Agr. Nat. Resour. 56 (2022) 673–686

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Antagonistic activity of *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* and *Lactobacillus paracasei* against growth and aflatoxin production of *Aspergillus flavus* in corn silage

Tassaporn Rungchaiwattanakul, Supat Chareonpornwattana, Cheewanun Dachoupakan Sirisomboon*

Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

Article Info

Article history:
Received 23 December 2021
Revised 28 March 2022
Accepted 25 April 2022
Available online 26 August 2022

Keywords:
Aflatoxins,
Aspergillus flavus,
Corn silage,
Lactic acid bacteria,
Yeasts

Abstract

Importance of the work: The contamination of *Aspergillus* section *Flavi* producing aflatoxins during ensiling is a main problem resulting in corn silage spoilage.

Objectives: To evaluate the antagonistic activities of yeasts and a lactic acid bacterium against *A. flavus*.

Materials & Methods: Individual yeast culture, mixed yeast culture (*Wickerhamomyces anomalus* MSCU 0652 and *Kluyveromyces marxianus* MSCU 0655) and *Lactobacillus paracasei* AN3 were tested for their antifungal activities against *A. flavus* growth and its aflatoxin production both *in vitro* and in corn silage.

Results: *In vitro* studies showed inhibitory activities of *Lactobacillus paracasei* and mixed yeasts against aflatoxin production but lower activity against fungal growth. The co-cultures of *L. paracasei* and mixed yeasts were used as starter for sterile and non-sterile corn ensiling. For the sterile condition, a substantial reduction of pH was observed 1 day after ensiling. The growth of mixed yeasts and *L. paracasei* was not mutually affected, but that of *A. flavus* was completely inhibited in 7 d with the reduction of aflatoxin production after 14 d of ensiling. For corn silage, mixed yeasts and *L. paracasei* at a ratio of 1×10^6:1×10^8 colony forming units/g inhibited fungal growth and reduced aflatoxin production at 5 d and 7 d of ensiling, respectively, with no effect on nutritional values.

Main finding: The mixed culture of selected yeasts and a lactic acid bacterium showed potential for use as a biocontrol agent in silage to improve silage quality.

* Corresponding author.
E-mail address: cheewanun.d@chula.ac.th (C.D. Sirisomboon)

https://doi.org/10.34044/j.anres.2022.56.4.02

Introduction

In the last decade, the quantity of feed consumed by livestock has increased but the production of feedstuffs has decreased due to climatic conditions and the reduction in cultivation area (Schader et al., 2015). To solve this problem, silage is a promising method to preserve the nutrition and quality of fresh forage, including corn and grass in ensiled form. Corn is an important forage with high dry matter and soluble carbohydrate contents and is suitable for use as potential feedstuff to make silage (Santos et al., 2013). The amount of air trapped while packaging the forage and the air exposure due to leakage as well as moisture, heat and insects result in the contamination of undesirable microorganisms, such as coliform bacteria and fungi, leading to increased pH and spoilage of silage (Chen et al., 2005; Dunière et al., 2013).

The contamination of mycotoxigenic fungi during stages of growing, harvesting, fermenting and storage leads to mycotoxin contamination in corn silage; among the mycotoxins, aflatoxins (AFs) are the most important mycotoxin in food and feed, including silage. They are mainly produced by *Aspergillus* section *Flavi*, especially *Aspergillus flavus* and *A. parasiticus* (Driehuis, 2013). In nature, there are around 20 types of aflatoxins; however, the most important are aflatoxin B_1, B_2, G_1 and G_2 (Blankson and Mill-Robertson, 2016). The presence of aflatoxin B_1 in the feed of dairy cattle leads to the emergence of its metabolite, aflatoxin M_1, in milk and dairy products (Zain, 2011). The daily handing of silage spoiled by aflatoxigenic fungi and aflatoxins could be a potential risk factor regarding animal and human safety. Aflatoxin B_1 is the most toxic mycotoxin known and is regarded to be mutagenic teratogenic, hepatotoxic and carcinogenic to many animal species, including humans (Bankole et al., 2010; Murphy et al., 2006).

Biological control using antagonistic microorganisms, including yeasts, molds and bacteria, is an effective method to control the contamination of mycotoxin in food and feed (Ehrlich, 2014; Fiori et al., 2014; Lavermicocca et al., 2000). Such a method is regarded as safe for the environment and for health, as well as stable in terms of the nutritive values in food and feed (Niba et al., 2014; Kumar et al., 2013). Among the microorganisms considered as potent biological control agents, yeasts and lactic acid bacteria (LAB) are particularly promising (El-Nezami et al., 1998; Haskard et al., 2000; Haskard et al., 2001; Peltonen et al., 2001; Shetty and Jespersen, 2006; Armando et al., 2012b). They play a key role in food fermentation as starter cultures and some species are probiotic and generally recognized as safe (Bintsis, 2018). Yeasts are also sources of proteins and vitamin B complex (Olvera-Novoa et al., 2002). Many yeast species have been shown to have promising antagonistic properties against the common filamentous fungi, including mycotoxigenic fungi generally contaminating food and feed. For example *Saccharomyces cerevisiae* RC008 and RC016 showed antagonistic activity against growth and mycotoxin production, including aflatoxin B_1, ochratoxin A, zearalenone and deoxynivalenol of *A. paraciticus*, *A. carbonarius* and *F. graminerum* (Armando et al., 2012a, b; Armando et al., 2013). Medina-Córdova et al. (2016) reported that *Debaryomyces hansenii* has the potential to inhibit growth of *Mucor circinelloides, Aspergillus* sp., *F. proliferatum* and *F. subglutinans* and to reduce fumonisin production of *F. subglutinans* in corn. Likewise, Hua et al. (2014) reported the inhibition of spore germination and aflatoxin production of *A. flavus* by *Wickerhamomyces anomalus* WRL-076 capable of producing 2-phenylethanol. Zearalenone (ZEA) and deoxynivalenol (DON) contamination in wheat flour and fodder could be removed by *S. cerevisiae*, *G. fermentans*, *K. marxianus*, and *M. pulcherrima* (Repečkienė et al., 2013). Recently, Jaibangyang et al. (2020) reported that *Candida nivariensis* DMKU-CE18 could inhibit the mycelial growth and conidial germination of *A. flavus* and reduce aflatoxin production in contaminated corn grains. In addition, they also found that *Kwoniella heveanensis* DMKU-CE82 was one of the most effective yeast strains producing antifungal volatile organic compounds against *A. flavus*.

The use of LAB has received significant attention as an alternative biocontrol agent and preservative in food and feed. Magnusson et al. (2003) found that *Lactobacillus coryniformis*, *L. plantarum* and *Pediococcus pentosaceus* showed strong inhibitory activities against *A. fumigatus*, *A. nidulans*, *Penicillium commune* and *F. sporotrichioides* by the production of organic acids and cyclic dipeptides. Prema et al. (2008) reported that antifungal compounds produced by *L. plantarum* isolated from grass silage could inhibit the growth of *A. fumigatus* and *R. stolonifera*. Rather et al. (2013) found that *L. plantarum* YML007 isolated from kimchi inhibited growth of *A. niger, A. oryzae, A. flavus* and *F. oxysporum* by production of various antifungal compounds. Bello et al. (2007) reported that *L. plantarum* FST1.7 could produce cyclic dipeptides—cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro)—to inhibit and retard the growth of *Fusarium* contamination in sourdough production. Sangmanee and Hongpattarakere

(2014) evaluated the antifungal activity of *Lactobacillus plantarum* K35 isolated from traditional Thai fermented rice noodle against the growth and aflatoxin production of *A. favus* and *A. parasiticus*. *L. plantarum* K35 supernatant caused irreversible damage and morphological alteration in various membrane-bound structures and cell wall of the fungi.

Lactobacillus paracasei AN3 and two yeasts strains (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) have been isolated from corn silage (Maroongrung et al., 2016). However, to date, there has been very limited information regarding the antagonistic activities of these species against aflatoxigenic fungi, such as *A. flavus*, or their interaction on silage. The aim of the current study was to evaluate the antagonistic activities of yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 against growth and aflatoxin production of *A. flavus* both *in vitro* and in corn silage.

Materials and Methods

Microorganism preparation

All microorganisms were previously deposited at the Culture Collection Center, the Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand. Yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 were maintained on yeast extract peptone dextrose (YPD; HiMedia Laboratories Pvt. Ltd.; India) agar and de Man Rogosa Sharpe (MRS; Difco Laboratories; USA) agar, respectively. Each yeast culture was prepared in yeast malt extract broth (YM; HiMedia Laboratories Pvt. Ltd.; India) at 30 °C for 9.5 hr to obtain a mid-log phase culture. The mid-log phase culture of *L. paracasei* AN3 was prepared in MRS broth and incubated at 30 °C for 14 hr.

