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INHIBITION OF MYCOTOXIN-PRODUCING *Aspergillus nomius* VSC 23 BY LACTIC ACID BACTERIA AND *Saccharomyces cerevisiae*

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ABSTRACT

The effect of different fermenting microorganisms on growth of a mycotoxin- producing *Aspergillus nomius* was assayed. Two lactic acid bacteria, *Lactobacillus fermentum* and *Lactobacillus rhamnosus*, and *Saccharomyces cerevisiae*, all of which are widely used in fermentation and preservation of food, were assayed on their fungus inhibitory properties. Assays were carried out by simultaneous inoculation of one of the possible inhibiting microorganisms and the fungus or subsequent inoculation of one of the microorganisms followed by the fungus. All three microorganisms assayed showed growth inhibition of the mycotoxin-producing *Aspergillus* strain. *L. rhamnosus* O236, isolated from sheep milk and selected for its technological properties, showed highest fungal inhibition of the microorganisms assayed. The use of antifungal LAB with excellent technological properties rather than chemical preservatives would enable the food industry to produce organic food without addition of chemical substances.

Key words: *Aspergillus nomius*, biocontrol, lactic acid bacteria, food preservation

INTRODUCTION

Food and feed spoilage moulds cause great economic losses worldwide. It is estimated that between 5 and 10% of the world's food production is wasted due to fungal deterioration (32). These fungi cause losses in dry matter or quality and some species can produce health-damaging mycotoxins. These toxins comprise a group of chemically diverse compounds originating from secondary metabolism by moulds (filamentous fungi) and are mainly produced by five genera: *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* (36). The compounds can be carcinogenic, hepatotoxic, teratogenic or immunosuppressing (12). Many mycotoxins are stable under normal food processing conditions and can therefore be present not only in food and feed but also in processed products (28). Some food additives as *Origanum vulgare* have an important inhibitory effect on Aspergillus, providing an inhibition of all assayed strains (10).

Mycotoxins could be eliminated after their production or by growth inhibition of the fungus-producing strain. Current strategies to destroy mycotoxins in food include heating, treatment with ammonia, screening and radiation, but they are too expensive, impractical for commercial application or destroy vital nutrients of the grain (17, 25). Therefore, reduction of mould growth during the production and storage of food and feed is of great importance. *Aspergillus* frequently

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produces mycotoxins in grains and consequently represents a direct threat to the crops. The primary method of control is the use of chemical fungicides. However, more of them are nowadays are been not authorized due the toxicological risks (Directive 91/414/CEE of the EU). After consumption of contaminated food, the microorganism also threatens human and animal health. The interaction between mycotoxin-producing fungi and other microorganisms is a common phenomenon in nature that can affect fungal growth and/or production of mycotoxins (25). For this reason, research is now focused, to some degree, on inhibition of fungal growth (2, 4, 19). For many years now it has been clear that the most effective means to prevent contamination of food with mycotoxins is to avoid growth of mycotoxigenic fungi (9). At the same time, the general public demands a reduced use of chemical preservatives and additives in food and feed (7). Some microorganisms have traditionally been used as biopreservatives in food and feed. Biopreservation allows prolonged shelf life and enhanced safety of foods through natural or supplementary microflora and their antimicrobial products (34). Among the different potential decontaminating microorganisms, *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) represent unique groups, which are widely used in food fermentation and preservation (35). Many LAB strains as well as some *S. cerevisiae* var. *bourardii* strains have shown probiotic activity (14). Lactic acid bacteria (LAB) are GRAS (generally recognized as safe) microorganisms that modify the food enhancing quality, and additionally they possess nutritional and therapeutic benefits (20). The majority of the numerous reports on antimicrobial activity by LAB have focused on antibacterial effects (30), whereas there are only few reports on antifungal effects. The adaptability of LAB and yeasts to sourdoughs prepared from cereals, was investigated, the dominant LAB was *Lactobacillus fermentum* and *S. cerevisiae* constituted the dominating yeast (40).

The aim of this work was to assess the effect of different LAB with excellent industrial and health properties and *S. cerevisiae* on growth of a mycotoxigenic *Aspergillus* strain. Assays were carried out using balanced poultry feed as trial medium, in order to resemble the effects of environmental conditions naturally occurring during microbial interaction.

