acetic acid production kinetic tests as well as substrate consumption and biomass formation. Structural changes were evaluated on the fungi through microscopic electronics. The LAB was molecularly identified as *Weissella cibaria*. It was found that after eight hours of fermentation, fractions S1 and S3 performed the highest fungistatic activity against *F. oxysporum* and *F. fujikoroii*. Porosities were observed in the cell walls of the fungi as an effect of the LAB's fungistatic activity. These results indicate that LABs, when isolated from the animal habitat, may have a biotechnological potential to be used in the development of biocides, useful to control *Fusarium* in crops; for instance, to control yellow pitahaya basal rot.

Keywords: *Fusarium*, Lactic Acid Bacteria, *Weissella cibaria*, Yellow Pitahaya

Introduction

Lactic-Acid Bacteria (LAB) biopreservative properties are attributable to the capacity they have to inhibit the growth of pathogenic bacteria and some fungus species (Magnusson *et al.*, 2003). These bacteria have drawn the attention of researchers as control agents in pathogenic bacteria and fungus causing illnesses in animals and plants. In previous studies conducted by Serna-Cock *et al.* (2010), a species of *Weissella* able to inhibiting pathogens causing illnesses in humans and animals, *Staphylococcus aureus*, *Streptococcus agalactiae*, *E. coli* and *K. neumoinc* was reported (Serna-Cock *et al.*, 2012). It has also been reported that *W. cibaria* and *Lactobacillus brevis* have fungistatic activity against *Fusarium* species (Mauch *et al.*, 2010). Baek *et al.* (2012) and found that *W. confusa* has an antagonistic effect against *Cadispodium* sp. YS1, *Penicillium crustosum* YS2, *Neurospora* sp. YS3.

LABs have been isolated from several sources such as herbs, fruit, vegetables, animals, human

beings and have been tested *in vitro* against plant pathogens. However, no studies are reported evaluating the potential of LABs isolated from the animal habitat to control plant pathogens in yellow pitahaya *Selenicereus megalanthus* (K. Schum. ex Vaupel) Moran. Yellow pitahaya growing in Colombia is an important economic activity since it offers market-appealing possibilities as dried and fresh fruit. However, Colombian producers have had some issues in taking advantage of these possibilities and opportunities as crops are mainly affected by *Fusarium* species causing basal rot in the fruit. To diminish economic loss caused by this microorganism, using fungicides and chemical fertilizers has been recommended (Fei *et al.*, 2004). Nevertheless, this type of treatments carries disadvantages to growers, consumers and the environment (Quintero, 1997). Therefore, it is necessary to have an effective strategy to avoid damages to the environment and the health of consumers. Biological control is one of the many options and can be achieved by using antagonistic

microorganisms, which is an environmentally friendly agricultural practice and may be incorporated to yellow pitahaya illnesses prevention programs.

Consequently, this paper evaluated the fungistatic activity of a lactic acid bacteria against two species of *Fusarium* causing basal rot in yellow pitahaya.

Materials and Methods

Lactic Acid Bacteria Growth

A LAB isolated from bovine rumen fluid was used in previous researches by Serna *et al.* (2010).

Molecular Characterization

Genomic DNA Extraction

To obtain LAB's deoxyribonucleic acid (DNA), the procedure described by Dimkic *et al.* (2013) was used with some modifications. 5 mL of bacterial culture was centrifuged at 10,000 rpm for 2 min at 4°C: the obtained pellet was re-suspended in 50 mM of Tris-HCl pH 8, 1 mM EDTA, 25% sucrose, 8mg/mL lysozyme) and it was incubated for 30 min at 37°C. Proteinase K and SDS was added at 10% and it was incubated for 10 min. at 65°C. A volume of Buffer solution was also added (50 mM of Tris-HCl pH 8, 1 mM EDTA and 25% sucrose) and two volumes of phenol chloroform (50:50) was mixed through inversion and was centrifuged at 14,000 rpm for 5 min at 4°C). 1 volume of chloroform was added to the supernatant and it was mixed and centrifuged at 14,000 rpm for 5 min at 4°C). 2 volumes of cold ethanol and a tenth part of sodium acetate 3 M was added to the recovered supernatant, it was mixed through inversion and the pill was recovered through centrifugation at 10,000 rpm for 10 min at 4°C. Salts from the DNA pill were eliminated using 1mL of ethanol 70%. The DNA pill was finally re-suspended in 50 µL of buffer TE (Tris 50 mM, EDTA 10mM) with RNasa. To see the purified genomic DNA's quality and quantity, an electrophoresis was conducted on agarose gel at 0.8% (p/v) and a photograph was taken using photodocumentation equipment (Molecular Imager Gel DocXR+ Systems Bio-rad®, USA).

16S Ribosomal RNA Gene Amplification

A piece of the 16S ribosomal gene was amplified by using primers W01 and W012 (Soler *et al.*, 2008). Amplification was made by using the Polymerase Chain Reaction (PCR) at a total volume of 50 µL containing buffer Taq 1X ((NO₄)₂ SO₄), Cl₂Mg₂ 0.2 mM, 0.4 µM of each primer W01 (5'-AGA GTT TGA TC(AC) TGG CTC-3') and W012 (5'-TAC GCA TTT CAC C(GT) C TAC A-3'), 0.025 U/µL of Taq DNA polymerase (Fermentas®, USA), 1 µL of genomic DNA and 0.2 mM dNTPs. The amplification was conducted in a Thermal

cycler (Bio-rad, USA), with denaturation cycle for 4 min at 94°C and 30 denaturation cycles at 94°C for 30 seconds, with the igniter at 50°C for 30 seconds and elongation at 72°C for 60 seconds and a final extension of 5 min at 72°C. The amplification products of 16S ribosomal RNA gene were analyzed through electrophoresis on agarose gel (0.8% p/v). 1kb molecular weight marker (Fermentas®, USA) was used. The visualization of the bands was made with a UV transilluminator and photographs were taken using photodocumentation equipment (Molecular Imager Gel DocXR+ Systems Bio-rad®, USA).

Bioinformatics Sequence and Analysis

The amplified PCR fragment was purified and sequenced in MacroGen-Korea, using primers W01 and W012. The sequence readings were assembled using CLC Main Workbench 7.0 (Qiagen, Germany). To know the identity of the nucleotide sequences, a bioinformatics analysis was conducted using Blastn software through the National Center for Biotechnology Information, using the TL/16S_ribosomal_RNA_Bacteria_and_Archaea database.

Fungistatic Activity and Fermentation Kinetics

Microorganisms Reproduction

The fungistatic activity was made against 2 strains of *F. oxysporum* and *F. fujikoro*i obtained in previous researches, which were related to basal rot in yellow pitahaya. Currently, the strains are preserved in the culture collection of the Cytogenetics Laboratory of Universidad Nacional de Colombia, Campus Palmira.