Spore suspensions of aflatoxigenic *A. flavus* M3T8R4G3 were prepared on potato dextrose agar plates (PDA; Difco Laboratories; USA) for 7 d at 25 °C. Then, the spores were harvested using sterile normal saline solution containing 0.01% Tween 80 and were adjusted after spore count using a haemocytometer to a final concentration of 1×10^8 spores/mL.

Determination of antifungal activity of yeasts and lactic acid bacterium using dual culture technique

Individual yeast culture, mixed yeast culture and *L. paracasei* AN3 were tested for their antifungal activities against *A. flavus* growth and its aflatoxin production using a dual culture technique according to Pantelides et al. (2015), with some modifications. PDA and MRS plates (9 cm diameter) were inoculated with yeast and LAB, respectively, in dual cultures with a distance of 2.5 cm between a drop of 10 µL of fungal spore suspension and a line of yeast or an LAB streak. The mixed yeast culture was obtained by mixing a mid-log phase culture of *W. anomalus* and *K. marxianus* at a ratio of 1:1 before streaking on a culture plate. The culture plates inoculated with only *A. flavus* served as the control. After incubation for 7 d at 25 °C, each inhibition zone was examined and the diameter of the *A. flavus* colony was measured. The inhibition of fungal growth was determined according to Equation 1:

$$\% \text{ Inhibition} = [(a - b) / a] \times 100 \quad (1)$$

where a is the diameter of the fungal colony on the medium without yeast and LAB (control plate) and b is the diameter of the fungal colony on medium with yeast and LAB (tested plate).

Determination of aflatoxin content in the PDA medium produced by *A. flavus* was carried out using enzyme-linked immunosorbent assay (ELISA) as described below.

One gram of agar plugs in the inhibition zone along the diameter of the mycelium was extracted with 5 mL of 70% methanol under sonication for 15 min and vigorously vortexed for 10 s. Then, the extract was passed through filter paper (Whatman No.1 filter paper; UK) and subjected to aflatoxin quantification using an ELISA test kit (Veratox®; USA) following the manufacturer's instruction against a toxin standard (Neogen; USA). Each experiment was quintuplicated and each result was reported as the average from three independent experiments. The inhibition of aflatoxin production was determined according to Equation 2:

$$\% \text{ Reduction} = [(a - b) / a] \times 100 \quad (2)$$

where a is the aflatoxin content from the fungal colony on medium without yeast and LAB (control plate) and b is the aflatoxin content from the fugal colony on medium with yeast and LAB (tested plate).

Determination of interaction of yeasts, lactic acid bacterium and A. flavus on fungal growth and aflatoxin production in silage

For corn silage preparation, old corn plants aged 70 d were harvested from a farm located in Nakhon Pathom, Thailand.

The whole plants were chopped and divided into two groups: sterile silage (heated at 121 °C for 45 min) and non-sterile silage (untreated). The plant materials were packed into two layers of plastic bags. The experiments were performed by mixing a mid-log phase culture of *W. anomalus* and *K. marxianus* at a ratio of 1:1 (either at 1×10^8 cells/mL or 1×10^{10} cells/mL each) to obtain the mixed yeast culture. The mid-log phase of *L. paracasei* AN3 was adjusted to 1×10^8 colony forming units (CFU)/mL and 1×10^{10} CFU/mL. Then, 5 mL of each initial inoculant was added to 1 kg of silage to obtain the final concentration of starter culture of 1×10^6 CFU/g or 1×10^8 CFU/g. The combinations of initial inoculants of LAB and mixed yeast culture in silage were 1×10^6:1×10^6 CFU/g in sterile silage and 1×10^6:1×10^8 CFU/g and 1×10^8:1×10^6 CFU/g in non-sterile silage in the presence of 1×10^6 CFU/g of *A. flavus* in all samples. Sterile silage and non-sterile silage inoculated with *A. flavus* without tested microorganisms were used as the control. After inoculation, each silage sample was thoroughly mixed in the bag before air-tight packaging and all samples were incubated at an ambient temperature of around 30–35 °C for 21 d. Samples at 0 d, 1 d, 3 d, 5 d, 7 d, 14 d and 21 d after ensiling were subjected to determination for pH value and microbial population. The aflatoxin contents were tested at 0 d, 7 d, 14 d and 21 d of ensiling. The experiment was performed in triplicate.

Enumeration of microorganisms and pH measurement

To estimate the amounts of fungi, yeasts, LAB and coliform bacteria in silage, 50 g of each silage sample were mixed with 450 mL of sterile 0.1% peptone solution and homogenized in a stomacher for 2 min. Then, 1 mL of the suspension was diluted in 9 mL of sterile 0.1% peptone solution and 0.1 mL of each dilution was spread onto MRS agar for LAB, a PDA plate for fungi and yeasts, and onto MacConkey agar (Difco Laboratories; USA) for coliform bacteria in the case of the non-sterile silage samples. The plates were incubated either at 30 °C for 48 hr (LAB), 25 °C for 5 d (fungi and yeast) or 37 °C for 24 hr (coliform bacteria). The results were expressed as colony-forming units per gram of silage. The pH of the suspension was immediately determined using a pH meter (Mettler Toledo; Switzerland).

Detection of aflatoxins using enzyme-linked immunosorbent assay

The levels of aflatoxins in all silage samples were determined using an ELISA test kit (Neogen's Veratox® for aflatoxin; USA). Ten grams of ground silage was mixed with 50 mL of methanol:water (70:30, volume per volume) and shaken vigorously for 5 min. Then, the extracts were passed through filter paper (Whatman No.1, UK). The filtrate was analyzed using the ELISA test kit according to the manufacturer's instructions.

Since the aflatoxin contents from each experimental condition at day 0 are different, it is difficult to compare the inhibitory efficiency of yeasts and LAB under such conditions. Therefore, the aflatoxin contents at day 0 were converted from all conditions to 100% and the aflatoxin contents afterward were converted to a percentage relative to the aflatoxin contents at day 0 to make result comparison within or between experimental conditions easier. The normalization aflatoxin content was calculated according to Equation 3:

$$\text{Normalized aflatoxin contents (\%)} = (a/b) \times 100 \quad (3)$$

Where a is the aflatoxin content at day X and b is the aflatoxin content at day 0.

Nutritional values of silage

The nutritional values were assessed of non-sterile corn silage inoculated with mixed culture of yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 (Test) with an initial concentration ratio 1×10^6:1×10^8 CFU/g and un-inoculated silage (control) incubated at room temperature of around 30–35 °C at 0 d, 7 d, 14 d and 21 d of ensiling. All samples were analyzed at the Animal Nutrition Laboratory of the Department of Animal Science, Kasetsart University, Bangkok, Thailand. The un-inoculated silage was naturally fermented silage and neither antagonistic microorganisms (yeasts and LAB) nor *A. flavus* were inoculated. The contents of dry matter, crude protein, crude fat, crude fiber, calcium and phosphorus were determined as described by Association of Official Analytical Chemists (2010). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using the forage fiber analysis procedures described by Goering and Van Soest (1970).

Statistical analysis

Statistical analyses were performed using the SPSS statistical software version 22 (SPSS Inc.; USA). The data were analyzed using one-way analysis of variance and a t test to compare means using least significant differences. Significance was tested at $p < 0.05$.

Results and Discussion

In vitro antagonistic activities of yeasts and lactic acid bacterium against growth and aflatoxin production of A. flavus

The antagonistic activities of individual yeast cultures, mixed yeast cultures and *L. paracasei* AN3 on growth and aflatoxin production of *A. flavus* were evaluated using a dual culture technique. Fig. 1 shows the *A. flavus* growth after 7 d in dual cultures on PDA medium (Figs. 1A–1D) and on MRS medium (Figs. 1E, 1F). From visual observation, the inhibition in all assays was clearly seen by the limited growth of fungal mycelia and inhibition of spore formation in the small zone surrounding the yeast streaks (Figs. 1B–1D) compared to the fungal colony on medium without yeast (Fig. 1A). Fig. 1F shows that the growth of *A. flavus* on medium with *L. paracasei* AN3 produced a smaller fungal colony than the fungal colony on medium without *L. paracasei* AN3 (Fig. 1E). The percentage inhibition of fungal colony diameter was low in all tests with 14–18% inhibition (Table 1). For aflatoxin production, the mixed yeast culture showed higher inhibitory activity compared to that of single yeast culture, with the highest aflatoxin reduction of 43.25%, followed by *L. paracasei* AN3 (35.64%) (Table 1). Although yeasts and LAB showed low efficiency against fungal growth, the mixed yeast culture and LAB showed high inhibitory activity against aflatoxin production.

