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ORIGINAL ARTICLE

Selection of lactic acid bacteria to promote an efficient silage fermentation capable of inhibiting the activity of *Aspergillus parasiticus* and *Fusarium graminearum* and mycotoxin production

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Abstract

Aims: To select lactic acid bacteria with potential silage inoculant properties. The bio-control activity against mycotoxicogenic fungi and the presence of antibiotics resistance gene were also evaluated.

Methods and Results: *Lactobacillus rhamnosus* RC007 and *Lactobacillus plantarum* RC009 were selected on the basis of growth rate and efficacy in reducing the pH of maize extract medium; therefore, they were evaluated for their bio-control ability against *Fusarium graminearum* and *Aspergillus parasiticus*. Studies on lag phase, growth rate and aflatoxin B1 (AFB1) and zearalenone (ZEA) production were carried out *in vitro* under different regimes of a_w (0·95 and 0·99); pH (4 and 6); temperature (25 and 37°C); and oxygen availability (normal and reduced). *Lactobacillus rhamnosus* RC007 was able to completely inhibit the *F. graminearum* growth at all assayed conditions, while *Lact. plantarum* RC009 only did it at pH 4. Both *Lactobacillus* strains were able to significantly reduce the *A. parasiticus* growth rate mainly at 0·99 a_w. A decrease in ZEA production was observed as result of *Lactobacillus* strains – *F. graminearum* interaction; however, the *A. parasiticus*- *Lact. plantarum* interaction resulted in an increased AFB1 production. *Lactobacillus rhamnosus* RC007 proved to have no genes for resistance to the tested antibiotics.

Conclusions: The ability of *Lact. rhamnosus* RC007 to rapidly drop the pH and to inhibit fungal growth and mycotoxin production and the absence of antibiotic resistance genes shows the potential of its application as inoculant and bio-control agent in animal feed.

Significance and Impact of the Study: This study demonstrated the importance of selecting bacteria for silage inoculants not only for the improvement of silage fermentation but also for their effects on mycotoxicogenic fungi and the resulting mycotoxin production due to the risk that they may involve.

Introduction

The fresh forage from cultures like maize, pulses, alfalfa and wheat can be preserved by ensilage. In many countries, the forage ensilages are very appreciated as animal food. The process is based on the fermentation of water-soluble carbohydrates by lactic acid bacteria (LAB) and air exclusion. The fermentation lowers the pH due to

lactic acid production that inhibits growth of many spoilage organisms (McDonald *et al.* 1991). Ensiling occurs naturally and is performed by the epiphytic flora of LAB present in the crop. In successful silage, the fermentation and consequent rapid decrease in pH value is the most important factor to avoid increasing of undesirable micro-organism.

Aerobic spoilage of ensilage is associated with oxygen penetration into the silage during storage or feeding. Thus, the presence of mycotoxins and mycotoxin-producing fungi in aerobically deteriorated silage forms a serious risk to the quality and safety of milk and to human and animal health (McDonald *et al.* 1991; Saarisalo *et al.* 2007).

Fungal spoilage of maize silage reduces the nutritional value and palatability of the feed, increases its allergenic potential and may result in mycotoxin contamination (Scudamore and Livesey 1998).

Mycotoxigenic fungi in silage are associated with animal health problems such as acute toxicoses, decreased productivity, fertility and increased disease susceptibility (CAST 2003). *Fusarium graminearum* is globally recognized as one of the most important fungal pathogens of grains and cereals, causing great agricultural losses (Franco *et al.* 2011). Zearalenone is a *Fusarium*-produced mycotoxin that has a chemical structure similar to oestrogen and can produce an oestrogenic response in animals. *Aspergillus parasiticus* is a post-harvest pathogen of several important food crops including maize, peanuts and several tree nut crops (Farr *et al.* 1989). Many strains of *A. parasiticus* belong to the major species of fungi-producing aflatoxins (Klich and Pitt 1988), which are known to be potent carcinogens and hepatotoxinogenic chemicals and represent a severe risk to animal and human health (van Egmond *et al.* 2007). Human health may also be affected because dust from contaminated silages has been implicated as a causal agent in organic dust toxic syndrome, a neurological and respiratory illness (Perry *et al.* 1998). Even with an effective fermentation step, the air can let into the system (e.g. during feeding) allowing the growth of aerobic spoilage organisms, such as filamentous fungi and yeasts (Woolford 1990). One way to overcome this problem and to make the ensiling process more effective could be adding LAB-inoculants with antifungal properties. Species and specific strains of LAB in commercial inoculants have been selected because they are able to improve silage fermentation. However, no studies have been designed to evaluate both, the antifungal and mycotoxin reduction properties in the same inoculant candidate strains.

The aims of the present work were as follows: (i) to screen native LABs capable of rapid growth and quickly reduction in the pH, (ii) to study their biocontrol activity against *A. parasiticus* and *F. graminearum* and (iii) to determine the resistance to antibiotics of veterinary medicine importance of the selected LAB strains. This study was conducted to the further selection of LAB for silage inoculants.