F. oxysporum and *F. fujikoro*i were grown in Potato Dextrose Agar (PDA) at 26°C for 8 days until sporulation. After that, spores were collected in test tubes, peptone water was added at 0.2% p/v and it was vigorously shaken (Magnusson *et al.*, 2003). The suspensions were adjusted between 10⁴ and 10⁵ spores per mL and were cryo preserved at -4°C with an addition of glycerol at 40% v/v up to its subsequent use. Spore count (to perform adjustments at 10⁴ or 10⁵) was made through microscopic determination of the number of cells by using a Neubauer count chamber.

The LAB was grown by tripled in batch-type ferments, using Man Rogosa Sharpe (MRS) supplemented broth (De Man *et al.*, 1960) as substrate with 40% sucrose (MRS + GLU). Fermentation was conducted at 37°C for 48 hours, non-stop shaking without ventilation at 100 rpm (shaker orbital model 5000I VWR, USA), with a work load of 500 mL. Fermentation was adjusted at pH 6.0 using NaOH 1M. 10% of inoculum was used in relation to the substrate volume.

Fungistatic Activity Kinetics

Ferment (S1) samples were collected at 0, 1, 2, 4, 6, 8, 12, 24 and 48 hours of fermentation and fungistatic activity kinetics was performed for (S1), as well as two fractions obtained through ferment centrifugation, supernatant (S2) and biomass (S3). S1 was bottled in a 50 mL falcon tube. S2 and S3 were obtained through ferment centrifugation, for 15 min. at 5,000 x g (Eppendorf Centrifuge-5804R, Germany). S2 contained the metabolites produced by the LAB, S2 was filtered twice, first with a 0.45 diameter filter and then with a 0.2 µm diameter filter. The filtered product was bottled. S3 corresponded to biomass free of metabolites. S1, S2 and S3 were used for the fungistatic activity tests against *F. oxysporum* and *F. fujikoroii*.

In the determination of the fungistatic activity kinetics for S1, S2 and S3 against *F. oxysporum* and *F. fujikoroii*, 2 growth mediums, agar MRS (favoring LAB's growth) and PDA (favoring *F. oxysporum* and *F. fujikoroii* growing), using the methodology reported by Uzair *et al.* (2008) with some modifications that are described below. 10 µL of spore suspension (10^5) of *F. oxysporum* and *F. fujikoroii* (separately) were collected and planted in the center of Petri dishes with 20 mL of agar PDA and MRS. Then, at a distance of 1.6 cm from the dishes inoculated with the fungus, a paper dish (Whatmann No. 1 with a diameter of 0.8 cm, Clifton, NJ, U.S.A.) was placed, containing 10 µL of S1, S2 and S3 at a concentration of 10^9 forming units of colonies per milliliter (CFU/mL). The Petri dishes were incubated at 37°C for 2 days, followed by a 26°C incubation for 6 days. Control procedures consisted of placing the Petri dishes containing agar PDA and paper MRS dishes without inoculating the LAB.

The growing of the fungus was monitored through photographs taken during the tests and the León *et al.* (2006) photography technique was used with some modifications. Measuring the growth area of the fungus was calculated using image evaluation software (Imagen j 1.40 g, Wayne Rasband, National Institutes of Health, USA). The fungistatic activity was measured in percentages of fungus growth inhibition (equation 1).

$$\%I = \frac{CHC - CH}{CHC} * 100 \quad (1)$$

where, % I: is the percentage of LAB inhibition against the Fusarium specie; CH: is the growth area of the Fusarium specie (cm²) when inoculated with the LAB; CHC: is the growth area of the Fusarium specie during the control test (cm²).

Lactic Acid and Acetic Acid Production Kinetics, Substrate Consumption and Biomass Formation

Fermented product samples were collected at 0, 1, 2, 4, 6, 8, 12, 24 and 48 h of fermentation and the

fungistatic activity was measured in each one of those hours, as well as lactic acid concentration, acetic acid concentration, substrate consumption, biomass concentration and cell viability.

To determine the concentration of lactic acid, a Reflectoquant (RQflex plus 10, Germany) was used, placing measuring strips, which had a measure interval between 3 and 60 mg/L of lactic acid. When the concentration of lactic acid in the samples was above the rank, dilution with distilled water was performed.

To determine the concentration of acetic acid, 50 mL samples were collected every time fermentation samples were also collected. Said samples were analyzed through gas chromatography, equipped with a flame ionization detector, using an Innowax column of 30m x 0.32 mm x 0.5 µm and helium as the carrier gas. Injector and detector temperatures were 250°C.

To determine biomass formation kinetics, 10 mL of fermentation substrate was collected each sample-collection hour and then centrifuged at 10,000 x g (Eppendorf Centrifuge-5804R, Germany) for 10 min and the supernatant was removed from each sample. The biomass was cleared with a salt solution and dried in a stove at 100°C (Standard AOAC 934.01, 1990), the results were showed in biomass grams per liter of fermentation broth.

To determine the viable cells concentration kinetics, dilutions of 10^{-1} to 10^{-10} in peptone water at 0.1% p/v were used each sample-collection hour. It was planted in depth and in duplicate in dishes with agar MRS and aniline blue at 0.3%. The dishes were inoculated with 1 mL of each one of the dilutions and were incubated at 37°C for 48 h. The viable cell count was expressed as CFU/mL.

To determine the substrate consumption kinetics, 5 mL samples were collected each sample-collection hour and total reducing sugars were determined using the DNS method based on reduction of 3,5-Dinitrosalicylic acid in presence of heat through the spectrophotometric method (Thermo Scientific, genesys 10 UV, USA) described by Millar (1959).

Based on the kinetics, the speed of the kinetics, the specific growth rate (μ), the biomass performance ($Y_{x/s}$) and the product performance ($Y_{p/s}$) were calculated for the LAB through equations 2 and 3. To measure the product, the production of lactic acid was taken as reference:

$$Y_{p/s} = \frac{P}{S_0 - S} (g \setminus g) \quad (2)$$

$$Y_{x/s} = \frac{X - X_0}{S_0 - S} (g \setminus g) \quad (3)$$

Where:

S_0 = Initial concentration of total sugars (g/L)
 S = Final concentration of total sugars (g/L), up to the moment in which P is the maximum
 P = Maximum concentration of lactic acid (g/L)
 X = Initial concentration of biomass (g/L)
 X_0 = Final concentration of biomass (g/L)

Microscopy Analysis

In order to see differences in the morphology of the fungus due to the effect of the fungistatic activity of lactic acid bacteria, samples were collected from the treatment that had produced the best results of the fungistatic activity and then analyzed through Scanning Electron Microscopy (SEM) (PhenomWorld, ProX, Holland).

