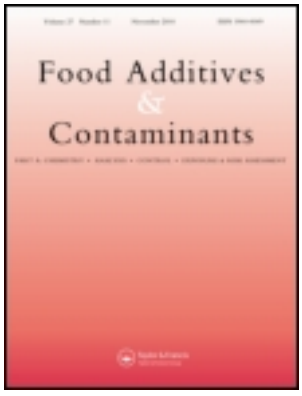
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Saccharomyces cerevisiae strains and the reduction of Aspergillus parasiticus growth and aflatoxin B_1 production at different interacting environmental conditions, in vitro

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Saccharomyces cerevisiae strains and the reduction of *Aspergillus parasiticus* growth and aflatoxin B_1 production at different interacting environmental conditions, *in vitro*

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The effect of *Saccharomyces cerevisiae* RC008 and RC016, previously selected based on their aflatoxin B_1 binding ability and beneficial properties, against *Aspergillus parasiticus* under different interacting environmental conditions was evaluated. Studies concerning the lag phase, growth rate and aflatoxin B_1 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). Both yeast strains showed great antagonistic activity at pH 4, decreasing growth rate compared with the control. The RC008 strain showed the greatest inhibitory activity at all assayed conditions. *A. parasiticus* produced large amounts of AFB_1 *in vitro*. A significant decrease of AFB_1 levels in comparison with the control were observed with yeast interaction. Differences between control and treatment values ranged from 130 to 5400 ng ml^{-1}. *S. cerevisiae* RC008 and RC016 could be considered as effective agents in reducing growth and AFB_1 production at different interacting environmental conditions, related to that found in stored feedstuff. The importance of the present work lies in the search for live strains with both probiotic and biocontrol properties able to prolong the safe storage of feedstuff and exert beneficial properties after animal consumption and which could be included in a novel product for animal feed.

Keywords: *Aspergillus parasiticus*; aflatoxin B_1 production; environmental conditions; *Saccharomyces cerevisiae*

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Introduction

Aflatoxin contamination of crops is a worldwide problem that compromises the safety of food and feed and devastates agricultural economies of specific commodity industries and regions where it occurs. Aflatoxin refers to a group of four mycotoxins (B_1, B_2, G_1 and G_2) produced in crops primarily by the closely related fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Council for Agricultural Science and Technology (CAST) 2003). Aflatoxins are potent hepatotoxins and carcinogens and their quantity in food and feed is closely monitored and regulated in most countries (van Egmond et al. 2007). Many strategies, including biological control, are being investigated to manage, reduce and, ultimately, eliminate aflatoxin contamination of crops (Abbas et al. 2006, 2011). Microbial biocontrol agents have shown great potential as an alternative to synthetic fungicides, and they offer an environmentally friendly alternative to the use of synthetic pesticides (Janisiewicz and Korsten 2002; Cavaglieri et al. 2004a).

Postharvest disease control by using biological agents has been extensively demonstrated (Aysan et al. 2003; Francés et al. 2006; Kotan et al. 2009). Yeasts may act as antagonistic microorganisms, thereby considerably decreasing the growth of filamentous spoilage fungi both *in vitro* and under storage conditions (Petersson and Shürer 1995; Petersson et al. 1998).

Food and feed are, in general, very diverse microbial growth substrates due to variation in both intrinsic factors such as pH, nutrients and water activity (a_w), and extrinsic factors such as storage temperature, among others. Forage stored in silos such as maize silage is subjected to a wide range of different environmental conditions. Temperature, humidity, oxygen availability and pH conditions vary during the silage process, and microbiota may also change from one stage to another. Several authors have informed that yeasts are the prevalent microorganisms present in feed ecosystems (González Pereyra et al. 2008, 2011; Pena et al. 2009; Pereyra et al. 2011). For a

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long time yeasts have been assigned as deteriorating agents in this substrate due to their ability to consume lactic acid in the presence of oxygen (Moon and Ely 1970). However, other studies have demonstrated the beneficial properties of yeast strains. Armando et al. (2011) demonstrated that *S. cerevisiae* RC008 and RC016, isolated from the animal environment, could adsorb AFB_1 and retained their mycotoxin-binding ability under gastrointestinal conditions. Furthermore, it was demonstrated that these strains were potentially probiotics with beneficial properties such as coaggregation and antimicrobial activity against pathogenic enterobacteria and were also able to improve ruminal fermentation (Armando et al. 2011; Dogi et al. 2011).