Several strains of *W. anomalus* and *K. marxianus* are non-pathogenic organisms and are classified as GRAS (Generally Recognized as Safe). These yeasts are already used in the food and feed industries (Fonseca et al., 2008; Walker, 2011). Many studies have reported the positive effect of *W. anomalus* and *K. marxianus* against fungal pathogens in different agricultural products. For example, Geng et al. (2011) reported that *K. marxianus* inhibited spore germination of *P. digitatum* in potato-dextrose broth. *K. marxianus* also showed a reduction of disease incidence in the green mold of citrus fruit caused by *P. digitatum* in artificial inoculation trials. Parafati et al. (2015) reported the ability of *S. cerevisiae, W. anomalus* and *M. pulcherrima* to produce volatile organic compounds inhibiting *B. cinerea* growth. Similarly, Oro et al. (2018) found that *W. anomalus* produced an antifungal compound, ethyl acetate, against *B. cinerea* growth. Mixed cultures of yeasts have been studied and showed greater inhibitory effect than of each strain individually. For example, Schisler et al. (2011) reported that co-cultures of *Papilioterma flavescens* OH182.9 and *P. aurea* OH71.4 markedly reduced fusarium head blight disease in wheat with the highest value of relative performance index compared to individual strains. Coda et al. (2011) reported that a sourdough starting with a combination of *L. plantarum* and *W. anomalus* did not produce observable fungal contamination until 28 d of storage.

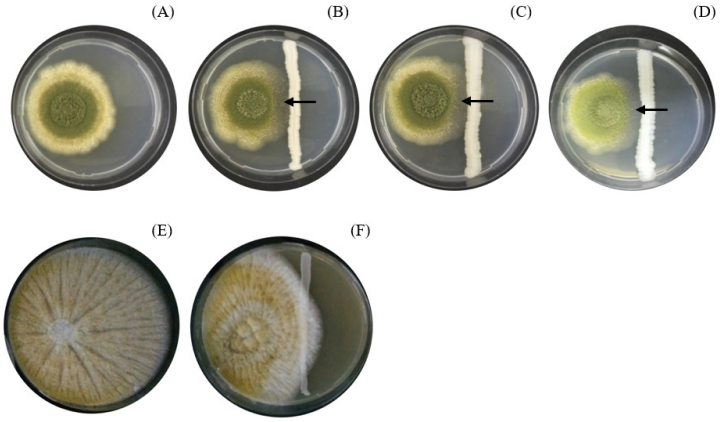

Fig. 1 Growth of *Aspergillus flavus* at 7 d in dual cultures on potato dextrose agar (A–D) and de Man Rogosa Sharpe medium (E, F); (A) control; (B) *Wickerhamomyces anomalus* MSCU 0652; (C) *Kluyveromyces marxianus* MSCU 0655; (D) mixed culture of *W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655; (E) control; (F) *Lactobacillus paracasei* AN3, where limited growth of fungal mycelia and inhibition of spore formation were observed in the small zone surrounding the yeast streaks (black arrows in B–D)

Table 1 Inhibitory effect of yeasts and lactic acid bacterium (LAB) on colony diameter and aflatoxin production of *Aspergillus flavus* by dual cultures technique on potato dextrose agar (yeast) and de Man Rogosa Sharpe (LAB) at 25 °C for 7 d

Yeast/LAB	Colony diameter (% inhibition)	Aflatoxin production (% reduction)
W. anomalus MSCU 0652	14.41±3.23[a]	5.20±6.72[a]
K. marxianus MSCU 0655	16.99±2.25[a]	2.93±9.71[a]
W. anomalus MSCU 0652 + *K. marxianus* MSCU 0655	15.11±4.49[a]	43.25±18.79[b]
L. paracasei AN3	17.83±2.51[a]	35.64±44.85[b]

Mean ± SD (derived from three independent experiments) within columns superscripted with different lowercase letters differ significantly ($p < 0.05$)

L. casei and *L. paracasei* are Gram-positive, non-spore forming, catalase-negative and facultative heterofermentative bacteria that grow under microaerophilic-to-strictly anaerobic conditions (Klein et al., 1998; Zhou and Li, 2015). In addition, LAB also are classified as GRAS and are widely used in food industries. The use of LAB has received considerable attention as a novel approach to the control of pathogens in foods, including mycotoxigenic fungi. Gomah and Zohri (2014) reported that *L. paracasei* subsp. *paracasei* reduced the production of deoxynivalenol, zearalenone and fumonisin produced by *Fusarium*. Li et al. (2014) reported that *L. casei* AST18 inhibited the cheese spoilage fungus, *P. chrysogenum,* by the production of antifungal compounds affecting mycelial morphology and fungal cytoplasm.

Inhibition of yeasts and L. paracasei AN3 on growth and aflatoxin production of A. flavus in sterile silage

A mixed starter culture consisting of mixed yeasts (*W. anomalus* MSCU 0655 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 with an initial inocula of 1×10^6 CFU/g (yeasts) and 1×10^6 CFU/g (*L. paracasei* AN3) was used for evaluating their ability to inhibit growth and aflatoxin production of *A. flavus* as well as their interaction in sterile silage. The changes in pH during the silage fermentation process are shown in Fig. 2A. Sterile corn silage inoculated with a mixed culture of yeasts and *L. paracasei* AN3 conferred a rapid reduction of pH in the first 24 hr after starting the fermentation process from 4.94±0.18 to 4.20±0.07, after which the pH value remained stable at around 4 until the end of fermentation. Conversely, the pH value of the control silage inoculated with only *A. flavus* did not decrease. During the ensiling process, LAB played the most important role in fermentation (Driehuis and Oude Elferink, 2000). LAB in silage could utilize the soluble carbohydrates to produce organic acids, particularly lactic acid, that could reduce the silage pH (3.6–4.5) and inhibit undesirable microorganisms (Kung, 2018).

The amount of *L. paracasei* AN3 rapidly increased within the first 24 hr of the incubation, with the highest number (8.67±0.23) on the third day of ensiling (9.01±0.12 log CFU/g) and remained stable at around 9 log CFU/g until the end of fermentation (Fig. 2B). Similarly, the amounts of the yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) increased on the first day of ensiling (7.77±0.23 log CFU/g) and stayed stable at around 7–8 log CFU/g until the end of ensiling (Fig. 2B). These results indicated that *W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655 were able to grow at a low pH in silage conditions. The growth of yeasts and the LAB did not affect each other. Yeast is a facultative anaerobic microorganism, some of which can utilize the lactic acid produced by LAB and sugar in silage to produce CO_2 and H_2O (Santos et al., 2017). However, the yeast population

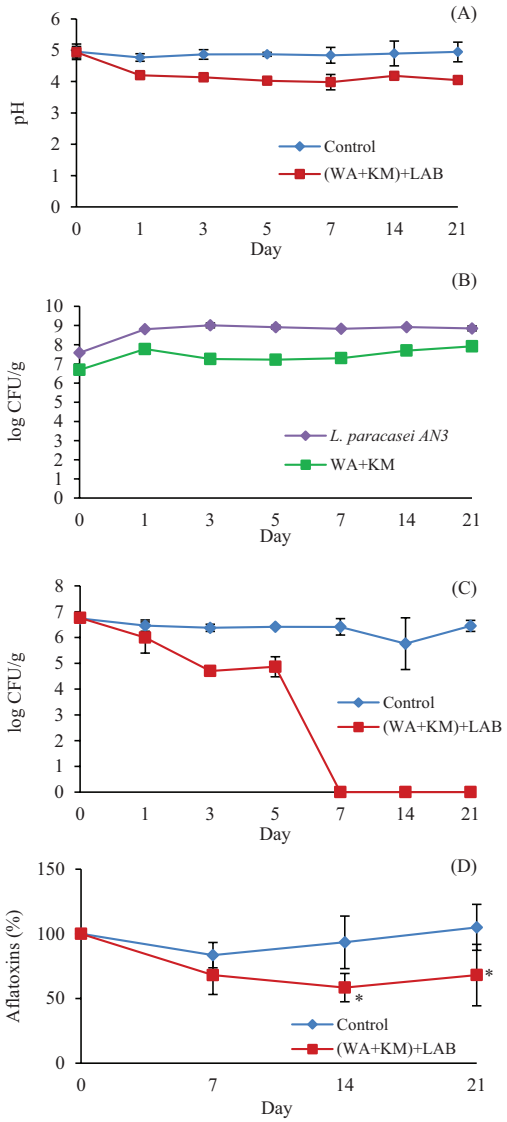

Fig. 2 Interaction of yeasts, *Lactobacillus paracasei* AN3, and *Aspergillus flavus* in sterile corn silage with combinations of initial inoculants of lactic acid bacterium (LAB) and mixed yeast culture in silage of 1×10^6:1×10^6 CFU/g: (A) pH; (B) number of mixed yeast culture (*Wickerhamomyces anomalus* MSCU 0652 (WA) and *Kluyveromyces marxianus* MSCU 0655 (KM)) and *L. paracasei* AN3 (LAB); (C) number of *A. flavus*; (D) aflatoxin production, sterile corn silage inoculated with only *A. flavus* used as control, where data were expressed as mean ± SD derived from three independent experiments, * indicates significant ($p < 0.05$) differences in aflatoxin reduction between the yeast/LAB treatment and control at each time point

was smaller than the *L. paracasei* AN3 population (Fig. 2B). Yeast as a facultative anaerobic microorganism tends to grow more slowly during the ensiling process when the oxygen content is depleted.