Materials and methods

Micro-organisms

The present work was performed on six LAB strains originally isolated from maize silage: *Lactobacillus casei* RC002 *Lact. casei* RC005; *Weisella paramesenteroides* RC006; *Lact. rhamnosus* RC007; *Lact. rhamnosus* RC008 and *Lact. plantarum* RC009. Strains were identified from both the fermentation pattern (API 50 CHL test) and the 16S rRNA gene sequence.

Aflatoxigenic *A. parasiticus* NRRL2999 was maintained at 4°C on slants of malt extract agar (MEA) and in 15% glycerol at −80°C. *Fusarium graminearum* Z3636 was maintained on V-8 juice agar slants.

Screening of lactic acid bacteria strains for silage inoculants

The candidate strains were assayed for growth rate and efficacy in reducing the pH of maize extract medium. The medium was prepared from fresh maize plants by extracting 1 kg of chopped plants (1–2 cm) in 5 l of water in a water bath for 2 h at 50°C, according to the methodology proposed by Saarisalo *et al.* (2007). For the screening assays, the maize extract medium was filtered (Whatman 40, Cambridge, UK) and sterilized (120°C, 15 min). Before use, the extract was supplemented with glucose (1%, w/v). Six batches of maize extract medium (300 ml each) were inoculated each with one LAB strain (10^6 CFU ml^{-1}). The inoculated batch cultures were incubated at 30°C. Samples from the growth medium were taken at regular intervals and analysed for pH and viable LAB counts (MRS Agar; Oxoid [Cambridge, UK]; 3 days, 30°C, 5–10% CO_2). The pH and viable cells were determined every 4 h until 12 h and then at 24 h. The growth was monitored until the pH of the medium had declined below pH 4. Based on the obtained results, two LAB strains (*Lact. rhamnosus* RC007 and *Lact. plantarum* RC009) were selected for the following experiments.

Assay of LAB – toxicogenic mould interactions

The basic medium used in this study was silage agar (containing 40 g extract of silage and 20 g agar in 1 l of distilled water). The water activity was adjusted at 0·95 or 0·99 by the addition of known amounts of the nonionic solute glycerol (Marin *et al.* 1995). The basic medium

with different levels of a_w was adjusted to pH 4·0 or pH 6·0 by adding the necessary quantity of HCl or NaOH. The medium was autoclaved for 20 min at 121°C. These conditions were selected to mimic the environmental conditions that can be found in the silage ecosystem.

Effect of the LAB-toxicogenic fungi interaction on growth and mycotoxin production

Growth rate and lag phase studies

Growth studies were carried out as previously described (Cavaglieri *et al.* 2004) with some modifications. For this assay, one millilitre (1 ml) of LAB strains inocula (1×10^6 CFU ml^{-1}) was inoculated in Petri plates and 20 ml of silage extract medium at different a_w and pH values was added. After solidification, plates were inoculated in the centre with spores of *A. parasiticus* or *F. graminearum* suspended in semisolid agar. Petri plates of the same a_w values were sealed in polyethylene bags. The plates were incubated at 25°C or 37°C (for LAB-*A. parasiticus* interaction) or at 25°C or 30°C (for LAB-*F. graminearum* interaction) under normal and reduced oxygen pressure conditions (microaerophilia) during 196 h. Control groups consisted of fungi growing alone, without LAB strain. The growing radius of the cultures containing both micro-organisms was compared with the control cultures. For each colony, two radii, measured at right angles to one another, were averaged to find the mean radius for that colony. All colony radii were determined using three replicates for each test fungus. The radial growth rate (mm per day) was subsequently calculated by linear regression of the linear phase for growth. The time at which the line intercepted the *x*-axis was used to calculate the lag phase in relation to LAB strain, water activity, temperature and oxygen pressure. All experiments were carried out with at least three separate replicate Petri plates per treatment.

Mycotoxin analysis

After growth was evaluated, all medium was taken from each plate of each treatment, transferred to an Erlenmeyer flask and 20 ml of chloroform was then added. The mixture was agitated at 200 rpm for 30 min. The chloroform extract was dried under nitrogen gas. The residue was redissolved in 1 ml of chloroform for AFB1 and ZEA quantification by high-performance liquid chromatography (HPLC). The HPLC with fluorescence detection (λexc 330 nm; λem 460 nm for AFB1 and λexc 280 nm; λem 460 nm for ZEA) consisted in a C18 column (Supelcosil LC-ABZ, Supelco [Sigma-Aldrich, Buenos Aires, Argentina]; 150 × 4·6 mm, 5-μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4·6 mm, 5-μm particle size).

Aflatoxin B1 was determined according to Trucksess *et al.* (1994). An aliquot (200 μl) was derivatized with 700 μl of trifluoroacetic acid/acetic acid/water (20 : 10 : 70). The mobile phase (water:acetonitrile:methanol, 4 : 1 : 1) was pumped at 1·5 ml min^{-1}. The injection volume was 100 μl, and the retention time was around 5 min.

Zearalenone was determined according to Cerveró *et al.* (2007). The mobile phase (water:methanol, 30 : 70) was pumped at 1 ml min^{-1}. The injection volume was 100 μl, and the retention time was around 6·5 min.