There is no available information on *S. cerevisiae* and *A. parasiticus* interactions on stored feed and on the impact on AFB_1 production under different interacting environmental conditions. Thus, it is necessary to elucidate the effectiveness of probiotic *S. cerevisiae* strains for control growth and AFB_1 production under different regimes of a_w, pH, temperature and oxygen availability.

The aim of this study was to investigate the efficacy of two *S. cerevisiae* strains isolated from maize silage in inhibiting the growth of *A. parasiticus* and AFB_1 production at different interacting conditions of temperature, pH, oxygen availability and water activity, *in vitro*. Therefore, the novel role that *S. cerevisiae* plays in the silage ecosystem could be explained.

Materials and methods

Microorganisms, growth medium and cultural conditions

Saccharomyces cerevisiae strains (RC008 and RC016), isolated from the animal ecosystem, were obtained from the collection centre at the University National of Rio Cuarto, Argentina. Stock cultures were maintained at −80°C in 30% (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers in yeast extract–peptone–dextrose (YPD broth – 5 g yeast extract, 5 g peptone, 40 g dextrose, 1000 ml water) and incubation at 37°C for 24 h.

Aflatoxigenic *A. parasiticus* NRRL2999 was used in this experiment. It was maintained at 4°C on slants of malt extract agar (MEA) and in 15% glycerol at −80°C.

Culture medium and in vitro *environmental conditions*

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 to 0.95 and 0.99 by the addition of known amounts of the non-ionic solute glycerol (Marín et al. 1995). The basic medium with different levels of a_w was adjusted to pHs 4 and 6 by adding the necessary quantity of HCl and NaOH. The medium was autoclaved at 121°C for 20 min. These environmental conditions were selected in order to mimic different regimes that could be found in stored feedstuff because biocontrol agent effectiveness below that found need to be evaluated.

S. cerevisiae *inoculum*

Inoculations of yeasts were made from the working cultures into YPD broth and incubated at 37°C for 24 h. After incubation, cells were collected by centrifugation (5000 rpm 10 min.), washed twice with phosphate-buffered saline (PBS, pH 7.2) and once with sterile double-distilled H_2O. Finally, the yeast pellets (1×10^7 cell ml^{-1}) were suspended in sterile PBS prior use in the assays.

A. parasiticus *inoculum*

Aspergillus parasiticus NRRL2999 was grown on MEA for 5 days at 25°C to obtain heavily sporulating cultures.

Effect of the interaction S. cerevisiae *and* A. parasiticus *on growth and aflatoxin B_1 production*

Growth rate and lag phase studies

Growth studies were tested as described by Cavaglieri et al. (2004a) with some modifications. A total of 1 ml of *S. cerevisiae* strains inoculums (1×10^7 CFU ml^{-1}) was cultured in silage agar medium plates at different a_w and pH values. After solidification, plates were inoculated in the centre with spores of *A. parasiticus* 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 under normal and reduced oxygen pressure conditions (micro-aerophilia). The growing radius of the cultures containing both microorganisms 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 by using three replicates for each test fungus. The radial growth rate (mm day^{-1}) 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 yeast strain, water activity, temperature and oxygen pressure. All experiments were carried out with at least three separate replicate Petri plates per treatment. After growth was evaluated, all samples were frozen for later extraction and AFB_1 quantification.

The time at which the line intercepted the *x*-axis was used to calculate the lag phase in relation to bacterial antagonists and water activity. The experiments were carried out three times for single and paired cultures.

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Aflatoxin B_1 analysis

All medium was taken from each plate of each treatment, transferred to an Erlenmeyer flask and 20 ml of chloroform were 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 AFB_1 quantification by high-performance liquid chromatography (HPLC), following the detection methodology proposed by Trucksess et al. (1994). An aliquot (200 µl) was derivatised with 700 µl of trifluoroacetic acid/acetic acid/water (20:10:70). The derivatised aflatoxins (50 µl solution) were analysed using a reversed-phase HPLC/fluorescence detection system. The HPLC with fluorescence detection ($\lambda_{exc} = 360$ nm; $\lambda_{em} = 440$ nm) consisted in a C18 column (Supelcosil LC-ABZ, Supelco, Bellefonte, PA, USA; 150×4.6 mm, 5 µm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 µm particle size). 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.

Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating the peak height of sample extracts and those of standard curves. The detection limit of the technique was 1 ng g^{-1}.

Statistical analyses

The influence of *S. cerevisiae* RC008 and RC016 strains on *A. parasiticus* growth and AFB_1 production was analysed by analysis of variance (ANOVA). Means obtained from growth rate, lag phase and AFB_1 production studies were compared using Fisher's protected LSD test (Quinn and Keough 2002). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

Results

Effect of S. cerevisiae *strains on* A. parasiticus *growth rate*

Statistical analyses of yeast strains (S), pH, temperature (T) oxygen availability (PO), water activity (a_W) on *A. parasiticus* growth rate showed that five-way interactions were statistically significant ($p < 0.0001$).

In general, *A. parasiticus* growing alone showed the best growth at pH 6, independently of the temperature, oxygen availability and water activity influences, except at 0.95_{aw}, reduced $P°O_2$ and 37°C, conditions at which *A. parasiticus* was not able to grow (Table 1). When *S. cerevisiae* strains' effect was studied, both yeast strains significantly reduced the *A. parasiticus* growth rate under all interacting factors influence ($p < 0.05$). Under certain interacting conditions, *S. cerevisiae* RC008 was more efficient than *S. cerevisiae* RC016 in reducing *A. parasiticus* growth rate.

Effect of S. cerevisiae *strains on* A. parasiticus *lag phase*

Statistical analyses of yeast strains (S), pH, temperature (T) oxygen availability (PO), water activity (a_W) on lag phase showed that five-way interactions were statistically significant.

Mean lag phases of *A. parasiticus* under different interacting environmental conditions are shown in Table 2. The lag phase for *A. parasiticus* growing alone was longer at pH 6 and at 0.95_{aw} (96.17; 98.32; 127.73; and 144.50 h). *S. cerevisiae* RC016 did not influence the lag phase of *A. parasiticus*, however a prolonged lag phase was observed in the presence of *S. cerevisiae* RC008 at pH 4 ($p < 0.05$).

Effect of S. cerevisiae *strains on aflatoxin B_1 production*

Statistical analyses of yeast strains (S), pH, temperature (T) oxygen availability (PO), water activity (a_W) on AFB_1 production showed that five-way interactions were statistically significant ($p < 0.0001$).

Aflatoxin B_1 production by *A. parasiticus* at different growth conditions is shown in Table 3. *A. parasiticus* alone was able to produce *in vitro* great amounts of AFB_1. A significant decrease of AFB_1 levels in comparison with the control ($p < 0.05$) were observed with yeast interaction mainly at pH 4. Both strains, *S. cerevisiae* RC008 and *S. cerevisiae* RC016, achieved the largest reduction percentages at 25°C (normal $P°O_2$ and 0.99 a_W) of 98% and 81% AFB_1, respectively and at 37°C (normal $P°O_2$ and 0.99 a_W) the reduction percentages were 87% and 96% for *S. cerevisiae* RC008 and RC016, respectively. The *A. parasiticus–S. cerevisiae* interaction on AFB_1 reduction at pH 6 was effective at normal oxygen availability and at both studied a_W's. *S. cerevisiae* RC016 achieved reduction percentages greater than 99% at 37°C (normal $P°O_2$ and 0.99 a_W). The interacting factors did not increase the AFB_1 production at any studied condition.

Discussion

Aflatoxin contamination of crops compromises the safety of food and feed supplies and causes significant economic losses each year. Among several approaches to reduce and, ultimately, eliminate aflatoxin contamination, biological control is one of the most promising. In this work, fungal growth of *A. parasiticus* and AFB_1 production were found to be significantly influenced by *S. cerevisiae* interactions.