The mixed culture of yeasts and *L. paracasei* AN3 showed great antifungal activity against *A. flavus* growth. *A. flavus* was completely inhibited in the early stage of ensiling on day 7 through to the end of the process. The sterile silage had a significant ($p < 0.05$) aflatoxin reduction of 37.49% at 14 d of fermentation, compared to the control inoculated only with *A. flavus* (Fig. 2D). Bello et al. (2007) reported that *L. plantarum* FST 1.7 increased the shelf-life of wheat bread and produced some antifungal compounds to inhibit the growth of *F. culmorum* and *F. graminearum* on bread. Dogi et al. (2015) found that *L. rhamnosus* RC007 was able to inhibit mycotoxicogenic fungi, including *A. fumigatus*, *A. flavus* and *A. parasiticus*, and rapidly reduced the pH in the corn silage fermentation process until exposure to air. The study of Ponsone et al. (2011) showed the capability of *K. thermotolerans* to reduce 3–100% of growth and 11–82.5% of ochratoxin A production of *A. carbonarius* and *A. niger* in grape. Etcheverry et al. (2009) showed that *Kluyveromyces* sp. L16 was able to reduce the growth rates of *F. verticillioides* and *A. flavus* in maize soil and to decrease the percentage of infection on maize ears. Niba et al. (2014) reported that inoculating maize with *W. anomalus* inhibited the growth of fungi, such as *Paecilomyces variotii* and *A. melleous,* after 2 mth of storage thus reducing the risk of mycotoxin contamination in maize.

Interaction of mixed culture of yeasts (W. anomalus MSCU 0652 and K. marxianus MSCU 0655), L. paracasei AN3, A. flavus and indigenous microorganisms in silage (non-sterile silage)

For the non-sterile silage condition, an experiment was conducted with the combination of initial inoculants of LAB and mixed yeast culture of 1×10^6:1×10^6 CFU/g as for the sterile silage condition. The preliminary results indicated the influence of indigenous microorganisms leading to unpromising results. Thus this ratio was not continued. Fig. 3A–B shows the changes in the pH of corn silage during the fermentation process. There were no significant differences in the pH of silage inoculated with mixed cultures of yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 at different initial concentration ratios of yeasts:*L. paracasei* AN3 (1×10^6:1×10^8 CFU/g and 1×10^8:1×10^6 CFU/g). The treated silage conferred a rapid reduction in pH from the first day of ensiling (from pH 5 to 4) and the pH value remained stable (at 4) throughout fermentation. These results were similar to those for the control silage (Figs. 3A–3B). Similarly, the study of Basso et al. (2012) reported that the reduction in the pH of corn silage inoculated with different doses of *L. buchneri* did not differ from the control silage. Muck (2004) reported that the pH values of silage treated with homofermentative and heterofermentative lactic acid bacteria were not significantly different from that of untreated silage.

The numbers of coliform bacteria, total yeasts and total LAB in silage during the fermentation process are shown in Table 2. The number of coliform bacteria in all treated silages was counted only before starting the fermentation process (day 0) and they were not detectable throughout ensiling. The counts of total LAB and total yeasts in all treated silages rapidly increased within 24 hr after starting the process. However, the numbers of these microorganisms in silage inoculated with mixed culture of yeasts and *L. paracasei* AN3 were higher than that in the control and slightly decreased at the end of ensiling.

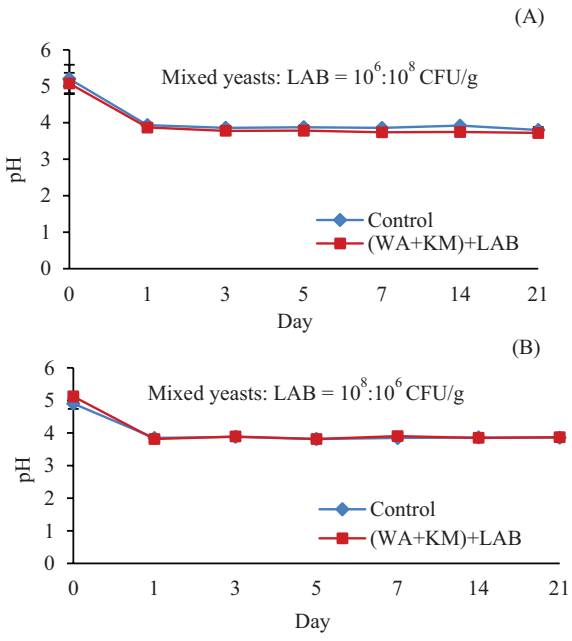

Fig. 3 pH of non-sterile corn silage inoculated with initial concentration ratio of yeasts (*Wickerhamomyces anomalus* MSCU 0652 (WA) and *Kluyveromyces marxianus* MSCU 0655 (KM)): *Lactobacillus paracasei* AN3 (LAB) during 21 d of ensiling, non-sterile corn silage inoculated with only *Aspergillus flavus* was used as control: (A) 1×10^6:1×10^8 CFU/g; (B) 1×10^8:1×10^6 CFU/g, where data were expressed as mean ± SD derived from three independent experiments

Table 2 Microbial populations in non-sterile corn silage inoculated with initial concentration ratio of yeasts (*Wickerhamomyces anomalus* MSCU 0652 and *Kluyveromyces marxianus* MSCU 0655): *Lactobacillus paracasei* AN3 (1×10^6:1×10^8 CFU/g and 1×10^8:1×10^6 CFU/g) during 21 d of ensiling incubated at room temperature as around 30–35 °C

Date	1×10^6:1×10^8 CFU/g						1×10^8:1×10^6 CFU/g					
	Coliform bacteria (logCFU/g)		LAB (logCFU/g)		Yeasts (logCFU/g)		Coliform bacteria (logCFU/g)		LAB (logCFU/g)		Yeasts (logCFU/g)	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
0	5.73±0.25	6.02±0.47	6.66±0.45	9.75±0.06	3.52±3.05	6.46±0.33	3.87±0.58	4.37±0.83	6.69±0.10	8.51±0.02	5.72±0.69	9.42±0.46
1	ND	ND	8.45±0.57	10.35±0.34	6.75±0.01	7.71±0.21	ND	ND	8.00±0.07	9.21±0.05	7.60±0.51	9.65±0.51
3	ND	ND	8.34±0.64	10.58±0.28	6.62±0.05	7.29±0.27	ND	ND	8.30±0.69	9.13±0.05	7.72±0.09	9.29±0.15
5	ND	ND	8.69±0.06	10.41±0.32	6.99±0.78	7.50±0.02	ND	ND	8.50±0.22	9.01±0.05	7.47±0.09	9.37±0.10
7	ND	ND	8.56±0.08	10.52±0.50	7.02±0.54	7.59±0.41	ND	ND	8.50±0.07	9.20±0.16	7.55±0.21	9.46±0.07
14	ND	ND	8.18±0.55	10.15±0.03	6.80±0.25	7.63±0.08	ND	ND	8.27±0.13	8.88±0.25	7.57±0.24	9.27±0.10
21	ND	ND	7.90±0.63	10.00±0.12	6.91±0.66	7.61±0.04	ND	ND	8.01±0.41	8.90±0.60	7.41±0.61	8.61±0.34

CFU = colony forming units; LAB = lactic acid bacterium; ND = not detected
Data expressed as mean ± SD derived from three independent experiments.