Statistical Analysis

To verify which of the S1, S2 and S3 components had the best fungistatic activity, a $2 \times 3 \times 2$ total random factorial design was used with repeated measurements in 9 times as follows:

Pathogen factor with 2 levels: *F. oxysporum* and *F. fujikoroii*.

Fermentation fraction factor with 3 levels: ferment (S1), supernatant (S2) and biomass (S3).

Culture medium factor to measure the fungistatic activity with 2 level: PDA and MRS.

Fermentation times were 0, 1, 2, 4, 6, 8, 12, 24 and 48 h.

The response variable was fungistatic activity. The results were analyzed through the statistics program SAS version 9.1.3 (Cary, NC). The comparison between the averages was conducted through the Tukey test with a probability of $p < 0.05$. For the generation of graphics, we used statistical software Excel module from Microsoft Office (USA, 2007).

In the production kinetics of lactic acid, acetic acid, biomass production, cell viability and substrate consumption, the standard deviations were calculated between the triplicates for each one of the fermentation times.

Results

Molecular Characterization

A 695 bp fragment of 16S ribosomal gene of the LAB biochemically identified as *W. confusa* in studies conducted by Serna *et al.* (2010) was amplified through PCR and then sequenced. Figure 1 shows the fragment that included the variable regions V1 to V4 of the 16S rRNA gene. A 695 pb sequence was obtained having an identity percentage of 100% of *W. Cibaria* strain II-I-59 16S ribosomal RNA (NR_036924) and 98.85% with *W. confusa* strain JCM 1093 16S ribosomal RNA (NR_113258). This analysis indicates that the LAB isolated from the rumen fluid is *W. cibaria*. The nucleotides sequence of *W. cibaria* has been deposited in GenBank under the accession number KU132362.

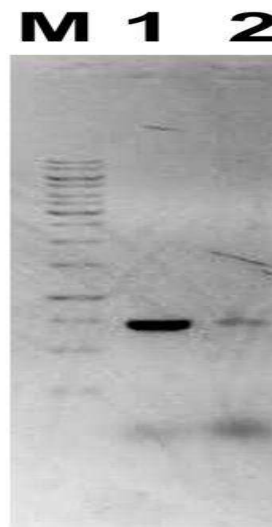


Fig. 1. PCR amplification of a fragment of 695 pb 16S ribosomal gene. M, molecular weight marker 1Kb; 1 and 2, genomic DNA of a LAB isolated from rumen fluid

This bacterium had been biochemically identified as *W. confusa*. Authors have reported through biochemical tests that *W. confusa* and *W. cibaria* ferment similar carbohydrates (Kang *et al.*, 2005); however, it is well known that biochemical identification leads to identification mistakes. There may be similarities in the rRNA 16S gene of *W. confusa* and *W. cibaria* (Malik *et al.*, 2009).

Fungistatic Activity and Fermentation Kinetics

Fungistatic Activity Kinetics

Figure 2 and 3 shows the fungistatic activity of *W. cibaria* conducted in agar MRS (favoring LAB growth). Figure 2 shows that inhibition of S1 and S3 against *F. oxysporum* increased as biomass increases, until maximum fungistatic activity is obtained, which happened at the 8th hour of the fermentation process. It means that it took place when the bacterium started its growing stationary phase, as shown in Figure 7. This indicates that the production of antifungal substances against *W. cibaria* is not related to the growing process of the bacterium. In the most active moment of the fungistatic activity, there were no statistical differences between S1 and S3. The S2 fraction had a fluctuating behavior. However, between the 8th and the 24th hour there was a trend to increase its fungistatic activity. This substance showed the lowest fungistatic activity.

Figure 3 shows the fungistatic activity of substances S1 and S3 of *W. cibaria*, against *F. fujikoroii*, during all the hour of fermentations and it was lower than 60%. It was possible to see that the maximum antifungal activity of substance S1 occurred at the 8th hour of fermentation, when the bacterium was in its growing stationary phase.

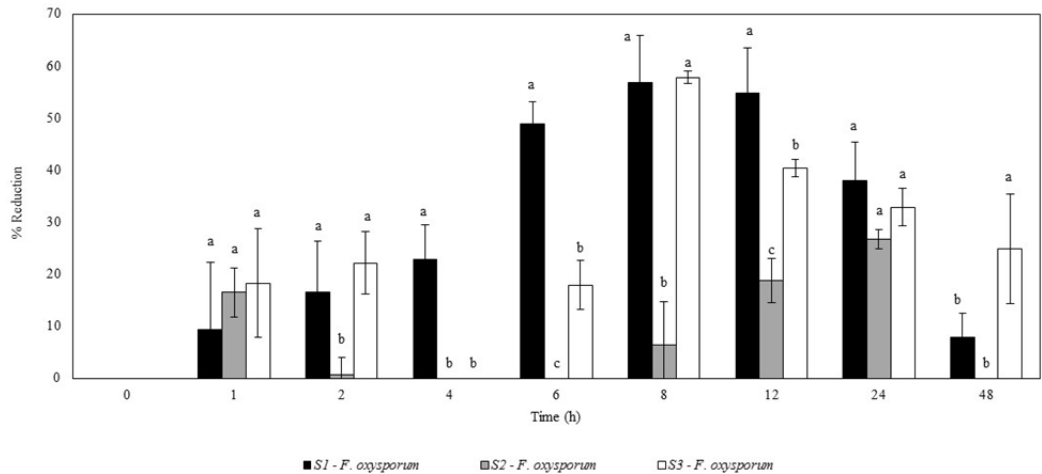


Fig. 2. Fungistatic activity kinetics for three fractions obtained through fermentation and centrifugation: ferment (S1), supernatant (S2) and biomass (S3) of *W. cibaria*, against *F. oxysporum* in substrate MRS (favorable for the growing of *W. cibaria*)