Standard curves were constructed with different levels of toxins. The toxins were quantified by correlating peak height of sample extracts and those of standard curves. The detection limit of the technique was 1 ng g^{-1}.

Antimicrobial resistance of *Lactobacillus rhamnosus* RC007

The presence of *vanA, vanB, mecA, blaZ and ermB* resistance genes was evaluated.

DNA was extracted from *Lact. rhamnosus* RC007 using an adaptation of the methodology described by Harju *et al.* (2004). Pellets were resuspended in 600 μl of extraction buffer (200 mmol l^{-1} Tris-HCl pH 8·0, 25 mmol l^{-1} EDTA pH 8·0, 25 mmol l^{-1} NaCl, 1% SDS) and incubated at 65°C for 30 min. Deproteinization was performed twice using equal volume of chloroform: isoamyl alcohol (24 : 1). For precipitation, it was added twice the volume of 100% cooled ethanol followed by incubation at −20°C for 2 h. Microtubes were centrifuged at 14 549 ***g*** for 30 min, and pellets were washed with 500 μl of dry 70% ethanol at room temperature and resuspended in 30 μl of Tris-EDTA (10 mmol l^{-1} Tris-HCl pH8·0, 1 mmol l^{-1} EDTA pH 8·0).

DNA concentration was evaluated by 0·8% agarose gel electrophoresis stained with SYBR® Safe DNA Gel Stain (Invitrogen, Buenos Aires, Argentina).

Polymerase chain reaction

The reaction mixtures contained 50 ng of total DNA of the analysed strain, 0,3 μmol l^{-1} of both forward and reverse primers, 200 μmol l^{-1} dNTPs, 1× Taq polymerase buffer and 1 U of Taq polymerase (Fermentas, Sao Paulo, Brasil) in a total volume of 20 μl. The primer sequences used in this assay are shown in Table 1.

All PCR reactions were performed in a MyCyclerTM thermal cycler (Bio-Rad, Sao Paulo, Brasil), by preheating at 94°C for 5 min followed by 40 cycles consisting of 30 s of denaturation at 94°C, 30 s of primers annealing at 60°C, 1 min of polymerase reaction at 72°C; and 5 min of final elongation at 72°C. Positive and negative controls were included. PCR products were separated by

Table 1 Primers used in PCR analysis to test the presence of the genes responsible for resistance to antimicrobial preparations

Gene	Primer nucleotide sequence, 5′–3′	Expected fragment length (bp)
mecA	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	512
blaZ	TACAACTGTAATATCGGAGGG CATTACACTCTTGGCGGTTTC	600–700
vanA	CTACAATGCGGCCGTTA GGCAAAACGACAATTGC	730
vanB	TCCACCCGATTCGTTC ACGGAATGGGAAGCCGA	630
ermB	GAAAAGGTACTCAAC AGTAACGGTACTTAAATTGTTTAC	400

electrophoresis at 100 V in horizontal 1·5% agarose gel containing 0·1% of SYBR in Tris-acetate buffer. To assess the DNA fragment size, 100 pb GeneRuler™ DNA (Fermentas) was used as a standard DNA marker.

Statistical analyses

Multifactor ANOVA and *post hoc* analysis of factors with more than two levels (Fisher's protected LSD test) were applied to determine the influence of LAB strains on *A. parasiticus* and *F. graminearum* growth and on mycotoxin production data. A 95% confidence level was used to assess influence of individual and interacting treatments (Quinn and Keough 2002). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

Results

Growth rate and pH drop in silage extract medium

All the studied strains proliferated rapidly in the silage extract medium and showed similar growth rate after 12 h (Table 2). *Lactobacillus rhamnosus* RC007 and *Lact. plantarum* RC009 were the most efficient in reducing pH ($P < 0·05$); both strains were able to achieve pH 4 or less after 12 h of fermentation.

According to these results, *Lact. rhamnosus* RC007 and *Lact. plantarum* RC009 were selected for the further studies.

Effect of *Lactobacillus rhamnosus* RC007 and *Lactobacillus plantarum* RC009 on *Fusarium graminearum* and *Aspergillus parasiticus* growth rate

Statistical analyses of *Lactobacillus* strains (S), pH, temperature (T) oxygen availability (PO), water activity (a_w) on *F. graminearum* growth rate showed that the five-way interactions were statistically significant ($P < 0·0001$). *Fusarium graminearum* growing alone (control group) showed a higher growth rate at pH 6, 30°C and 0·99 a_w, whereas fungal growth was not observed at pH 4, 25°C and 0·95 a_w, independently of the oxygen availability condition (Table 3). *Lactobacillus rhamnosus* RC007 was able to completely inhibit the *F. graminearum* growth at all assayed conditions ($P < 0·05$), while *Lact. plantarum* RC009 only did it at pH 4. At pH 6, *Lact. plantarum* RC009 was able to reduce fungal growth at most of the interacting conditions.