Silage is a method for forage preservation based on lactic acid fermentation under anaerobic conditions by indoginous microorganisms, predominantly LAB including *Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc* (Muck, 2010), involving the conversion of the soluble carbohydrates to organic acids, predominantly lactic acid (Bolsen et al., 1996). The coliform bacteria cannot tolerate low pH conditions, so they are inhibited in silage fermentation (Chen et al., 2005). Driehuis (2013) reported that bacteria in the *Enterobacteriaceae* family were inhibited in the early stage of ensiling when the pH rapidly decreased below 4.5. Yeasts, for example, *S. cerevisiae*, *W. anomalus* and species of *Torulopsis, Candida*, *Issatchenkia*, *Hansenula* and *Endomycopsis*, are commonly found in silage (Santos et al., 2017). The study of Olstorpe and Passoth (2011) found that *W. anomalus* was capable of producing ethyl acetate from glucose metabolism and subsequently reduced the amount of *Enterobacteriaceae* growth in grain during storage. Welin et al. (2015) reported the impact of *W. anomalus* and LAB starter cultures in crimped barley. Their results showed that LAB were found in high proportions and became dominant until the end of fermentation. On the other hand, the proportion of *W. anomalus* decreased and was outgrown by yeast-flora. However, the growth of yeast-flora did not affect storage stability.

As shown in Fig. 4, the number of total fungi in silage inoculated with a mixed culture of yeasts and *L. paracasei* AN3 at the ratio of 1×10^6:1×10^8 CFU/g rapidly reduced in the first day (3.78±0.95 log CFU/g), and fungi were completely inhibited after 5 d of ensiling. This reduction was greater than in silage inoculated at the ratio of 1×10^8:1×10^6 CFU/g, in that the fungi were still observed during the fermentation process (Fig. 4). The inhibition of fungal growth at the beginning of the fermentation process is due to yeast activity occurring with the remaining air in the system. After that, the silage runs out of oxygen causing anaerobic conditions that induce LAB activity to inhibit the fungal growth (Cheli et al., 2013). However, in the silage inoculated with a mixed culture of yeasts and *L. paracasei* AN3 with the ratio of 1×10^8:1×10^6 CFU/g (Fig. 4B), the initial number of *L. paracasei* AN3 was significantly lower than in the silage inoculated with the ratio 1×10^6:1×10^8 CFU/g (Fig. 4A). Therefore, it was possible that the low initial number of *L. paracasei* AN3 could not completely inhibit the growth of *A. flavus*, which allowed the fungus to rebound in the stage of ensiling, as seen in Fig. 4B. These results indicated that the LAB played a key role in fungal inhibition. Lactic acid bacteria can produce a variety of metabolites to inhibit the growth of fungi, such as hydrogen peroxide, organic acids (such as lactic and propionic

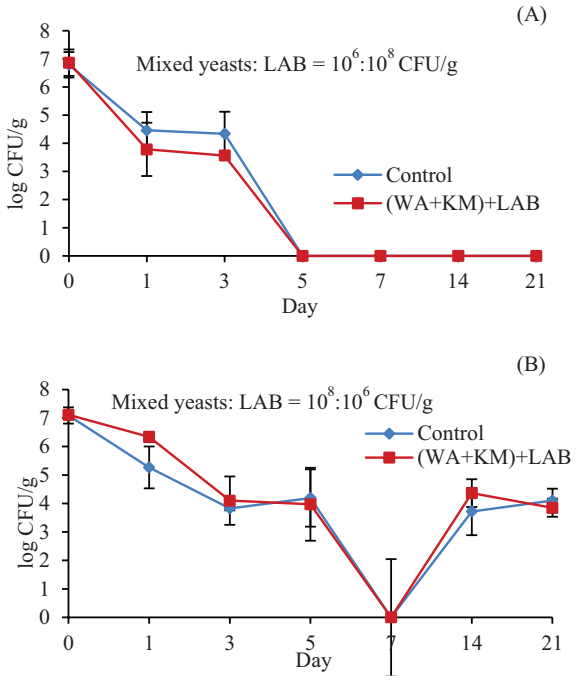

Fig. 4 Number of total fungi in non-sterile corn silage inoculated with initial concentration ratio of yeasts (*Wickerhamomyces anomalus* MSCU 0652 (WA) and *Kluyveromyces marxianus* MSCU 0655 (KM)): *Lactobacillus paracasei* AN3 (LAB) during 21 d of ensiling, non-sterile corn silage inoculated with only *Aspergillus flavus* was used as control: (A) 1×10^6:1×10^8 CFU/g; (B) 1×10^8:1×10^6 CFU/g where data were expressed as mean ± SD derived from three independent experiments

acid), phenyllactic acid (3-phenyllacticacid), cyclic dipeptides, such as cyclo(L-Leu-L-Pro)) and cyclo(L-Phe-L-Pro), and proteinaceous compounds (Magnusson and Schnurer, 2001; Bello et al., 2007; Trias et al., 2008; Arasu et al., 2013). However, the results for the treated silage were not different from the control silage. The inoculum size of the mixed culture of yeasts and *L. paracasei* AN3 could affect the inhibitory activity. Pereyra et al. (2008) reported that the adjustment of concentration of the yeast inoculum was related to the antifungal activity and metabolites of yeasts. Wang et al. (2008) reported that the percentage of infection in cherry tomato fruits treated by a yeast inoculum size of 1×10^9 cells/mL was reduced to 37% compared to the control after 5 d of incubation. In addition, the growth of fungi in silage could be inhibited by antagonistic yeasts that produced metabolites, such as volatile organic compounds (2-phenylethanol, 1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol), and enzymes (β-1,3-glucanase, protease, phytase and pectinase) or by competition for nutrition and space to reduce fungal growth (Wilson et al., 1991; Huang et al., 2011; Olstorpe and Passoth, 2011; Hua et al., 2014). One of the most important approaches to maintaining good quality of silage is to exclude oxygen while packing the silage into bags (Elferink et al., 2000), which prevents poor storage that leads to undesirable growth of aerobic microorganisms (Garon et al., 2006). Yeasts can grow in a low oxygen environment and can tolerate the low pH conditions of silage and reduce the level of oxygen trapped on the surface of the silage (Druvefors et al., 2002; Niba et al., 2014).

The aflatoxin contents in all tests were reduced compared to the control silage, particularly the silage inoculated with the mixed culture of yeasts and *L. paracasei* AN3 at the ratio of 1×10^6:1×10^8 CFU/g that conferred the significantly highest aflatoxin reduction of 63.94% at day 7 of ensiling (Fig. 5). The antifungal activity against growth and aflatoxin production of LAB also depended on their number in the inoculum (Gerez et al., 2010). Bueno et al. (2007) reported that the potential of aflatoxin binding of lactic acid bacteria and *S. cerevisiae* depended on the toxin and microbial concentrations in the sense that the higher LAB concentration could remove more aflatoxin. Similar to the current study, the silage inoculated with the higher concentration of the lactic acid bacterium *L. paracasei* AN3 showed the greatest antifungal activity and capability of reducing aflatoxin production in silage.

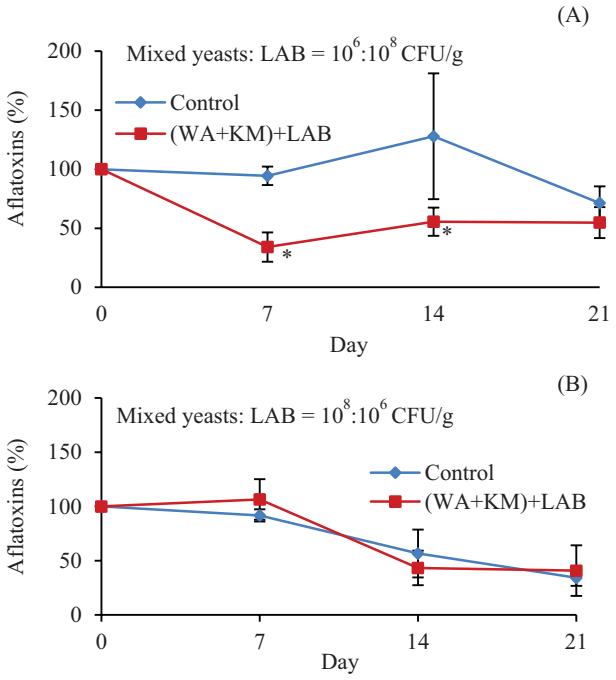

Fig. 5 Aflatoxins in non-sterile corn silage inoculated with initial concentration ratio of yeasts (*Wickerhamomyces anomalus* MSCU 0652 (WA) and *Kluyveromyces marxianus* MSCU 0655 (KM)): *Lactobacillus paracasei* AN3 (LAB) during 21 d of ensiling, non-sterile corn silage inoculated with only *Aspergillus flavus* was used as control: (A) 1×10^6:1×10^8 CFU/g; (B) 1×10^8:1×10^6 CFU/g, where data expressed as mean ± SD and derived from three independent experiments, * indicates significant ($p < 0.05$) differences of aflatoxin reduction between the yeast/lactic acid bacterium(LAB) treatment and control at each time point