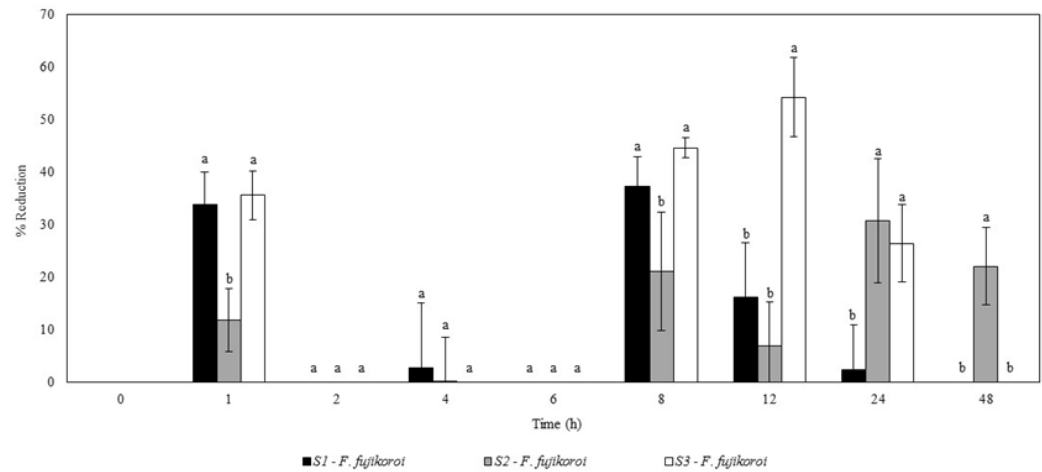


Fig. 3. Fungistatic activity kinetics for three fractions obtained through fermentation and centrifugation: ferment (S1), supernatant (S2) and biomass (S3) of *W. cibaria* against *F. fujikuroi* in MRS substrate (favorable for the growing of *W. cibaria*)

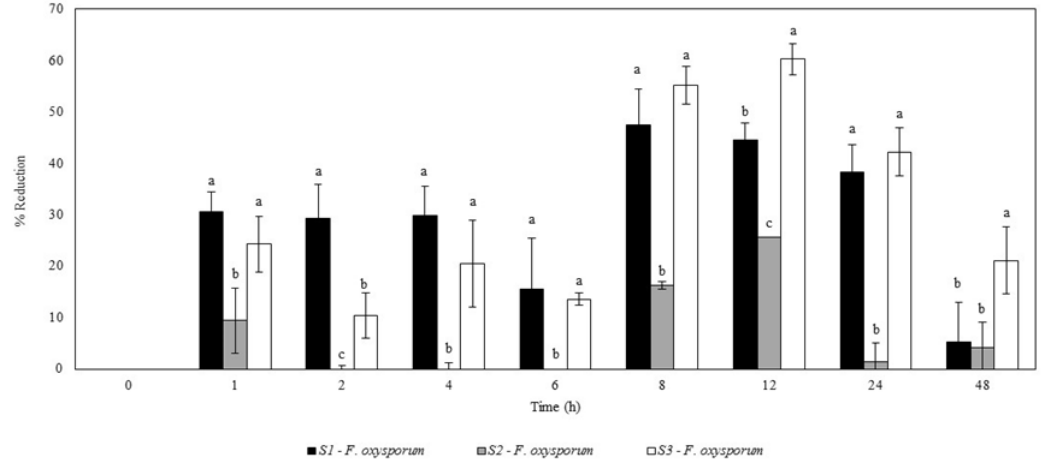


Fig. 4. Fungistatic activity kinetics for three fractions obtained through fermentation and centrifugation: ferment (S1), supernatant (S2) and biomass (S3) of *W. cibaria* against *F. oxysporum* in PDA substrate (favorable for the growing of fungi)

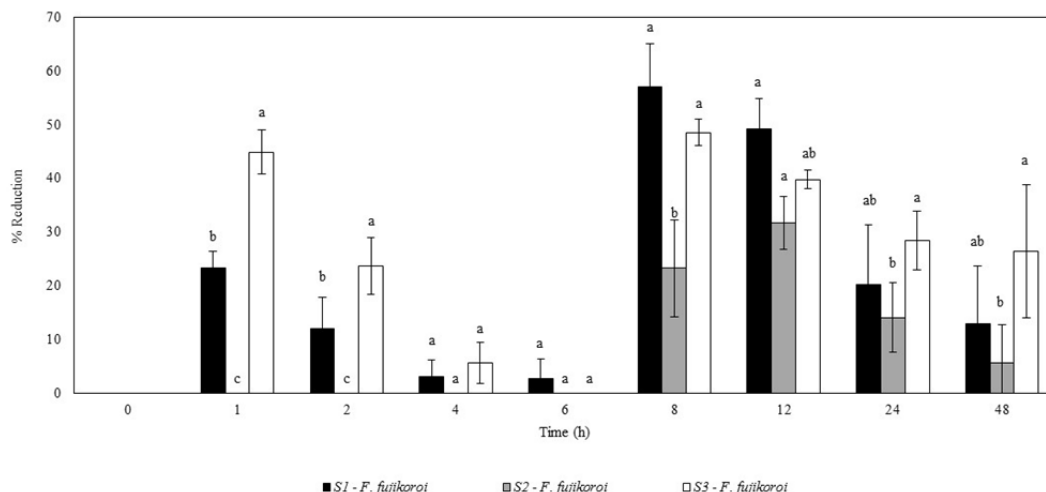


Fig. 5. Fungistatic activity kinetics for three fractions obtained through fermentation and centrifugation: ferment (S1), supernatant (S2) and biomass (S3) of *W. cibaria* against *F. fujikoroii* in PDA substrate (favorable for the growing of fungi)

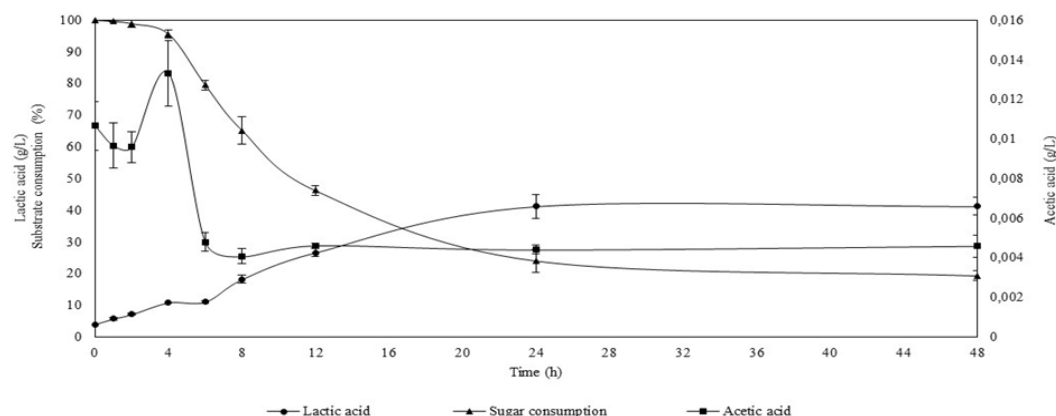


Fig. 6. Lactic acid and acetic acid production kinetics, and sugars consumption of *W. cibaria* (S1) during 48 hour of fermentation

On the other hand, substance S3 had its maximum fungistatic activity at the 12th hour; which means that such activity was not associated to the growth of *W. cibaria*. With substance S2, reduction percentages lower than 40% were obtained, in which the maximum fungistatic activity occurred at the 24th hour. With these results, it is possible to affirm that the antifungal activity is due to the production of secondary metabolites.