MATERIAL AND METHODS

Strains

Lactobacillus fermentum ssp. *cellobiosus* 408 and *Lactobacillus fermentum* 27A (isolated from feces of healthy chickens) and *Lactobacillus rhamnosus* O236 (isolated from sheep milk), selected for their potential probiotic or technological properties (24), were obtained from the CERELA culture collection. *Aspergillus nomius* VSC 23, a mycotoxin-producing fungus, was provided by Dr. D.J. Bueno (INTA Concepcion del Uruguay, Entre Rios, Argentina). *Saccharomyces cerevisiae*, with a wide application in feed as a protein source of high biological value (Bueno D, Doctoral Thesis, UNT, 2003), was supplied by Calsa quality control laboratory, Tucuman, Argentina.

Growth media

LAB and *S. cerevisiae* were grown in LAPTG, containing (g/L): Yeast extract 10, Peptone 15, Glucose 10, Triptone 10 and Tween 80 1 mL/L, purchased from Britania, Bs As, Argentina; pH 6.5 (control medium). *A. nomius* VSC 23 was grown on potato glucose agar medium at pH 5.5. In order to resemble natural conditions, HBA (avian balanced meal, commercial name Vitosan at BB), a commercial balanced food for birds, was dissolved in distilled water at different concentrations (1, 3 and 5%; hereafter called HBA medium). The medium was heated at 80°C for 30 min, filtered and centrifuged at 13,700 x g for 30 min and pH was adjusted to 6.5 prior to sterilization. Growth of the different microorganisms was assayed at the different concentrations. HBA supplemented with 1.5% agar was used to quantify fungal growth.

Inhibitory effect in HBA medium

Fungal growth was determined at the end of the incubation period by measuring the colony diameter. Growth in the

absence of any of the other microorganisms was considered 100% (control). The percentage of inhibition was calculated by comparing the diameter of the colonies in mixed cultures with the control.

The inhibitory effect was determined on plates using an overlay technique; the potentially inhibitory microorganisms were inoculated first on solid agar and then *A. nomius* was inoculated on top in two different ways: simultaneous and consecutive.

Simultaneous inoculation

LAB and *S. cerevisiae* strains were inoculated at a concentration of 10^6 CFU/mL, mixed with HBA agar and poured into Petri dishes. The center of recently solidified agar containing LAB was inoculated with *Aspergillus* isolated from a solid culture with a calibrated inoculation loop. Petri dishes were incubated at 28 and 37°C during 7 days.

Consecutive inoculation

LAB and *S. cerevisiae* strains were inoculated at a concentration of 10^6 CFU/mL, mixed with HBA agar and poured into Petri dishes as above. Cultures were incubated for 3 days at 37°C, after which a loopful of *Aspergillus* was spread over the agar medium on the dishes, which were then incubated at 28 and 37°C during 7 days.

Sweep Electronic Transmission

In order to identify possible morphologic alterations after growth inhibition of fungal cells obtained from mixed cultures, scanning and transmission electron microscopy was carried out. Cells were fixed by successive immersion in Karnowsky reagent (26) and an osmium tetroxide - uranyl acetate combination.

Transmission Electron Microscopy

Cells were first fixed by immersion in a solution of 2.5% glutaraldehyde and then dehydrated with alcohol solutions of increasing concentration and finally by immersion in acetone for 12 h. Micrographs were performed at the LAMENOA Institute, Universidad Nacional de Tucumán.

RESULTS

Growth in HBA

Growth of LAB and *S.cerevisiae* in different concentrations of experimental HBA medium (1, 3 and 5%) was compared with that in LAPTG medium (Data not show). Highest growth was observed with 3 and 5% HBA medium for both LAB and *Saccharomyces*, and for this reason the lower concentration (3%) was used for further assaying. All LAB strains assayed grew in HBA medium, although growth was lower than in LAPTG medium. Growth of *L. fermentum* 27A in HBA was four times lower than in LAPG at 37°C and 2.5 times lower at 28°C. In contrast, growth of *L. fermentum* ssp *cellobiosus* 408 was only 2-fold lower in HBA compared to LAPTG at 37°C, and at 28° C no difference was observed. Results for *L. rhamnosus* O236 were similar. *S. cerevisiae* grew in 3% HBA at 28 and 37°C, but only showed a decrease of 30% (± 4) in growth at 28°C compared to LAPTG.