Gourama (1997) reported that some metabolites in supernatant from *L. casei* culture had the potential to inhibit patulin and citrinin production of *P. citrinum* and *P. expansum*. Not only could antifungal metabolites of LAB inhibit fungal growth and reduce mycotoxin production, but the cell wall of LAB also had potential to remove contaminated mycotoxin by physical mechanisms, such as aflatoxin binding to the peptidoglycan of LAB cell walls (Lahtinen et al., 2004). Haskard et al. (2001) reported that the *L. rhamnosus* strain GG and *L. rhamnosus* strain LC-705 had the greatest capacity to rapidly removed aflatoxin B_1 from the solution, at about 80% (El-Nezami et al., 1998). The mixed culture of yeasts in the current study might have supported the antagonistic microorganism with the LAB to inhibit the growth and aflatoxin content on the surface of silage. Petersson et al. (1998) found that a co-culture of *P. anomala* and *S. cerevisiae* reduced the accumulation of ochratoxin A of *P. verrucosum in vitro* and in wheat. *S. cerevisiae*, *G. fermentans*, *K. marxianus* and *M. pulcherrima* were able to completely detoxify aflatoxins in wheat flour and a composite fodder for sucker pigs (Repečkienė et al., 2013). Hence, yeasts and lactic acid bacteria have a high mycotoxin binding capacity to minimize the mycotoxin content in food and feed exposed to humans and animals (Shetty and Jespersen, 2006).

Nutritional values of silage

The nutritional values of silage inoculated with mixed culture of yeasts and *L. paracasei* AN3 are shown in Table 3. The dry matter (DM) content at the end of the process for the control and tested silages slightly decreased during silage fermentation compared to the control silage at day 0 (fresh corn). There were no significant differences in any of the nutrition parameters between the inoculated silage and the control at each particular time, until the end of ensiling on day 21. During LAB fermentation, the soluble carbohydrates were converted to lactic acid, ethanol, CO_2 and water, as observed by the slight losses of DM and energy. Jatkauskas and Vrotniakiene (2011) reported that the inoculum of homofermentative LAB conferred a lower final pH and reduced the DM loss in silage. Likewise, Clavero and Razz (2002) found that LAB increased the DM content while lowering the NDF and ADF contents, resulting in enhanced silage quality. In contrast, some studies reported that microbial additives did not affect the NDF and ADF contents in silage (Ridla and Uchida, 1999; Kung, 2018; Nowak et al., 2004). In addition, the total energy was determined using a bomb calorimeter according to the manufacturer's instructions. The total energy did not differ between the control and tested silages within the entire range of 4,000–42,000 cal/g DM (data not shown).

Table 3 Nutritional values of non-sterile corn silage inoculated with mixed culture of yeasts (*Wickerhamomyces anomalus* MSCU 0652 and *Kluyveromyces marxianus* MSCU 0655) and *Lactobacillus paracasei* AN3 (test) with initial concentration ratio 1×10^6:1×10^8 CFU/g and un-inoculated silage (control) incubated at room temperature at around 30–35 °C for 21 d

Variable (%)	Sample							
	Day0		Day 7		Day 14		Day 21	
	Control (fresh corn)	Test	Control	Test	Control	Test	Control	Test
Dry matter	24.65±0.01	24.13±0.17	21.67±1.12	21.66±0.61	23.00±2.14	22.13±2.28	21.89±2.73	21.13±1.48
Crude protein	8.73±0.87	8.68±0.67	8.80±0.28	9.17±0.11	9.56±0.90	9.14±0.32	9.30±0.46	9.78±0.76
Crude fat	1.40±0.25	1.56±0.22	2.12±0.60	1.96±0.49	1.94±0.94	1.80±0.22	2.14±0.66	2.04±0.49
Crude fiber	26.42±0.40	26.63±0.33	27.31±0.91	27.62±0.02	28.64±1.10	29.59±3.03	29.55±0.98	31.00±0.59
Neutral detergent fiber	56.64±0.23	58.53±3.49	62.44±0.80	62.41±2.74	63.85±3.04	63.15±0.91	64.82±2.91	65.77±4.45
Acid detergent fiber	35.50±4.59	37.81±8.12	37.65±6.52	37.59±4.53	38.75±4.12	39.24±0.83	39.22±2.69	39.52±2.29
Calcium	0.35±0.09	0.30±0.04	0.36±0.01	0.39±0.05	0.49±0.03	0.42±0.04	0.43±0.04	0.47±0.02
Phosphorus	0.20±0.06	0.20±0.08	0.25±0.09	0.21±0.05	0.27±0.01	0.24±0.01	0.24±0.01	0.27±0.05

Data expressed as mean ± SD derived from three independent experiments.
Non-significant ($p > 0.05$) differences between control and test means for each variable at each time point

From the results, the mixed culture of yeasts (*W. anomalus* MSCU 0652 and *K. marxianus* MSCU 0655) and *L. paracasei* AN3 showed antifungal activity both in *in vitro* and in corn silage. The concentration ratio of 1×10^6: 1×10^8 CFU/g was the optimal concentration ratio with the greatest inhibition against growth and aflatoxin production of *A. flavus* in silage. The high concentration of *L. paracasei* AN3 played a key role in the ensiling process and enhanced the antifungal activity in corn silage. The mixed culture of yeasts could reduce the oxygen content and competed with fungi for nutrition and space in silage. Therefore, such co-culture at the optimal concentration ratio of 1×10^6:1×10^8 CFU/g had potential to be used as a biocontrol agent in silage to improve silage quality and to reduce the risk of fungal and mycotoxin contamination in feed that could affect human and animal health.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by the Agricultural Research Development Agency (Public Organization), ARDR (CRP5805010710) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphort Endowment Fund) under Grant GCUGR1125612073M no. 64.

References

Arasu, M.V., Jung, M.W., Ilavenil, S., et al. 2013. Isolation and characterization of antifungal compound from *Lactobacillus plantarum* KCC-10 from forage silage with potential beneficial properties. J. Appl. Microbiol. 115: 1172–1185. doi.org/10.1111/jam.12319

Armando, M.R., Dogi, C.A., Poloni, V., Rosa, C.A., Dalcero, A.M., Cavaglieri, L.R. 2013. *In vitro* study on the effect of *Saccharomyces cerevisiae* strains on growth and mycotoxin production by *Aspergillus carbonarius* and *Fusarium graminearum*. Int. J. Food Microbiol. 161: 182–188. doi.org/10.1016/j.ijfoodmicro.2012.11.016

Armando, M.R., Dogi, C.A., Rosa, C.A, Dalcero, A.M., Cavaglieri, L.R. 2012a. *Saccharomyces cerevisiae* strains and the reduction of *Aspergillus parasiticus* growth and aflatoxin B1 production at different interacting environmental conditions, *in vitro*. Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess. 29: 1443–1449. doi.org/10.1080/19440049.2012.698655

Armando, M.R., Pizzolitto, R.P., Dogi, C.A., Cristofolini, A., Merkis, C., Poloni, V., Dalcero, A.M., Cavaglieri, L.R. 2012b. Adsorption of ochratoxin A and zearalenone by potential probiotic *Saccharomyces cerevisiae* strains and its relation with cell wall thickness. J. Appl. Microbiol. 113: 256–264. doi.org/10.1111/j.1365-2672.2012.05331.x

Association of Official Analytical Chemists. 2010. Official Methods of Analysis of AOAC International Volume I, Agricultural Chemicals, Contaminants, Drugs. Gaithersburg, MD, USA.

Bankole, S.A., Adenusi, A.A., Lawal, O.S., Adesanya, O.O. 2010. Occurrence of aflatoxin B1 in food products derivable from 'egusi' melon seeds consumed in southwestern Nigeria. Food Control 21: 974–976. doi.org/10.1016/j.foodcont.2009.11.014

Basso, F.C., Bernardes, T.F., Roth, A.P.T., Lodo, B.N., Berchielli, T.T., Reis, R.A. 2012. Fermentation and aerobic stability of corn silage inoculated with *Lactobacillus buchneri*. R. Bras. Zootec. 41: 1789–1794. doi.org/10.1590/S1516-35982012000700032

Bello, F.D., Clarke, C.I., Ryan, L.A.M., et al. 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. J. Cereal Sci. 45: 309–318. doi.org/10.1016/j.jcs.2006.09.004

Bintsis, T. 2018. Lactic acid bacteria as starter culture: An update in their metabolism and genetics. AIMS Microbiol. 4 :665–684.