Figure 4 and 5 show the fungistatic activity of *W. cibaria* conducted in agar PDA, (favoring the growth of fungi) against *F. oxysporum* and *F. fujikoroii* respectively. Figure 4 shows that the first fungistatic activity hour of fermentations, both for S1 and S3, was low and variable. However, as of the 8th and 12th hours, respectively, the maximum reductions in the fungus growing process occurred. Fraction S2 showed the lowest fungistatic activity. It was also seen that fractions S1, S2 and S3 were not related to the growth of the bacteria. The inhibitory

power of fraction S3 was more significant ($p > 0.05$) and higher than S1 at the 12th and 48th hours.

Figure 5 shows the reduction percentages of *F. fujikoroii* in the PDA substrate as lower than 60%. Loss of fungistatic activity was observed during the evaluated times. S1 and S3 showed a high antifungal activity at the 8th hour. The highest percentages of inhibition did not show significant statistical differences, which indicates that substance S1 or S3 will exert the same effect against *F. fujikoroii*. The fungistatic activity of S2 fluctuated and lower in comparison to S1 and S3. There were significant differences ($p < 0.0005$) between the fungistatic activity (against *F. oxysporum*), measured in PDA and in MRS, which indicates the culture medium affects the fungistatic activity of the analyzed substances.

The results obtained in this research indicate that when S1, S2 and S3 are obtained from *W. cibaria* during the stationary phase, high fungistatic activity levels are obtained against *F. oxysporum* and *F. fujikoroii* species.

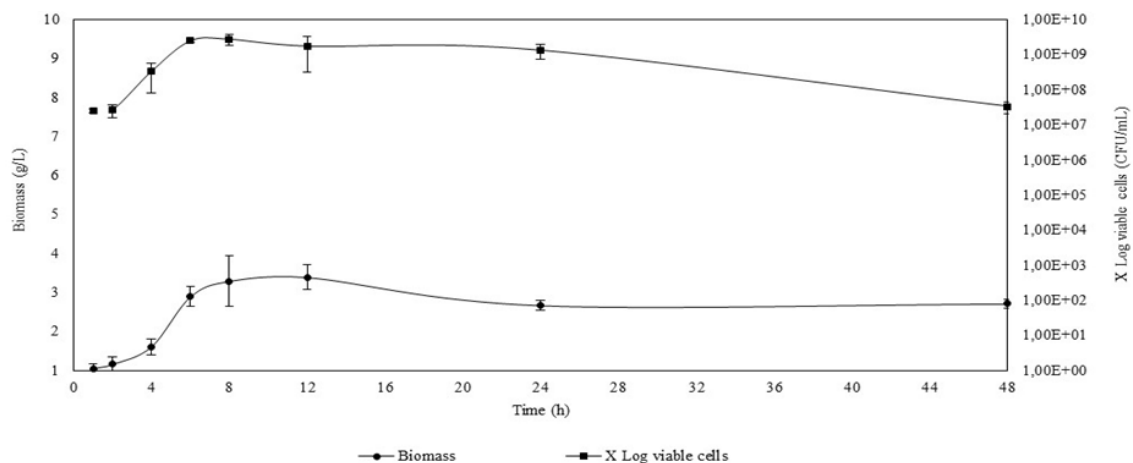


Fig. 7. Biomass production kinetics and forming units of colonies per milliliter of *W. cibaria* (S1) during 48 hour of fermentations