Table 1. Effect of *Saccharomyces cerevisiae* strains on *Aspergillus parasiticus* growth rate under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*A. parasiticus* growth rate (mm h^{-1})					
				Control[a]		Interaction 1[b]		Interaction 2[c]	
pH	T°	P°O_2	a_w	Media ± SD		Media ± SD		Media ± SD	
4	25	Normal	0.99	0.30 ± 0.01	defg	0.02 ± 0.01	r	0.23 ± 0.015	ijkl
			0.95	0.36 ± 0.005	cde	0.02 ± 0.01	r	0.23 ± 0.006	ijkl
		Reduced	0.99	0.34 ± 0.005	def	0.05 ± 0.00 5	qr	0.27 ± 0.006	ghij
			0.95	0.36 ± 0.005	cde	0.11 ± 0.01	opq	0.17 ± 0.005	lmno
	37	Normal	0.99	0.39 ± 0.06	cde	0.14 ± 0.06	klmn	0.16 ± 0.035	mno
			0.95	0.35 ± 0.006	def	0.21 ± 0.01	jklmn	0.22 ± 0.02	jkl
		Reduced	0.99	0.35 ± 0.005	def	0.17 ± 0.005	lmno	0.16 ± 0.015	mno
			0.95	0.35 ± 0.015	def	0.14 ± 0.1	no	0.04 ± 0.04	r
6	25	Normal	0.99	0.39 ± 0.065	cd	0.12 ± 0.006	op	0.37 ± 0.006	cde
			0.95	0.55 ± 0.15	ab	0.15 ± 0.005	no	0.06 ± 0.05	pqr
		Reduced	0.99	0.52 ± 0.006	b	0.12 ± 0.006	op	0.42 ± 0.05	c
			0.95	0.38 ± 0.006	dce	0.16 ± 0.015	mno	0.14 ± 0.01	no
	37	Normal	0.99	0.52 ± 0.006	b	0.13 ± 0.02	o	0.32 ± 0.01	efgh
			0.95	0.61 ± 0.006	a	0.26 ± 0.005	hij	0.29 ± 0.015	fghi
		Reduced	0.99	0.34 ± 0.025	def	0.2 ± 0.01	klmn	0.15 ± 0.03	mno
			0.95	0	r	0.13 ± 0.07	o	0.26 ± 0.015	hij

Notes: [a]*A. parasiticus* growing alone.
[b]*A. parasiticus–S. cerevisiae* RC008 interaction.
[c]*A. parasiticus–S. cerevisiae* RC016 interaction.
Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 2. Effect of *Saccharomyces cerevisiae* 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)					
				Control[a]		Interaction 1[b]		Interaction 2[c]	
pH	T°	P°O_2	a_w	Media ± SD		Media ± SD		Media ± SD	
4	25	Normal	0.99	16.45 ± 2.08	mnop	9.50 ± 0.50	nop	54.79 ± 9.79	ghijkl
			0.95	34.76 ± 1.33	klmno	80.38 ± 13.62	cdefg	44.88 ± 5.32	hijklm
		Reduced	0.99	9.51 ± 2.54	nop	85.10 ± 5.90	cdef	22.96 ± 1.48	lmnop
			0.95	33.78 ± 1.49	klmno	55.67 ± 33.16	fghij	48.21 ± 24.14	hijklm
	37	Normal	0.99	22.57 ± 7.42	mnop	80.66 ± 27.34	cdefg	36.47 ± 10.63	klmno
			0.95	25.56 ± 2.14	klmnop	43.3 ± 16.45	ijklm	25.31 ± 0.65	klmnop
		Reduced	0.99	22.30 ± 0.83	mnop	40.48 ± 3.63	jklmn	29.47 ± 0.53	klmnop
			0.95	28.41 ± 3.26	klmnop	103.19 ± 55.06	bcde	81.23 ± 0.025	cdefg
6	25	Normal	0.99	32.93 ± 19.64	klmnop	48.22 ± 12.86	ghijklm	41.30 ± 0.70	ijklm
			0.95	96.17 ± 17.41	bcde	109.62 ± 54.29	bc	96.80 ± 0.20	bcde
		Reduced	0.99	36.84 ± 0.01	klmno	31.23 ± 0.02	klmnop	40.89 ± 13.36	jklmn
			0.95	98.320.42	bcde	109.99 ± 0.70	bc	86.87 ± 13.80	cdef
	37	Normal	0.99	34.45 ± 0.16	klmno	37.82 ± 4.185	klmn	43.34 ± 16.365	ijklm
			0.95	127.73 ± 0.27	ab	71.72 ± 6.35	efghij	75.28 ± 0.26	defgh
		Reduced	0.99	18.33 ± 9.19	mnop	74.52 ± 3.98	defghi	10.88 ± 7.12	nop
			0.95	144.50 ± 0.50	a	136.30 ± 37.70	ab	132.25 ± 0.25	ab

Notes: [a]*A. parasiticus* growing alone.
[b]*A. parasiticus–S. cerevisiae* RC008.
[c]*A. parasiticus–S. cerevisiae* RC016.
Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

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Table 3. Effect of *Saccharromyces cerevisiae* strains on aflatoxin B_1 production by *Aspergillus parasiticus* under interacting pH, temperature (T°), oxygen availability ($P°O_2$) and water activity (a_w) conditions.