Blankson, G.K., Mill-Robertson, F.C. 2016. Aflatoxin contamination and exposure in processed cereal-based complementary foods for infants and young children in greater Accra, Ghana. Food Control 64: 212–217. doi.org/10.1016/j.foodcont.2015.12.032

Bolsen, K.K., Ashbell, G., Weinberg, Z.G. 1996. Silage fermentation and silage additives- Review. Asian Australas. J. Anim. Sci. 9: 483–494. doi.org/10.5713/ajas.1996.483

Bueno, D.J., Casale, C.H., Pizzolitto, R.P., Salvano, M.A., Oliver, G. 2007. Physical adsorption of aflatoxin B1 by lactic acid bacteria and *Saccharomyces cerevisiae*: A theoretical model. J. Food Prot. 70: 2148–2154. doi.org/10.4315/0362-028X-70.9.2148

Cheli, F., Campagnoli, A., Dell'Orto, V. 2013. Fungal populations and mycotoxins in silage: from occurrence to analysis. Anim. Feed Sci. Technol. 183: 1–16. doi.org/10.1016/j.anifeedsci.2013.01.013

Chen, Y., Sela, S., Gamburg, M., Pinto, R., Weinberg, Z.G. 2005. Fate of *Escherichia coli* during ensiling of wheat and corn. Appl. Environ. Microbiol. 71: 5163–5170. doi: 10.1128/AEM.71.9.5163-5170.2005

Clavero, T., Razz, R. 2002. Effects of biological additives on silage composition of mott dwarf elephantgrass and animal performance. Revista Científica de Verarunaria 12: 313–316.

Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M. 2011. Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: Identification of novel compounds and long-term effect during storage of wheat bread. Appl. Environ. Microbiol. 77: 3484–3492. doi.org/10.1128/AEM.02669-10

Dogi, C.A., Pellegrino, M., Poloni, V., et al. 2015. Efficacy of corn silage inoculants on the fermentation quality under farm conditions and their influence on *Aspergillus parasitucus*, *A. flavus* and *A. fumigatus* determined by q-PCR. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 32: 229–235. doi.org/10.1080/19440049.2014.986223

Driehuis, F. 2013. Silage and the safety and quality of dairy foods: A review. Agr. Food Sci. 22: 16–34.

Driehuis, F., Elferink, S.J.O. 2000. The impact of the quality of silage on animal health and food safety: A review. Vet. Q. 22: 212–216. doi.org/10.1080/01652176.2000.9695061

Druvefors, U., Jonsson, N., Boysen, M.E., Schnürer, J. 2002. Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. FEMS Yeast Res. 2: 389–394. doi.org/10.1016/S1567-1356(02)00091-0

Dunière, L., Sindou, J., Chaucheyras-Durand, F., Chevallier, I., Thévenot-Sergentet, D. 2013. Silage processing and strategies to prevent persistence of undesirable microorganisms. Anim. Feed Sci. Technol. 182: 1–15. doi.org/10.1016/j.anifeedsci.2013.04.006

Ehrlich, K.C. 2014. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: Advantages and limitations. Front. Microbiol. 5: 50. doi.org/10.3389/fmicb.2014.00050

El-Nezami, H., Kankaanpaa, P., Salminen, S., Ahokas, J. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. Food Chem. Toxicol. 36: 321–326. doi.org/10.1016/S0278-6915(97)00160-9

Elferink, S., Driehuis, F., Gottschal, J.C., Spoelstra, S.F. 2000. Silage fermentation processes and their manipulation. FAO Plant Production and Protection Papers. 161: 17–30.

Etcheverry, M.G., Scandolara, A., Nesci, A., Vilas Boas Ribeiro, M.S., Pereira, P., Battilani, P. 2009. Biological interactions to select biocontrol agents against toxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides* from maize. Mycopathologia 167: 287–295. doi.org/10.1007/s11046-008-9177-1

Fiori, S., Urgeghe, P.P., Hammami, W., Razzu, S., Jaoua, S., Migheli, Q. 2014. Biocontrol activity of four non- and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. Int. J. Food Microbiol. 189: 45–50. doi.org/10.1016/j.ijfoodmicro.2014.07.020

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K. 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79: 339–354. doi.org/10.1007/s00253-008-1458-6

Garon, D., Richard, E., Sage, L., Bouchart, V., Pottier, D., Lebailly, P. 2006. Mycoflora and multimycotoxin detection in corn silage: Experimental study. J. Agric. Food Chem. 54: 3479–3484. doi.org/10.1021/jf060179i

Geng, P., Chen, S., Hu, M., Rizwan-Ul-Haq, M., Lai, K., Qu, F., Zhang, Y. 2011. Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit. Int. J. Food Microbiol. 151: 190–194. doi.org/10.1016/j.ijfoodmicro.2011.08.023

Gerez, C.L., Carbajo, M.S., Rollan, G., Torres Leal, G., Font de Valdez, G. 2010. Inhibition of citrus fungal pathogens by using lactic acid bacteria. J. Food Sci. 75: M354–M359. doi.org/10.1111/j.1750-3841.2010.01671.x

Goering, H.K., Van Soest, P.J. 1970. Forage Fiber Ananlysis: Apparatus, Reagents, Pocedures and Applications. USDA Agricultural Research Service. Washington DC, USA.

Gomah, N., Zohri, A. 2014. Inhibition of fungal growth and *Fusarium* toxins by selected cultures of lactic acid bacteria. J. Microbial. Biochem. Technol. https://doi.org/10.4172/1948-5948.S7-001

Gourama, H. 1997. Inhibition of growth and mycotoxin production of *Penicillium* by *Lactobacillus* species. LWT Food Sci. Technol. 30: 279–283. doi.org/10.1006/fstl.1996.0183

Haskard, C., Binnion, C., Ahokas, J. 2000. Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chem-Biol. Interact. 128: 39–49. doi.org/10.1016/S0009-2797(00)00186-1

Haskard, C.A., El-Nezami, H.S., Kankaanpaa, P.E., Salminen, S., Ahokas, J.T. 2001. Surface binding of aflatoxin B(1) by lactic acid bacteria. Appl. Environ. Microbiol. 67: 3086–3091. doi.org/10.1128/AEM.67.7.3086-3091.2001

Hua, S.S., Beck, J.J., Sarreal, S.B., Gee, W. 2014. The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. Mycotoxin Res. 30: 71–78. doi.org/10.1007/s12550-014-0189-z

Huang, R., Li, G., Zhang, J., Yang, L., Che, H., Jiang, D., Huang, H. 2011. Control of postharvest Botrytis fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. Phytopathology 101: 859–869. doi.org/10.1094/PHYTO-09-10-0255

Jaibangyang, S., Nasanit, R., Limtong, S. 2020. Biological control of aflatoxin-producing *Aspergillus flavus* by volatile organic compound-producing antagonistic yeasts. BioControl 65: 337–386. doi.org/10.1007/s10526-020-09996-9

Jatkauskas, J., Vrotniakiene, V. 2011. The effects of silage inoculants on the fermentation and aerobic stability of legume-grass silage. Zemdirbyste Agriculture 98: 367–374.

Klein, G., Pack, A., Bonaparte, C., Reuter, G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. 41: 103–125. doi.org/10.1016/S0168-1605(98)00049-X

Kumar, S.N., Mohandas, C., Nambisan, B. 2013. Purification of an antifungal compound, cyclo(l-Pro-d-Leu) for cereals produced by *Bacillus cereus* subsp. *thuringiensis* associated with entomopathogenic nematode. Microbiol. Res. 168: 278–288. doi.org/10.1016/j.micres.2012.12.003

Kung, L. 2018. Silage fermentation and additives. Latin American Archives of Animal Production 26: 61–66.

Lahtinen, S.J., Haskard, C.A., Ouwehand, A.C., Salminen, S.J., Ahokas, J.T. 2004. Binding of aflatoxin B1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Addit. Contam. 21: 158–164. doi.org/10.1080/02652030310001639521

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M. 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. 66: 4084–4090. doi.org/10.1128/AEM.66.9.4084-4090.2000

Li, H., Zhang, S., Lu, J., et al. 2014. Antifungal activities and effect of *Lactobacillus casei* AST18 on the mycelia morphology and ultrastructure of *Penicillium chrysogenum*. Food Control 43: 57–64. doi.org/10.1016/j.foodcont.2014.02.045

Magnusson, J., Schnurer, J. 2001. *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67: 1–5. doi.org/10.1128/AEM.67.1.1-5.2001

Magnusson, J., Strom, K., Roos, S., Sjogren, J., Schnurer, J. 2003. Broad and complex antifungal activity among environmental isolates of lactic

acid bacteria. FEMS Microbiol. Lett. 219: 129–135. doi.org/10.1016/S0378-1097(02)01207-7

Maroongrung, P., Chareonpornwattana, S., Sirisomboon Dachoupakan, C. 2016. *In vitro* study on the effect of antagonistic yeast from corn silage on growth and aflatoxin B1 production by *Aspergillus flavus*. In: Proceeding of the 28th Annual Meeting of the Thai Society for Biotechnology. Chiang Mai, Thailand.