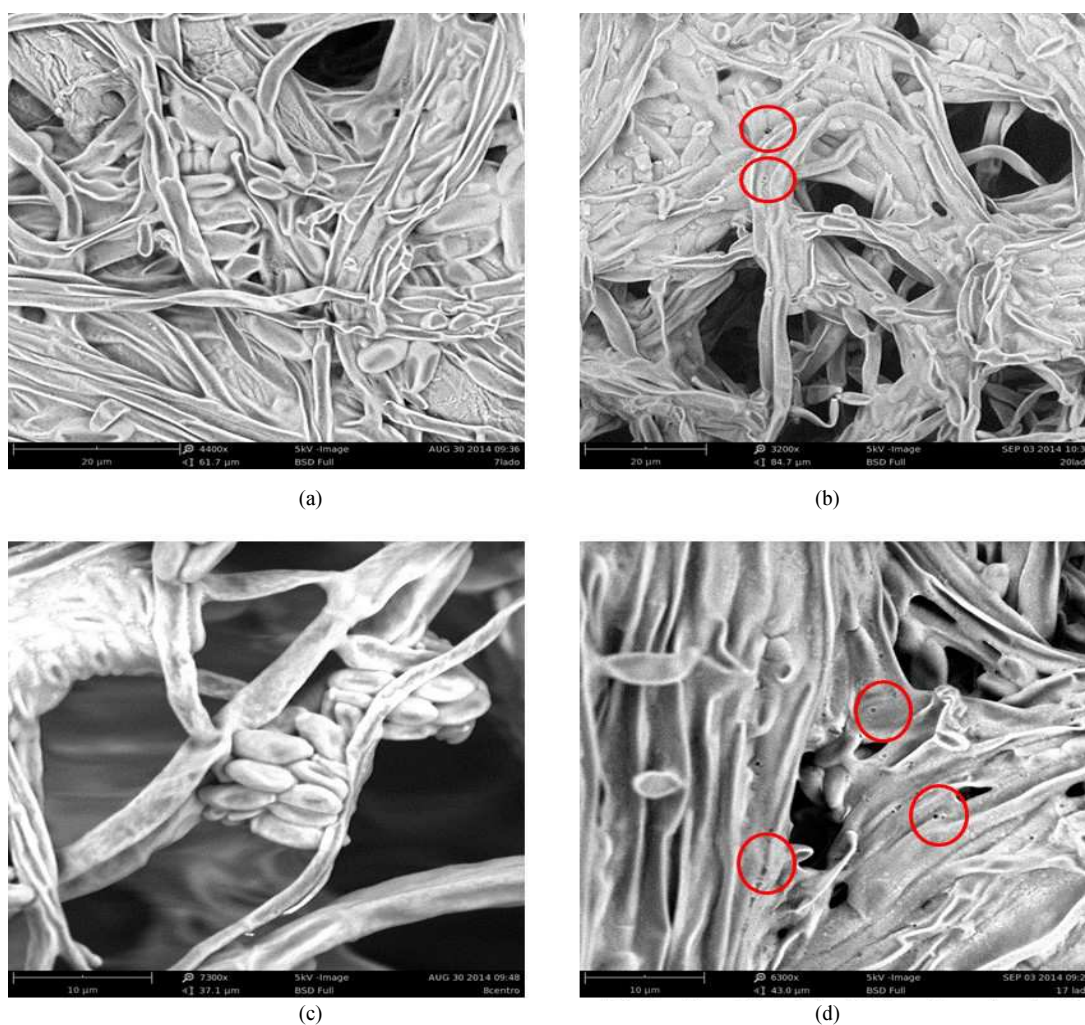


Fig. 8. Morphological Changes in *F. oxysporum* and *F. fujikoroii* during fungistatic activity tests with S1, S2 and S3 of *W. cibaria* (a) control of *F. oxysporum* (b) *W. cibaria* biomass (S3) obtained at the 8th hour of fermentation against *F. oxysporum* (c) control of *F. fujikoroii* (d) *W. cibaria* (S3) obtained at the 8th hour of fermentation against *F. fujikoroii*

Lactic Acid and Acetic Acid Production Kinetics, Substrate Consumption and Biomass Formation

Figure 6 shows the production kinetics of lactic acid, acetic acid and substrate consumption and Fig. 7 shows the production kinetics of biomass and the count of living cells, respectively.

Lactic acid concentration in MRS+glucose broth varied between 5.8 and 41.3 g/L, obtaining a considerable decrease in the production as of the 24th hour of fermentation. Acetic acid production was lower than the one obtained with the lactic acid, with a variable behavior; in which the maximum production of this metabolite was 0.013 g/L during the 4th hour of fermentation.

The consumption of sugars had a quick decrease as of the 4th hour of fermentation (95.63%) until the 24th hours (24.12%), with residual sugars of 12.9 g/L corresponding to 19.43% of the non-consumed substrate.

The biomass concentration curve is shown in Fig. 7, where a typical microbial growth curve is shown, without adaption phase and exponential growth until the 6th hour, stationary phase at the 13th hour and as of the 14th hour, cell death initiated.

In the cell viability figure it is possible to see an exponential growth until the 6th hour of fermentation, the stationary phase until the 12th hour and cell death as of the 24th hour, as shown in Fig. 7. This behavior is correlated to the production of biomass. In addition, the kinetic parameters of *W. cibaria* were measured: specific growth speed μ , glucose conversion (%), cell performance ($Y_{x/s}$) and performance in product ($Y_{p/s}$), obtaining the following values: 0.22 h⁻¹, 80.5%, 0.037 g/g and 0.69 g/g, respectively.

Microscopy Analysis

Figure 8 shows the morphological changes (7 days of incubation) of *F. oxysporum* and *F. fujikoroii* exposed to S1, S2 and S3 of *W. cibaria*. The SEM observation shows that the regular morphology of *F. oxysporum* and *F. fujikoroii* present hyphas with a tubular shape and soft cell walls, septate and conidia with central or curved depressions, as shown in Fig. 8a and c. After treatment, the fungi showed morphologies with damages in the cell walls of the hyphas and some tests showed porosities (see red circles in Fig. 8), milling, scarce conidia and with changes in their shape. Figure 8b shows that S3 (obtained at the 8th hour of fermentation) caused changes in the structure of *F. fujikoroii*. S1 and S2 at the 24th hour also affected the morphology of *Fusarium* (results not shown). Likewise, graph 2d shows that S3 (obtained at the 8th hour of fermentation) caused changes in the structure of *F. oxysporum*. This shows that the metabolites produced by *W. cibaria* not only affect the growth of fungi in Petri dishes, but also induce structural changes in fungi hyphas and conidia.

Discussion

Several researches have reported the antifungal abilities of LAB on fungal growth (Cizeikiene *et al.*, 2013). Our results indicate that the metabolite responsible for the highest fungistatic activity was produced during the stationary phase of the microbial growth, being then, secondary metabolites. Similar results were obtained with Ahmadova *et al.* (2013) in which antifungal activity was obtained for strain *L. curvatus* A61, isolated from Azerbaijani cheese against *Cladosporium* and *Fusarium* ssp; however, this same activity was reduced when supernatants of the LAB culture were used.

Authors have demonstrated that the antifungal activity is the result of a different nature compounds. Ryan *et al.* (2011); Ström *et al.* (2002), found that the antifungal power of bacteria occurs due to the production of phenylactic acid, cycle (l-Phe-l-Pro), cycle (l-Phe-trans-4-OH-l-Pro), propionic acid, coumaric acid, phenylpropanoic acid, 2-methylcinnamic acid, salicylic acid and sodium decanoate.

Other authors report bacteriocins and low molecular weight compounds with antifungal activity produced by *L. coryniformis* (Magnusson *et al.*, 2001). It has been reported that these substances do not cause any antagonistic effect when separated since the produced quantities in a fermentation process do not reach the minimum concentrations to inhibit fungus (Ryan *et al.*, 2009; Vermeulen *et al.*, 2006). Ahmadova *et al.* (2013) reported that the low antifungal activity of supernatant is because of the low concentration of the compounds responsible for such activity. This fungistatic activity, according to Schnürer and Magnusson (2005); Corsetti *et al.* (1998), is reinforced by the final products of the fermentation process, such as organic acids.

The high fungistatic activity for S1 and S3 may be explained by the communication mechanisms between bacteria, called Quorum Sensing (QS). This mechanism is responsible for the production of Gram positive bacteria's bacteriocins in which the genes expression and the protein synthesis depend on the cell concentration (Kuipers *et al.*, 1998). It means that the increase of the population may cause the synthesis of bacteriocins in S1 and S3.

The results may also be explained by the production of bacteriocins, *Fusarium* induced, a fungus that causes stress situations and competence for nutrients. These bacteriocins generated through stress are generally produced during the exponential growing phase or during the early stationary phase (Moshood *et al.*, 2012). Bacteriocins mode of action depends on factors such as strain, growing conditions (pH, temperature and nutrients), competence against other microorganisms, among other (Barboza-Corona *et al.*, 2007; Parada *et al.*, 2007; Diep *et al.*, 2000) and that means that their antagonistic activity is variable, unstable and inconsistent (Schillinger *et al.*, 1996).

It has been reported that bacteriocins act by forming pores through the cytoplasmic potential differential (Ghrai *et al.*, 2012). In some cases, it has a bacteriostatic effect and in other cases its effect is bactericide, in which cell lysis may occur or not (Cintas *et al.*, 2001). The absence or exhaustion of the bacteria may limit the production of the peptide that induces the production of the antimicrobial substance; which is indirectly correlated to cell growth. This occurs in case the inhibition is attributable to the QS mechanism.