Growth conditions				*A. parasiticus* AFB_1 production ($ng\,ml^{-1}$)					
				Control[a]		Interaction 1[b]		Interaction 2[c]	
pH	T°	$P°O_2$	a_w	Media ± SD		Media ± SD		Media ± SD	
4	25	Normal	0.99	347.5 ± 0.28	d	331.9 ± 12.5	d	44.4 ± 0.36	gh
			0.95	793.6 ± 0.23	a	17.3 ± 0.4	h	150.9 ± 121	ef
		Reduced	0.99	680.0 ± 1.15	b	18.7 ± 9.45	h	517.5 ± 0.29	c
			0.95	88.5 ± 23.4	fgh	28.9 ± 7.85	h	58.2 ± 2.15	fgh
	37	Normal	0.99	597.4 ± 0.34	bc	76.9 ± 34	fgh	24.2 ± 5.15	h
			0.95	183.4 ± 44.7	e	123.9 ± 110.4	efg	54.4 ± 34	gh
		Reduced	0.99	607.5 ± 0.29	bc	46.1 ± 0.52	gh	7.6 ± 0.23	h
			0.95	203.17 ± 184.7	e	31.8 ± 0.11	gh	10.0 ± 7.0	h
6	25	Normal	0.99	2658 ± 813.2	c	791 ± 0.58	d	18.8 ± 0.12	d
			0.95	2583 ± 0.58	c	378.5 ± 42.4	d	189.1 ± 128	d
		Reduced	0.99	67.1 ± 0.58	d	18.5 ± 0.29	d	55.0 ± 0.58	d
			0.95	50.0 ± 1.15	d	1.8 ± 1.8	d	28.67 ± 16	d
	37	Normal	0.99	4163 ± 4.84	b	703.6 ± 0.23	d	43.67 ± 57.5	d
			0.95	5532 ± 1.15	a	124.2 ± 59	d	70.55 ± 40	d
		Reduced	0.99	429.9 ± 94.35	d	56.9 ± 34.4	d	23.9 ± 0.06	d
			0.95	0	i	11.0 ± 0.58	d	42.15 ± 2.25	d

Notes: [a]AFB_1 production by *A. parasiticus* growing alone.
[b]*A. parasiticus–S. cerevisiae* RC008.
[c]*A. parasiticus–S. cerevisiae* RC016.
Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$). Statistical analysis compared means from each pH separately.

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Several authors have demonstrated the safety of *S. cerevisiae* for nutritional and pharmaceutical use in animal feed in the European Community and Japan (Nitta and Kobayashi 1999). As *S. cerevisiae* strains have GRAS status (http://www.fda.gov/Food/FoodIngredientsPackaging/ucm078956.htm), the presence of an environmentally safe microorganism to prevent aflatoxin contamination should be of considerable value.

In the storage feedstuff, complex interactions occur between biotic and abiotic factors that are influenced by temperature, pH and water activity. The present work was carried out at different environmental factors since the biocontrol agent effectiveness under environmental conditions that could be found in stored feedstuff needs to be evaluated. Silage native *S. cerevisiae* RC008 and RC016 were able to inhibit *A. parasiticus* growth *in vitro* and strongly reduced AFB_1 production at different environmental conditions according to that found in stored feedstuff.

Since mycotoxins are secondary metabolites that can be produced in response to a stress factor, growth reduction caused by the presence of bacterial or yeast strains can influence mycotoxin production (Boyacioglu et al. 1992; Gareis and Ceynowa 1994). However, no stimulation of AFB_1 production was observed with *S. cerevisiae* RC008 or RC016–*A. parasiticus* interaction. Different results were reported by Wicklow et al. (1980) and Cuero et al. (1987) with other interacting yeast–fungi cultures.