Table 2 Growth strains and drop in pH after 12 and 24 h

	Drop in pH		Log CFU ml^{-1}	
Strain	12 h	24 h	12 h	24 h
RC005	1·14 ± 0·07 b	1·93 ± 0·5 a	8·50 ± 0·02	9·6 ± 0·01
RC006	1·16 ± 0·06 b	1·86 ± 0·23 a	8·04 ± 0·01	9·6 ± 0·01
RC007	1·65 ± 0·22 d	3·77 ± 0·6 b	8·04 ± 0·01	10·4 ± 0·01
RC008	0·84 ± 0·04 a	1·86 ± 0·55 a	7·80 ± 0·02	9·5 ± 0·01
RC009	1·38 ± 0·02 c	2·10 ± 0·15 a	7·70 ± 0·1	9·4 ± 0·01
RC010	1·24 ± 0·01 b,c	2·10 ± 0·25 a	7·30 ± 0·15	9·5 ± 0·01

Six batches of maize extract medium were inoculated each with one LAB strain (10^6 CFU ml^{-1}). The inoculated batch cultures were incubated at 30°C. Samples from the growth medium were taken at regular intervals and analysed for pH and viable LAB counts. Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

Statistical analyses of S, pH, T, PO, a_w on *A. parasiticus* growth rate showed that five-way interactions were statistically significant ($P < 0·0001$). *Aspergillus parasiticus* growing alone showed the higher growth rate at pH 4, 37°C, reduced PO and 0·99 a_w (1·31 ± 0·05) (Table 4). While no growth was observed at pH 4, 37°C, reduced PO and 0·95 a_w.

Both *Lactobacillus* strains were able to significantly reduce the *A. parasiticus* growth rate mainly at 0·99 a_w ($P < 0·05$). Under certain interacting conditions, *Lact. rhamnosus* RC007 was more efficient than *Lact. plantarum* RC009 to reduce *A. parasiticus* growth rate.

Effect of *Lactobacillus* strains on *Fusarium graminearum* and *Aspergillus parasiticus* lag phase

Statistical analyses of S, pH, T, PO, a_w on lag phase of *F. graminearum* showed that five-way interactions were statistically significant ($P < 0·0001$). Mean lag phases of *F. graminearum* under different interacting environmental conditions are shown in Table 5. The lag phase in the control (*F. graminearum* growing alone) ranged between 45·06 and 196 h at pH 4, and between 11·92 and 99·51 h

Table 3 Effect of *Lactobacillus strains* on *Fusarium graminearum* growth rate under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions

Growth condition				*F. graminearum* growth rate (mm h^{-1})		
				Media ± SD		
pH	T°	P°O_2	a_w	Control*	Interaction 1†	Interaction 2‡
4	25	Normal	0·99	0·08 ± 0·01 h,j	0·0 k	0·0 k
			0·95	0·0 k	0·0 k	0·0 k
		Reduced	0·99	0·10 ± 0·01 g,h,i,j	0·0 k	0·0 k
			0·95	0·0 k	0·0 k	0·0 k
	30	Normal	0·99	0·30 ± 0·006 b	0·0 k	0·0 k
			0·95	0·06 ± 0·01 j	0·0 k	0·0 k
		Reduced	0·99	0·31 ± 0·01 b	0·0 k	0·0 k
			0·95	0·14 ± 0·017 d,e,f	0·0 k	0·0 k
6	25	Normal	0·99	0·11 ± 0·01 g,h,i	0·0 k	0·09 ± 0·001 h,i,j
			0·95	0·12 ± 0·001 e,f,g,h	0·0 k	0·0 k
		Reduced	0·99	0·15 ± 0·01 d,e	0·0 k	0·11 ± 0·01 f,g,h,i
			0·95	0·12 ± 0·003 e,f,g	0·0 k	0·0 k
	30	Normal	0·99	0·43 ± 0·025 a	0·0 k	0·17 ± 0·1 d
			0·95	0·11 ± 0·01 f,g,h,i	0·0 k	0·12 ± 0·01 e,f,g,h,i
		Reduced	0·99	0·43 ± 0·005 a	0·0 k	0·27 ± 0·015 B
			0·95	0·20 ± 0·015 c	0·0 k	0·0 k

*Control: *F. graminearum* growing alone.
†Interaction 1: *F. graminearum* – *Lact. rhamnosus* RC007.
‡Interaction 2: *F. graminearum* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

Table 4 Effect of *Lactobacillus strains* on *Aspergillus parasiticus* growth rate under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions

Growth conditions				*A. parasiticus* growth rate (mm h^{-1})		
				Media ± SD		
pH	T°	P°O_2	a_w	Control*	Interaction 1†	Interaction 2‡
4	25	Normal	0·99	0·41 ± 0·01 b	0·18 ± 0·001 c	0·46 ± 0·02 b
			0·95	0·30 ± 0·04 c	0·29 ± 0·01 c	0·37 ± 0·03 b
		Reduced	0·99	0·39 ± 0·001 b	0·20 ± 0·01 c	0·26 ± 0·11 c
			0·95	0·28 ± 0·001 c	0·30 ± 0·01 c	0·26 ± 0·01 c
	37	Normal	0·99	0·20 ± 0·01 c	0·0 ± 0·0 d	0·03 ± 0·01 d
			0·95	0·15 ± 0·04 c	0·17 ± 0·02 c	0·06 ± 0·04 d
		Reduced	0·99	1·31 ± 0·05 a	0·0 ± 0·0 d	0·08 ± 0·02 d
			0·95	0·0 ± 0·0 d	0·0 ± 0·0 d	0·0 ± 0·0 d
6	25	Normal	0·99	0·39 ± 0·1 b	0·11 ± 0·03 d	0·22 ± 0·04 c
			0·95	0·30 ± 0·13 c	0·22 ± 0·2 c	0·32 ± 0·02 c
		Reduced	0·99	0·44 ± 0·1 b	0·26 ± 0·14 c	0·35 ± 0·04 b
			0·95	0·20 ± 0·05 c	0·28 ± 0·03 c	0·28 ± 0·02 c
	37	Normal	0·99	0·21 ± 0·01 c	0·06 ± 0·1 d	0·08 ± 0·01 d
			0·95	0·10 ± 0·03 d	0·12 ± 0·01 d	0·12 ± 0·01 d
		Reduced	0·99	0·03 ± 0·01 d	0·0 ± 0·0 d	0·0 ± 0·0 d
			0·95	0·02 ± 0·01 d	0·17 ± 0·02 c	0·18 ± 0·01 c

*Control: *A. parasiticus* growing alone.
†Interaction 1: *A. parasiticus* – *Lact. rhamnosus* RC007.
‡Interaction 2: *A. parasiticus* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

at pH 6. Mean lag phase of 196 h indicates that no fungal growth was observed at the end of the incubation period. The lag phases were increased when a_w decreased (0·95 a_w). The *F. graminearum-Lact. rhamnosus* RC007 interaction resulted in a completely inhibition of *F. graminearum* growth, so the mean lag phase was 196 h at all the assayed conditions. The same results were observed in the *F. graminearum-Lact. plantarum* RC009 interaction at pH 4. At pH 6, mean lag phase ranged between 28·31 and 196 h.

Statistical analyses of S, pH, T, PO, a_w on *A. parasiticus* lag phase showed that five-way interactions were statistically significant ($P < 0·0001$). Mean lag phases of *A. parasiticus* under different interacting environmental conditions are shown in Table 6. The incubation period for *A. parasiticus-Lactobacillus* strains was 124 h; therefore, mean lag phase of 124 h indicates that no fungal growth was observed at the end of the incubation period. The lag phase for *A. parasiticus* growing alone was longer at pH 6, 37°C, reduced PO and 0·95 a_w (87 h), whereas the shorter lag phase was observed at pH 4, 37°C, reduced PO and 0·99 a_w (0·72 h); these results are consistent with those obtained for the highest and the smallest growth rate, respectively. A prolonged lag phase was observed in the presence of *Lact. rhamnosus* RC007 at almost all the interacting conditions. In some cases although there was not any inhibition on growth rate, there was influence on the lag phase (Table 4 and 6). On the contrary, *Lact. plantarum* RC009 had only limited influence on the lag phase of *A. parasiticus*.

Effect of *Lactobacillus* strains on aflatoxin B1 and zearalenone production

Statistical analyses of S, T, PO, a_w on ZEA production showed that four-way interactions were statistically significant ($P < 0·0001$). Zearalenone production was only analysed at pH 6, because no growth was observed at pH 4 with both *Lactobacillus* strains.

Zearalenone production by *F. graminearum* at different growth conditions is shown in Table 7. The higher ZEA production by *F. graminearum* growing alone was observed at 30°C, normal PO and 0·95 a_w (21·38 ng ml^{-1}). A decrease in ZEA production ($P < 0·05$) was observed in *F. graminearum-Lact. plantarum* RC009 at all the interacting conditions assayed.

Statistical analyses of S, pH, T, PO, a_w on AFB1 production showed that five-way interactions were statistically significant ($P < 0·0001$).

Aflatoxin B1 production by *A. parasiticus* at different growth conditions is shown in Table 8. *Aspergillus*

Table 5 Effect of *Lactobacillus strains* on *Fusarium graminearum* lag phase under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions

Growth conditions				*F. graminearum* lag phase (h)		
				Media ± SD		
pH	T°	P°O_2	a_w	Control*	Interaction 1†	Interaction 2‡
4	25	Normal	0·99	45·1 ± 3·0 d,e,f,g,h,i	196 ± 0·0 a	196 ± 0·0 a
			0·95	196·0 ± 0·1 a	196 ± 0·0 a	196 ± 0·0 a
		Reduced	0·99	63·4 ± 1·5 c,d,e	196 ± 0·0 a	196 ± 0·0 a
			0·95	196 ± 0·0 a	196 ± 0·0 a	196 ± 0·0 a
	30	Normal	0·99	61·4 ± 5 c,d,e,f	196 ± 0·0 a	196 ± 0·0 a
			0·95	127·8 ± 10 b	196 ± 0·0 a	196 ± 0·0 a
		Reduced	0·99	56·7 ± 5 d,e,f,g,h	196 ± 0·0 a	196 ± 0·0 a
			0·95	102·8 ± 10 b	196 ± 0·0 a	196 ± 0·0 a
6	25	Normal	0·99	11·9 ± 3 i	196 ± 0·0 a	28·3 ± 9 e,f,g,h,i
			0·95	48·8 ± 5 d,e,f,g,h	196 ± 0·0 a	196 ± 0·0 a
		Reduced	0·99	23·1 ± 1·5 h,i	196 ± 0·0 a	63·9 ± 5 c,d
			0·95	60·6 ± 5 d,e,f,g	196 ± 0·0 a	196 ± 0·0 a
	30	Normal	0·99	26·8 ± 3 f,g,h,i	196 ± 0·0 a	118·2 ± 30 b
			0·95	99·5 ± 8 b	196 ± 0·0 a	96·8 ± 15 b,c
		Reduced	0·99	25·6 ± 5 g,h,i	196 ± 0·0 a	196 ± 0·0 a
			0·95	47·1 ± 3 d,e,f,g,h,i	196 ± 0·0 a	196 ± 0·0 a

*Control: *F. graminearum* growing alone.

†Interaction 1: *F. graminearum* – *Lact. rhamnosus* RC007.

‡Interaction 2: *F. graminearum* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

Table 6 Effect of *Lactobacillus strains* on *Aspergillus parasiticus* lag phase under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions

Growth conditions				*A. parasiticus* lag phase (h)		
				Media ± SD		
pH	T°	P°O_2	a_w	Control*	Interaction 1†	Interaction 2‡
4	25	Normal	0·99	36·7 ± 0·4 c	68·3 ± 0·4 b	63·9 ± 0·01 b
			0·95	55·7 ± 10 c	45·8 ± 2·3 c	55·8 ± 8·2 c
		Reduced	0·99	31·1 ± 0·7 c	70·2 ± 1·1 b	36·1 ± 15 c
			0·95	51·1 ± 0·4 c	50·7 ± 0·6 c	50·3 ± 1·6 c
	37	Normal	0·99	53·9 ± 3 c	124 ± 0·9 a	103·9 ± 17 a
			0·95	46·8 ± 7·6 c	69·7 ± 8 b	40·2 ± 8·6 c
		Reduced	0·99	0·7 ± 0·2 d	124 ± 0·0 a	67·1 ± 8·5 b
			0·95	124 ± 0·5 a	124 ± 0·0 a	124 ± 0·0 a
6	25	Normal	0·99	52·8 ± 9 c	95·2 ± 10 b	47·1 ± 2·3 c
			0·95	56·4 ± 9·2 c	106·2 ± 30 a	47·1 ± 2·1 c
		Reduced	0·99	40·5 ± 8·6 c	19·7 ± 9 d	29·6 ± 6 c
			0·95	46·5 ± 3·7 c	49·3 ± 3·5 c	57·1 ± 7·3 c
	37	Normal	0·99	69·5 ± 8·7 b	106·8 ± 29 a	70·4 ± 4·6 b
			0·95	18·4 ± 5·8 d	43·8 ± 10 c	49·8 ± 5·3 c
		Reduced	0·99	50·7 ± 2·1 c	124 ± 0·5 a	124 ± 0·5 a
			0·95	87 ± 1·2 b	86·5 ± 0·9 b	86·2 ± 0·3 b

*Control: *A. parasiticus* growing alone.
†Interaction 1: *A. parasiticus* – *Lact. rhamnosus* RC007.
‡Interaction 2: *A. parasiticus* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

Table 7 Effect of *Lactobacillus plantarum* RC009 on ZEA production under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions

Growth condition				Zearalenone production (ηg ml^{-1})	
				Media ± SD	
pH	T°	P°O_2	a_w	Control*	Interaction 1†
6	25	Normal	0·99	2·86 ± 0·01 d	2·35 ± 0·2 c
			0·95	6·60 ± 0·8 b	0·0 ± 00 f
		Reduced	0·99	0·14 ± 0·01 f	0·03 ± 0·001 f
			0·95	3·97 ± 0·5 c	0·0 ± 00 f
	30	Normal	0·99	1·37 ± 0·01 e	1·20 ± 0·01 e
			0·95	21·38 ± 1·5 a	2·35 ± 0·1 d
		Reduced	0·99	4·47 ± 0·01 c	0·0 ± 00 f
			0·95	1·24 ± 0·01 e	0·0 ± 00 f

*Control: *Fusarium graminearum* growing alone,
†Interaction 1: *F. graminearum* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

parasiticus growing alone was able to *in vitro* produce great amounts of AFB1, except at pH 4, 37°C, where AFB1 production was little or no production was observed. A significant decrease in AFB1 levels in comparison with the control ($P < 0·05$) was observed with *Lact. rhamnosus* RC007 interaction at almost all the interacting conditions assayed. The reduction percentages ranged between 53 and 95%. On the contrary, the *A. parasiticus*- *Lact. plantarum* RC009 interaction resulted in an increased AFB1 production, compared with the control (*A. parasiticus* growing alone) at almost all the interacting conditions assayed.