The production of bacteriocins may be conducted through *in vitro* fermentation processes in optimal temperature conditions, pH, nutrients availability, among other (Leroy and De Vuyst, 2005); it means that any variation in such conditions may be determining in the synthesis of such compounds and therefore in the production of biomass (Verluyten *et al.*, 2004) since the production of bacteriocins depends on the cell growth of the bacteria (Leroy and De Vuyst, 1999).

Results for the lactic and acetic acids may be different depending on the LAB and the substrate. Salmerón *et al.* (2014) found that *L. plantarum*, *L. acidophilus* and *L. reuteri* in different substrates had lactic and acetic acid concentrations higher than 3 and 0.2 g/L, respectively. Zalán *et al.* (2010) reported that the profiles of the organic acids may change according to the fermentation substrate and the type of LAB used, even between strains of the same species. This indicates that the concentrations of lactic and acetic acids may increase if other carbon and nitrogen sources were used for the LABs.

The results for the organic acids are important for the analysis of the fungistatic activity of *W. cibaria*, since it has been reported that the production of metabolites from acid nature have an inhibitory effect against the growing of fungi (Wang *et al.*, 2013).

Our results indicate that MRS substrate supplemented with 40% glucose provides the necessary nutritional requirements for the growing of the LAB, which can be correlated to the high concentrations of lactic acid. However, it is necessary to evaluate other growth substrates that help increase the carbon source performance to obtain a lower quantity of residual sugars. Patel *et al.* (2013), in tests with *W. confusa* and *W. cibaria* glucose, xylose and xylooligosaccharides were used as growth substrates and it was found that the best results in the production of lactic and acetic acid were achieved using xylooligosaccharides in comparison to the growth obtained with glucose.

To conclude, a LAB taken from an animal niche, such as *W. cibaria*, has a great potential as a biocontrol agent for basal rot in yellow pitahaya, since the results indicated that S1 and S3 fractions obtained during the stationary growth phase of *W. cibaria* have an antagonistic effect against *F. oxysporum* and *F. fujikoroii*. It was evident that the fungistatic activity of *W. cibaria*

is performed both in a medium favoring the growth of the bacteria and in mediums favoring the growth of fungi. SEM analysis showed that the bacteria causes damages in the cell wall of hyphas, porosities and milling in hyphas, scarce conidia and changes in the conidia shape. The growth medium of microorganisms only affected the fungistatic activity of S1, S2 and S3 on *F. fujikoroii*, indicating that this type of fungus react in several ways before competence and stress situations.

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Author's Contributions

All authors of this research article have directly participated in the planning, execution and analysis of this study.

Ethics

The authors confirm that this work is original and has not been published elsewhere. The authors declare that they have no conflict of interest.

References

- Ahmadvova, A., S.D. Todorov, I. Hadji-Sfaxi, Y. Choiset and H. Rabesona *et al.*, 2013. Antimicrobial and antifungal activities of *Lactobacillus curvatus* strain isolated from homemade Azerbaijani cheese. *Anaerobe*, 20: 42-49. DOI: 10.1016/j.anaerobe.2013.01.003
- AOAC 934.01, 1990. Official methods of analysis of the Association of Official Analytical Chemists: Method. Print Official Methods of Analysis, Virginia, EE.UU.
- Baek, E., H. Kim, H. Choi, S. Yoon and J. Kim, 2012. Antifungal activity of *Leuconostoc citreum* and *Weissella confusa* in rice cakes. *J. Microbiol.*, 50: 842-848. DOI: 10.1007/s12275-012-2153-y
- Barboza-Corona, J.E., H. Vázquez-Acosta, D.K. Bideshi and R. Salcedo-Hernández, 2007. Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*. *Archives Microbiol.*, 187: 117-126. DOI: 10.1007/s00203-006-0178-5

- Cintas, L.M., M.P. Casaus, C. Herranz, I.F. Nes and P.E. Hernández, 2001. Review: Bacteriocins of lactic acid bacteria. *Food Sci. Technology Int.*, 7: 281-305. DOI: 10.1106/R8DE-P6HU-CLXP-5RYT
- Cizeikiene, D., G. Juodeikiene, A. Paskevicius and E. Bartkiene, 2013. Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control*, 31: 539-545. DOI: 10.1016/j.foodcont.2012.12.004
- Corsetti, A., M. Gobetti, J. Rossi and P. Damiani, 1998. Antimould activity of sourdough lactic acid bacteria: Identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. *Applied Microbiol. Biotechnol.*, 50: 253-256. DOI: 10.1007/s002530051285
- De Man, J.C., M. Rogosa and M.E. Sharpe, 1960. A medium for the cultivation of *Lactobacilli*. *J. Applied Microbiol.*, 23: 130-135. DOI: 10.1111/j.1365-2672.1960.tb00188.x
- Diep, D.B., L. Axelsson, C. Grefslis and I.F. Nes, 2000. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three component regulatory system. *Microbiology*, 146: 2155-2160. DOI: 10.1099/00221287-146-9-2155
- Dimkic, I., S. Zivkovic, T. Beric, Z. Ivanovic and V. Gavrilovic *et al.*, 2013. Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents for the control of phytopathogenic bacteria and fungi. *Biological Control*, 65: 312-321. DOI: 10.1016/j.biocontrol.2013.03.012
- Fei, W.C., Y.M. Wang, F.C. Chen, C.M. Lin and Y.H. Lee, 2004. *Plant Protection Manual*. In: Taiwan Agriculture Chemicals and Toxic Substances Research Institute, Wufeng, Taichung Press, China, pp: 825.
- Ghrai, T., N. Chafar and K. Hani, 2012. Bacteriocins: Recent Advances and Opportunities. In: *Progress in Food Preservation*, Bhat, R., A. Karim Alias and G. Paliyath (Eds.), WileyBlackwell, Oxford, UK, ISBN-10: 9781119962045, pp: 485.
- Kang, M.S., H.S. Na and J.S. Oh, 2005. Coaggregation ability of *Weissella cibaria* isolates with *Fusobacterium nucleatum* and their adhesiveness to epithelial cells. *FEMS Microbiol. Lett.*, 253: 323-329. DOI: 10.1016/j.femsle.2005.10.002
- Kuipers, O.P., P.G.G.A. De Ruyter, M. Kleerebezem and W.M. De Vos, 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnology*, 64: 15-21. DOI: 10.1016/S0168-1656(98)00100-X
- León, K., M. Domingo, P. Franco and J. León, 2006. Color measurement in L*a*b* units from RGB digital images. *Food Res. Int.*, 39: 1084-1091. DOI: 10.1016/j.foodres.2006.03.006
- Leroy, F. and L. De Vuyst, 1999. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the Antilisterial Bacteriocin Sakacin K. *Applied Environ. Microbiol.*, 65: 974-981.
- Leroy, F. and L. De Vuyst, 2005. Bacteriocin-Producing Strains in a Meat Environment. In: *Microbial Processes and Products*, Barredo, J.L. (Ed.), Humana Press, Totowa, NJ, pp: 369-380, ISBN-10: 978-1-59259-847-2
- Magnusson, J. and J. Schnürer, 2001. *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Applied Environ. Microbiol.*, 67: 1-5. DOI: 10.1128/AEM.67.1.1-5.2001
- Magnusson, J., K. Ström, S. Roos, J. Sjögren and J. Schnürer, 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol. Lett.*, 219: 129-135. DOI: 10.1016/S0378-1097(02)01207-7
- Malik, A., M. Radji, S. Kralj and L. Dijkhuizen, 2009. Screening of lactic acid bacteria from Indonesia reveals glucansucrase and fructansucrase genes in two different *Weissella confusa* strains from soya. *FEMS Microbiol. Lett.*, 300: 131-138. DOI: 10.1111/j.1574-6968.2009.01772.x
- Mauch, A., F. Dal Bello, A. Coffey and E.K. Arendt, 2010. The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. *Int. J. Food Microbiol.*, 141: 116-121. DOI: 10.1016/j.ijfoodmicro.2010.05.002
- Millar, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chem.*, 31: 426-428. DOI: 10.1021/ac60147a030
- Moshood, A.Y., A. Tengku Haziya and A.H. Tengku, 2012. Optimization of temperature and pH for the growth and bacteriocin production of *Enterococcus faecium*. B3L3. *IOSR J. Pharmacy*, 2: 49-59.
- Parada, J.L., C.C. Ricoy, A.P.M. Bianchi and C.R. Soccol, 2007. Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. *Brazilian Archives Biol. Technol.*, 50: 521-542. DOI: 10.1590/S1516-89132007000300018
- Patel, A., P. Falck, N. Shah, P. Immerzeel and P. Adlercreutz *et al.*, 2013. Evidence for xylooligosaccharide utilization in *Weissella* strains isolated from Indian fermented foods and vegetables. *FEMS Microbiol. Lett.*, 346: 20-28. DOI: 10.1111/1574-6968.12191
- Quintero, D.R., 1997. Fertilización nitrogenada en caña de azúcar. In: *El cultivo de la caña en la zona azucarera de Colombia*. Centro de Investigaciones de la Caña de Azúcar de Colombia-Cenicaña, (Eds.), CENICAÑA, Florida, Valle del Cauca, pp: 11-13.