A. nomius VSC 23 showed similar growth on 3% HBA agar and potato glucose agar at both temperatures.

Inhibition of *Aspergillus nomius*. Simultaneous inoculation.

Growth inhibition of *A. nomius* VSC 23 after simultaneous inoculation with the microorganisms assayed using the overlay technique is shown in Fig. 1.

At both assay temperatures, *L. rhamnosus* O236 was the most effective microorganism in controlling fungal growth with 75% and 25% inhibition at 37°C and 28°C, respectively (Fig. 2).

At 37°C the probiotic strains from chicken origin, *L. fermentum* 27A and *L. fermentum ssp. cellobiosus* 408, showed fungal inhibition between 36 and 40%, whereas at 28°C inhibition was less than 10% for either strain.

Inhibition of fungal growth by *S. cerevisiae* was between 20 and 10% at both temperatures assayed.

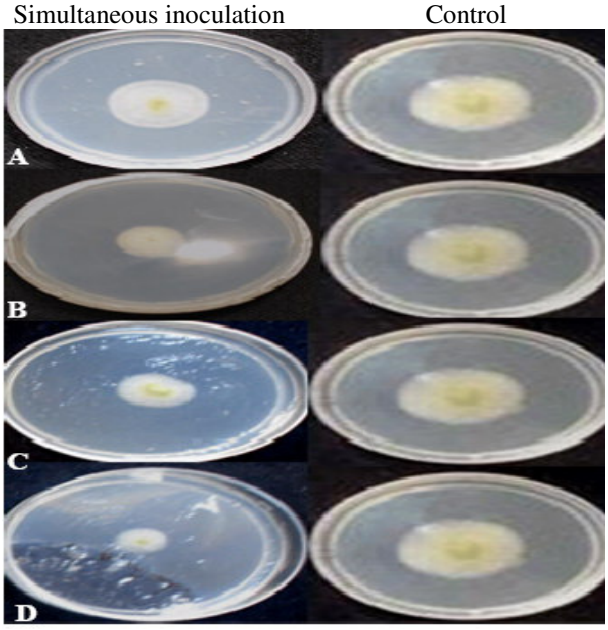

Figure 1. Effect of simultaneous inoculation of LAB and *S. cerevisiae* on *A. nomius* growth. Simultaneous inoculation of *A. nomius* VSC 23 and A) *L. fermentum ssp. cellobiosus* 408, B) *L. fermentum* 27 A, C) *S. cerevisiae* and D) *L. rhamnosus* O236. Plates were incubated at 37°C. Growth of a monoculture of *A. nomius* VSC 23 was used as control.

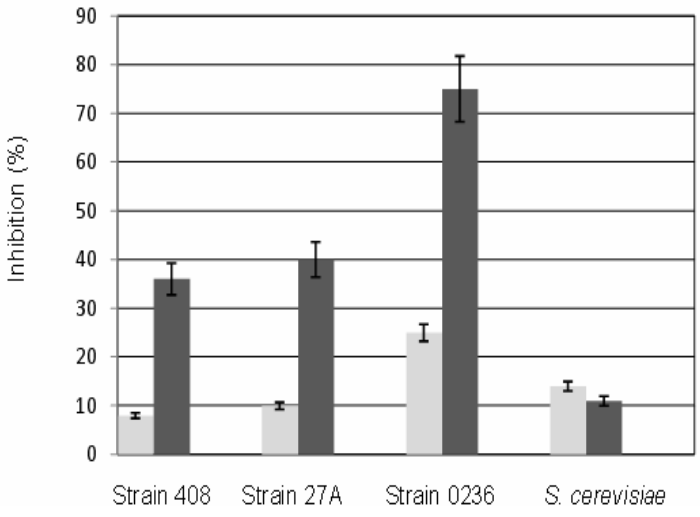

Figure 2. Growth inhibition (%) of *A. nomius* VSC 23 after simultaneous inoculation with *L. fermentum* ssp. *cellobiosus* 408, *L. fermentum* 27A, *L. rhamnosus* O236 and *S. cerevisiae* compared to growth of a monoculture of *A. nomius* VSC 23 growing on the same medium (control). Assays were carried out at 28°C ( ) and at 37°C ( ).