Antibiotic resistance of *Lactobacillus rhamnosus* RC007

The above results demonstrated that *Lactobacillus rhamnosus* RC007 was more effective bio-control agent than *Lact. rhamnosus* RC009; thus, antibiotic resistance assay was carried out only with this strain.

The presence of resistance gene of veterinary importance antibiotics was determined by PCR, and *Lact. rhamnosus* RC007 proved to possess none of the tested genes (Fig. 1).

Discussion

The present work was designed to select LAB strains with potential as an inoculant added to herbage at ensiling. Moreover, the bio-control activity and resistance gene towards antibiotics of veterinary importance were also evaluated. A range of selection criteria has been suggested for an ideal silage inoculant such as rapid growth rate and a consequent rapid decrease in pH value (Saarisalo

Table 8 Effect of *Lactobacillus strains* on AFB1 production under interacting pH, temperature (T°), oxygen availability ($P°O_2$) and water activity (a_w) conditions

Growth conditions				AFB1 production (Log_{10} ppb)		
				Media ± SD		
pH	T°	$P°O_2$	a_w	Control*	Interaction 1†	Interaction 2‡
4	25	Normal	0·99	2·33 ± 0·1 d	0·98 ± 0·01 i	3·51 ± 0·1 a
			0·95	1·52 ± 0·1 g	1·55 ± 0·1 g	2·29 ± 0·1 d
		Reduced	0·99	2·16 ± 0·1 e	0·61 ± 0·01 j	3·60 ± 0·5 a
			0·95	0·66 ± 0·01 j	0·31 ± 0·01 k	1·27 ± 0·01 h
	37	Normal	0·99	0·05 ± 0·01 l	0·0 ± 0·0 l	0·0 ± 0·0 l
			0·95	0·64 ± 0·01 j	0·68 ± 0·01 j	242·8 ± 0·5 d
		Reduced	0·99	0·05 ± 0·01 l	0·0 ± 0·0 l	0·0 ± 0·0 l
			0·95	0·0 ± 0·0 l	0·0 ± 0·0 l	0·0 ± 0·0 l
6	25	Normal	0·99	2·61 ± 0·1 c	1·09 ± 0·01 i	3·43 ± 0·3 a
			0·95	1·95 ± 0·1 f	0·60 ± 0·01 j	1·21 ± 0·1 h
		Reduced	0·99	2·15 ± 0·1 e	1·04 ± 0·01 i	2·98 ± 0·1 b
			0·95	1·27 ± 0·1 h,g	1·08 ± 0·05 i	1·38 ± 0·1 h
	37	Normal	0·99	0·04 ± 0·01 l	0·0 ± 0·0 l	1·40 ± 0·5 h
			0·95	2·25 ± 0·3 d	0·11 ± 0·01 l	0·13 ± 0·01 l
		Reduced	0·99	0·06 ± 0·01 l	0·0 ± 0·0 l	0·0 ± 0·0 l
			0·95	0·26 ± 0·01 k	0·08 ± 0·01 l	0·22 ± 0·01 k

*Control: *Aspergillus parasiticus* growing alone.

†Interaction 1: *Aspergillus parasiticus* – *Lact. rhamnosus* RC007.

‡Interaction 2: *Aspergillus parasiticus* – *Lact. plantarum* RC009, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

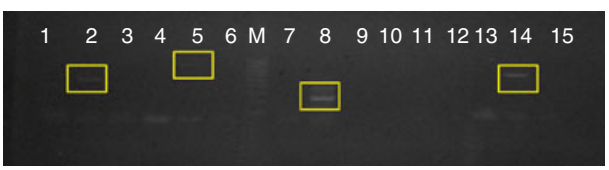

Figure 1 Electrophoresis of PCR product on 1·5% agarose gel. Primers mecA (lane 1–3), blaZ (lane 4–6), vanA (line 7–9), van B (lane 10–12), ermB (lane 13–15). Lane 1, 4, 7, 10 and 13: Blank; Lane 2, 5, 8, 11 and 14: Positive control; Lane 3, 6, 9, 12 and 15: *Lactobacillus rhamnosus* RC007.

et al. 2007). Among the six LAB strains assayed, *Lact. rhamnosus* RC007 and *Lact. plantarum* RC009 showed the fastest growth and a rapid drop in pH after 12 h as a result of lactic acid production. In the selection of LAB for silage inoculants, it would be of interest if such an inoculant produce antifungal metabolites during the fermentation and prevent the reactivation of fungal micro-organisms during feedout.

In the present work, the bio-control activity of LAB strains was evaluated against two of the most prevalent mycotoxicogenic fungi present in silage reported previously from the central region of Argentina (González Pereyra *et al.* 2008).

The bio-control activity of *Lact. rhamnosus* RC007 and *Lact. plantarum* RC009 against *A. parasiticus* and *F. graminearum* was determined under different environmental conditions, according to those found in silage ecosystem. Both LAB strains were able to *in vitro* significantly reduce or inhibit *F. graminearum* growth. Similarly, other authors have reported that *F. graminearum* strains were inhibited by commercial LAB cultures and by different LAB strains (Lavermicocca *et al.* 2000; Franco *et al.* 2011). In the LAB strains -*A. parasiticus* interactions, LAB strains were less effective as antagonistic agents, reducing fungal growth rate only at certain interacting conditions, mainly at the higher water activity tested. Other authors observed similar results, because three *Lactobacillus acidophilus* strains and two *Bifidobacterium* strains were not effective in preventing the growth of *A. parasiticus* NRRL2999 (Kabak and Var 2004).