- Ryan, L.A.M., E. Zannini, F. Dal Bello, A. Pawlowska and P. Koehler *et al.*, 2011. Lactobacillus amylovorus DSM 19280 as a novel food-grade antifungal agent for bakery products. *Int. J. Food Microbiol.*, 146: 276-283.
 DOI: 10.1016/j.ijfoodmicro.2011.02.036
- Ryan, L.A.M., F. Dal Bello, M. Czerny, P. Koehle and E.K. Arendt, 2009. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. *J. Agric. Food Chem.*, 57: 1060-1064.
 DOI: 10.1021/jf802578e
- Salmerón, I., K. Thomas and S.S. Pandiella, 2014. Effect of substrate composition and inoculum on the fermentation kinetics and flavour compound profiles of potentially non-dairy probiotic formulations. *LWT-Food Sci. Technol.*, 55: 240-247.
 DOI: 10.1016/j.lwt.2013.07.008
- Schillinger, U., R. Geisen and W.H. Holzapfel, 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends Food Sci. Technol.*, 7: 158-164.
 DOI: 10.1016/0924-2244(96)81256-8
- Schnürer, J. and J. Magnusson, 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci. Technol.*, 16: 70-78.
 DOI: 10.1016/j.tifs.2004.02.014
- Serna-Cock, L., L.F. Rubiano-Duque, N.B. Loaiza-Castillo and C.E. Enríquez-Valencia, 2012. Weissella confusa como un agente bioprotector en la inocuidad alimentaria contra patógenos Gram negativos. *Revista Alimentos Hoy*, 21: 102-114.
- Serna-Cock, L., L.J. Valencia-Hernández and R. Campos-Gaona, 2010. Kinetic of fermentation and antimicrobial activity of Weissella confusa against Staphylococcus aureus and Streptococcus agalactiae. *Revista de la Facultad de Ingeniería de la Universidad de Antioquia*, 55: 55-65.
- Soler, J.J., M. Martín-Vivaldi, M. Ruiz-Rodríguez, E. Valdivia and A.M. Martín-Platero *et al.*, 2008. Symbiotic association between hoopes and antibiotic producing bacteria that live in their uropygial gland. *Functional Ecol.*, 22: 864-871.
 DOI: 10.1111/j.1365-2435.2008.01448.x
- Ström, K., J. Sjögren, A. Broberg and J. Schnürer, 2002. Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-Phenyllactic Acid. *Applied Environ. Microbiol.*, 68: 4322-4327.
 DOI: 10.1128/AEM.68.9.4322-4327.2002
- Uzair, B., N. Ahmed, F.V. Mohammad and V.U. Ahmad, 2008. Detection, Isolation and partial characterization of antifungal compounds produced by Pseudomonas aeruginosa CMG1055. *J. Chem. Society Pak.*, 30: 649-653.
- Verluyten, J., W. Messens and L. De Vuyst, 2004. Sodium chloride reduces production of curvacin A, a bacteriocin produced by Lactobacillus curvatus strain LTH 1174, originating from fermented sausage. *Applied Environ. Microbiol.*, 70: 2271-2278.
 DOI: 10.1128/AEM.70.4.2271-2278.2004
- Vermeulen, N., M.G. Ganzle and R.F. Vogel, 2006. Influence of peptide supply and cosubstrates on phenylalanine metabolism of Lactobacillus sanfranciscensis DSM20451T and Lactobacillus plantarum TMW1.468. *J. Agric. Food Chem.*, 54: 3832-3839. DOI: 10.1021/jf052733e
- Wang, H., Y. Sun, C. Chen, Z. Sun and Y. Zhou *et al.*, 2013. Genome shuffling of Lactobacillus plantarum for improving antifungal activity. *Food Control*, 32: 341-347. DOI: 10.1016/j.foodcont.2012.12.020
- Zalán, Z., J. Hudáček, J. Stetina, J. Chumchalova and A. Halász, 2010. Production of organic acids by Lactobacillus strains in three different media. *Eur. Food Res. Technol.*, 230: 395-404.
 DOI: 10.1007/s00217-009-1179-9.