Medina-Córdova, N., López-Aguilar, R., Ascencio, F., Castellanos, T., Campa-Córdova, A.I., Ângulo, C. 2016. Biocontrol activity of the marine yeast *Debaryomyces hansenii* against phytopathogenic fungi and its ability to inhibit mycotoxins production in maize grain (*Zea mays* L.). Biol Contr. 97: 70–79. doi.org/10.1016/j.biocontrol.2016.03.006

Muck, R.E. 2004. Effects of corn silage inoculants on aerobic stability. Transactions of the ASAE 47: 1011–1016. doi: 10.13031/2013.16571

Muck, R.E. 2010. Silage microbiology and its control through additives. R. Bras. Zootec. 39: 183–191. doi.org/10.1590/S1516-35982010001300021

Murphy, P.A., Hendrich, S., Landgren, C., Bryant, C.M. 2006. Food mycotoxins: An update. J. Food Sci. 71: R51–R65. doi.org/10.1111/j.1750-3841.2006.00052.x

Niba, A.T., Su-lin, L.L., Olstorpe, M. 2014. Biocontrol efficacy of *Wickerhamomyces anomalus* in moist maize storage. Afr. J. Biotechnol. 13: 4208–4214. doi.org/10.5897/AJB2014.13755

Nowak, W., Potkanski, A., Wylegala, S. 2004. The effect of additives on quality and nutrient degradability and digestibility of round bale silage. S. Afr. J. Anim. Sci. 34: 123–129.

Olstorpe, M., Passoth, V., 2011. *Pichia anomala* in grain biopreservation. Antonie Van Leeuwenhoek 99: 57–62. doi.org/10.1007/s10482-010-9497-2

Olvera-Novoa, M.A., MartÍNez-Palacios, C.A., Olivera-Castillo, L. 2002. Utilization of torula yeast (*Candida utilis*) as a protein source in diets for tilapia (*Oreochromis mossambicus* Peters) fry. Aquacult. Nutr. 8: 257–264. doi.org/10.1046/j.1365-2095.2002.00215.x

Oro, L., Feliziani, Eb, Ciani, M., Romanazzi, G., Comitini, F. 2018. Volatile organic compounds from *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* inhibit growth of decay causing fungi and control postharvest diseases of strawberries. Int. J. Food Microbiol. 265: 18–22. doi.org/10.1016/j.ijfoodmicro.2017.10.027

Pantelides, I.S., Christou, O., Tsolakidou, M.D., Tsaltas, D., Ioannou, N. 2015. Isolation, identification and *in vitro* screening of grapevine yeasts for the control of black aspergilli on grapes. BiolControl 88: 46–53. doi.org/10.1016/j.biocontrol.2015.04.021

Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G. 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiol. 47: 85–92. doi.org/10.1016/j.fm.2014.11.013

Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J., Salminen, S. 2001. Aflatoxin B_1 binding by dairy strains of lactic acid bacteria and bifidobacteria. J. Dairy Sci. 84: 2152–2156. doi.org/10.3168/jds.S0022-0302(01)74660-7

Petersson, S., Hansen, M.W., Axberg, K., Hult, K., SchnÜRer, J. 1998. Ochratoxin A accumulation in cultures of *Penicillium verrucosum* with the antagonistic yeast *Pichia anomala* and *Saccharomyces cerevisiae*. Mycol. Res. 102: 1003–1008. doi.org/10.1017/S0953756297006047

Pereyra, M.L.G, Alonso, V.A., Sager, R., et al. 2008. Fungi and selected mycotoxins from pre- and postfermented corn silage. J. Appl. Microbiol. 104: 1034–1041. doi.org/10.1111/j.1365-2672.2007.03634.x

Ponsone, M.L., Chiotta, M.L., Combina, M., Dalcero, A., Chulze, S. 2011. Biocontrol as a strategy to reduce the impact of ochratoxin A and *Aspergillus* section *Nigri* in grapes. Int. J. Food Microbiol. 151: 70–77. doi.org/10.1016/j.ijfoodmicro.2011.08.005

Prema, P., Smila, D., Palavesam, A., Immanuel, G. 2008. Production and characterization of an antifungal compound (3-phenyllactic acid) produced by *Lactobacillus plantarum* strain. Food Bioprocess Technol. 3: 379–386. doi.org/10.1007/s11947-008-0127-1

Rather, I.A., Seo, B.J., Rejish Kumar, V.J., Choi, U.H., Choi, K.H., Lim, J.H., Park, Y.H. 2013. Isolation and characterization of a proteinaceous antifungal compound from *Lactobacillus plantarum* YML007 and its application as a food preservative. Lett. Appl. Microbiol. 57: 69–76. doi.org/10.1111/lam.12077

Repečkienė, J., Levinskaitė, L., Paškevičius, A., Raudonienė ,V. 2013. Toxin-producing fungi on feed grains and application of yeasts for their detoxification. Pol. J. Vet. Sci. 16: 391–393. doi: 10.2478/pjvs-2013-0054

Ridla, M., Uchida, S. 1999. Comparative study on the effects of combined treatments of lactic acid bacteria and cellulases on the fermentation characteristic and chemical composition of Rhodesgrass (*Chloris gayana* Kunth.) and Italian ryegrass (*Lolium multiflorum* Lam.) silages. Asian Australas. J. Anim. Sci. 12: 525–530. doi.org/10.5713/ajas.1999.531

Sangmanee, P., Hongpattarakere, T. 2014. Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus*and *Aspergillus parasiticus.* Food Control 40: 224–233. doi.org/10.1016/j.foodcont.2013.12.005

Santos, A.O., Avila, C.L., Schwan, R.F. 2013. Selection of tropical lactic acid bacteria for enhancing the quality of maize silage. J. Dairy Sci. 96: 7777–7789. doi.org/10.3168/jds.2013-6782

Santos, M.C., Golt, C., Joerger, R.D., Mechor, G.D., Mourao, G.B., Kung, L. 2017. Identification of the major yeasts isolated from high moisture corn and corn silages in the United States using genetic and biochemical methods. J. Dairy Sci. 100: 1151–1160. doi.org/10.3168/jds.2016-11450

Schader, C., Muller, A., El-Hang Scialabba, N., et al. 2015. Impacts of feeding less food-competing feedstuffs to livestock on global food system sustainability. J. R. Soc. Interface. 12: 20150891. doi.org/10.1098/rsif.2015.0891

Schisler, D., Slininger, P., Boehm, M.J., Paul, P. 2011. Co-culture of yeast antagonists of fusarium head blight and their effect on disease development in wheat. Plant Pathology J. 10: 128–137. doi: 10.3923/ppj.2011.128.137

Shetty, P.H., Jespersen, L. 2006. *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends Food Sci. Tech. 17: 48–55. doi.org/10.1016/j.tifs.2005.10.004

Trias, R., Baneras, L., Montesinos, E., Badosa, E. 2008. Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. Int. Microbiol. 11: 231–236. doi: 10.2436/20.1501.01.66

Walker, G.M. 2011. *Pichia anomala*: Cell physiology and biotechnology relative to other yeasts. Antonie Van Leeuwenhoek 99: 25–34. doi.org/10.1007/s10482-010-9491-8

Wang, Y., Bao, Y., Shen, D., Feng, W., Yu, T., Zhang, J., Zheng, X.D. 2008. Biocontrol of *Alternaria alternata* on cherry tomato fruit by use of marine yeast *Rhodosporidium paludigenum* Fell & Tallman. Int. J. Food Microbiol. 123: 234–239. doi.org/10.1016/j.ijfoodmicro.2008.02.002

Welin, J.B., Lyberg, K., Passoth, V., Olstorpe, M. 2015. Combined moist airtight storage and feed fermentation of barley by the yeast *Wickerhamomyces anomalus* and a lactic acid bacteria consortium. Front. Plant Sci. 6: 270. doi.org/10.3389/fpls.2015.00270

Wilson, C.L., Wisniewski, M.E., Biles, C.L., McLaughlin, R., Chalutz, E., Droby, S. 1991. Biological control of post-harvest diseases of fruits and vegetables: Alternatives to synthetic fungicides. Crop Prot. 10: 172–177. doi.org/10.1016/0261-2194(91)90039-T

Zain, M.E. 2011. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc. 15: 129–144. doi.org/10.1016/j.jscs.2010.06.006

Zhou, X., Li, Y. 2015. Supragingival microbes. In: Atlas of Oral Microbiology. Academic Press. Chengdu, China, pp. 41–65.