Due to lactic acid degradation by the mainly lactic acid-utilising yeasts of the genera *Pichia* and *Candida*, yeast strains have been often considered as spoilage microorganisms in silage (Ramon-Portugal et al. 1998). The results obtained in the present work demonstrate for the first time a beneficial role that *S. cerevisiae* plays in the silage ecosystem. They could be applied at the assembly stage of the silage; previous studies have demonstrated *S. cerevisiae*'s ability to survive at low pH without showing an increase in cells number (Armando et al. 2011). Consequently, these strains could not use lactic acid at this moment. Later, when the silage cover is damaged or opened for use, the viable *S. cerevisiae* strains RC008 and RC016 could prevent AFB_1 producers' growth and AFB_1 contamination.

In this work, *A. parasiticus* NRRL2999 was used as an aflatoxin B_1 producer. The ability of this fungus to produce large amounts of aflatoxin B1 and the stability in its production has been widely demonstrated (Ram and Shamsudeen 2008). Silage-born *A. flavus* were not stable during AFB_1 production (data not shown).

Different assays have been conducted to study the influence of environmental factors on the growth and mycotoxin production by filamentous fungi (Borreani et al. 2007; Cavallarin et al. 2011). Some studies aimed

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to study the ecophysiological behaviour of toxicogenic fungi; while others applied strategies to prevent fungal growth and mycotoxin production by using antioxidants or bacteria. Barberis et al. (2010) and Passone et al. (2007) studied the effect of food-grade antioxidants on the lag phase, growth rate and ochratoxin A production by *Aspergillus* section *Nigri* strains and on the AFB_1 production by *Aspergillus* section *Flavi* (under different a_W and temperature conditions), respectively. They observed that in some conditions the antioxidants effectively inhibit growth and mycotoxin production. Cavaglieri et al. (2004b) studied the biocontrol effect of rhizobacteria upon *Fusarium verticillioides* and observed that fumonisin B_1 production was significantly inhibited at ranges between 70% and 100%. These results are comparable with those observed in this work, where both strains of *S. cerevisiae* were able achieve a significant growth inhibition of *A. parasiticus* and AFB_1 production.

None of the abovementioned studies were ever carried out using potentially probiotic strains able to prolong the safe storage of feedstuff as well as to exert beneficial properties after animal consumption. Previous studies demonstrated that *S. cerevisiae* RC008 and RC016 can adsorb AFB_1, zearalenone and ochratoxin A *in vitro*. In addition, these strains showed coaggregation and antimicrobial activity against pathogens (Armando et al. 2011), were able to retain their viability and mycotoxin binding ability under gastrointestinal conditions, and improved ruminal fermentation (Dogi et al. 2011).

Several important properties of yeasts make them useful for biocontrol purposes; yeasts do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotic metabolites as possibly produced by bacterial antagonists. Yeasts generally have simple nutritional requirements and can colonise dry surfaces for long periods of time (Hussein et al. 1996).

For most biocontrol organisms several modes of action have been suggested, and no single mechanism has been shown to be responsible for the whole biocontrol effect (Janisiewicz and Korsten 2002).

Several possible modes of action for biocontrol by yeasts such as competition for space and nutrients and the production of cell wall-degrading enzymes have been suggested. Magliani et al. (1997) reported that certain strains of *S. cerevisiae* were able to produce 'killer' toxins. Moreover, many killer toxins can affect other yeasts and even bacteria and filamentous fungi (Polonelli and Morace 1986; Polonelli et al. 1987). Both, *S. cerevisiae* RC008 and RC016 strains used in this work showed great antagonistic activity against bacterial pathogens in previous studies (Armando et al. 2011). Preliminary assays carried out as described in the present work, but using the growth supernatants of yeast cultures, demonstrated a significant reduction of AFB_1 production by *A. parasiticus* (data not shown). This fact suggests that there is an AFB_1 production inhibitor secreted by the growing yeast cells, whose nature is under study.

Understanding the consequence of *S. cerevisiae–A. parasiticus* interactions under different environmental conditions may be helpful to know the nature and extent of fungal growth in the stored feedstuff ecosystem. This may have important implications for biological control with these yeast strains. The addition of *S. cerevisiae* RC008 and RC016 yeast strains to animal feeds could prevent mould spoilage and AFB_1 production at different interacting environmental conditions prevailing in stored feedstuff.

The importance of the present work lays in the search for live strains, with both probiotic and biocontrol properties, able to prolong the safe storage of feedstuff and exert beneficial properties after animal consumption, to be included in a novel product to animal feed.

Functional and technological studies related to the inclusion of yeast strains into animal feed are in progress.

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