It has been suggested that the inhibitory activity of LAB may result from the production of organic acids (in particular, lactic, propionic and acetic acids), carbon dioxide, ethanol, hydrogen peroxide, diacetyl, competitive growth; decrease in the pH caused by acid production, proteinaceous compounds (Magnusson and Schnurer 2001) low-molecular weight compounds (phenyllactic acid, reuterin, cyclic dipeptides, benzoic acid, hydroxylated fatty acids, methylhydantoin and mevalonolactone) (Niku-Paavola *et al.* 1999; Schnurer and Magnusson 2005) and bacteriocin-like substances (Okkers *et al.*

1999). Therefore, the antifungal activity of LAB is a complex phenomenon that has not been fully elucidated because it is related to a number of factors including the synergistic effects among antagonistic compounds (Okkers *et al.* 1999; Laitila *et al.* 2002; Yang and Clausen 2004; Hassan and Bullerman 2008; Voulgari *et al.* 2010; Yang and Chang 2010).

When a bio-control agent is evaluated to be used against to mycotoxicogenic fungi, it is important to determine the mycotoxin production. Because mycotoxins are secondary metabolites that can be produced in response to a stress factor, growth reduction caused by the presence of LAB strains could influence mycotoxin production (Boyacioglu *et al.* 1992; Gareis and Wolff 2000). In the LAB strains – *F. graminearum* interaction, ZEA production was not increased, and on the contrary, it was always reduced in the presence of LAB strains. However, the *A. parasiticus* – *Lact. plantarum* RC009 interaction resulted in a higher AFB1 production, at almost all the different interacting assayed conditions. Aflatoxins are potent hepatotoxins and carcinogens, and their presence in feeds is a health hazard for animals and a potential public health concern due to the subsequent excretion of M1 in dairy milk (Diaz *et al.* 2004; Alonso *et al.* 2011).

Many authors have reported the antifungal activity of different LAB strains, but in these studies, the mycotoxin production resulting of this interaction is absent (Laitila *et al.* 2002; Strom *et al.* 2002; Saarisalo *et al.* 2007) or the mycotoxin reduction is evaluated in artificially contaminated liquid media (Franco *et al.* 2011).

The results showed in Table 8 demonstrated an increase in AFB1 production as a result of *Lact. plantarum* RC009-*A. parasiticus* interaction. Therefore, this LAB strain was eliminated as possible silage inoculant. An increase in mycotoxin production caused by a bio-control agent is expected. The challenge is to find agents that are not only able to reduce the fungal growth but at the same time are able to reduce the mycotoxin levels produced as a result of fungi-LAB interaction. In the present work, this requirement was met only by *Lact. rhamnosus* RC007. Future studies should determine the mechanism of mycotoxin reduction involved such as biodegradation or influence at bio-synthesis level.

By the above explained, *Lact. rhamnosus* RC007 constitutes a promissory inoculant strain.

The 'generally recognized as safe' (GRAS) status of LAB offers the potential to use these bacteria in commercial applications as biological control agents in foods or feeds to prevent mould growth, improves the shelf life of fermented products and reduces the health hazards associated with mycotoxins. Inoculated silages sometimes improve cattle performance, possibly because of probiotic effects of LAB silage inoculants (Weinberg *et al.* 2004). However, it is well known that Lactobacilli harbour natural resistances against antibiotics and chemotherapeutics (Tynkkynen *et al.* 1998). Therefore, they have been considered as potential vectors of resistances via the food chain or the environment from animal production to the consumer, because this resistance could be transferred to pathogenic bacteria or to the gut microbiota (Morelli and Wright 1997; Salminen *et al.* 1998; Saarela *et al.* 2000). Veterinary antibiotics are widely used in many countries worldwide to treat disease and protect the health of animals. Some of them mainly used to treat and prevent infectious diseases (e.g. tetracycline, b-lactams, aminoglycosides and others) (Teuber 2001; Sarmah *et al.* 2006). In the present study, *Lactobacillus rhamnosus* RC007 proved to have no genes for resistance to the tested antibiotics of veterinary medicine importance.

Conclusions

This study demonstrates the importance of using good criteria to select bacteria as silage inoculants. Therefore, the efficacy of inoculant use should be evaluated not only for the improvement of silage fermentation but also for their effects on micotoxicogenic fungi and the resulting mycotoxin production due to the risk that they may involve.

In summary, the ability of *Lact. rhamnosus* RC007 to inhibit fungal growth and mycotoxin production along with the status of these bacteria as safe and the absence of antibiotic resistance genes shows its potential as bio-control inoculant agent in animal feed.

Studies in mini silos to evaluate the improvement of silage fermentation and the effect on silage microbial communities and mycotoxin production are in progress.

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