Consecutive inoculation

The effects of consecutive inoculation are shown in Fig. 3 and 4. In the presence of *L. rhamnosus* O236, growth of *Aspergillus* decreased 73% at 37°C. Under the same conditions the inhibitory effect of *L. fermentum* 27a and *L. fermentum ssp. cellobiosus* 408 on the fungus was 47% and 38%, respectively.

At 28°C all LAB strains demonstrated lower inhibitory effects compared with 37ºC. Inhibition by *L. rhamnosus* O236 was 36%, whereas the other two LAB strains showed only a decrease in fungal growth of about 10%.

Neither the incubation temperature nor the trial procedures (simultaneous or consecutive inoculation) seemed to influence the effect of *S. cerevisiae*. The yeast showed similar inhibition values under all the conditions assayed.

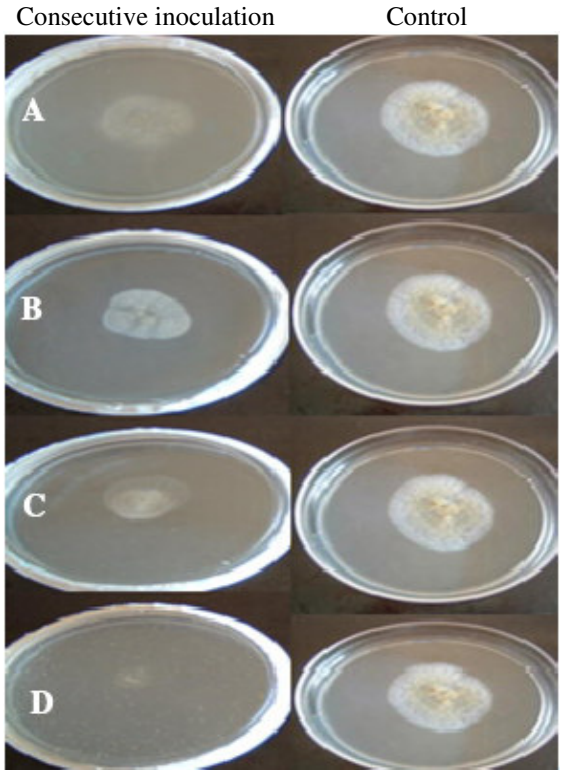

Figure 3. Effect of consecutive inoculation of LAB and *S. cerevisiae* on *A. nomius* growth.
Consecutive inoculation of A) *L. fermentum ssp. cellobiosus* 408; B) *L. fermentum* 27 A; C) *S.cerevisiae* and D) *L. rhamnosus* O236 and *A.s nomius VSC* 23. Plates were incubated at 37°C. See text for inoculation details. Growth of a monoculture of *A. nomius* VSC 23 was used as control.

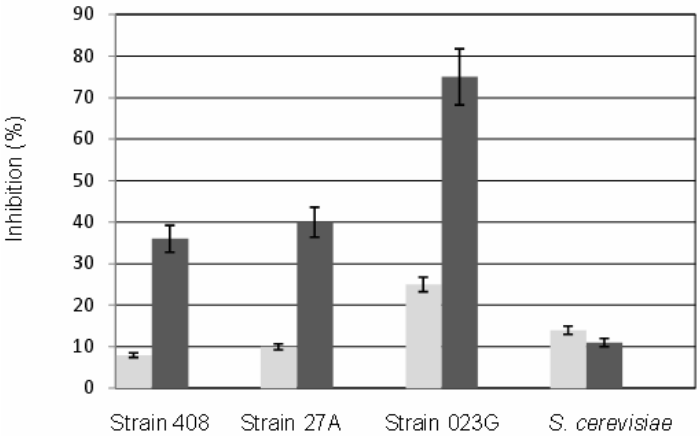

Figure 4. Inhibition (%) of *A. nomius* VSC 23 growth after consecutive inoculation with *L. fermentum* ssp. *cellobiosus* 408, *L. fermentum* 27A, *L. rhamnosus* O236 and *S. cerevisiae* compared with growth of a monoculture of *A. nomius* VSC 23 growing on the same medium (control). Assays were carried out at 28°C ( ) and at 37°C ( ).

Electron microscopy studies

Scanning electron micrographs shown in Fig. 5 did not reveal structural changes in the morphology of *A. nomius* VSC 23 during any of the inhibitory experiments.

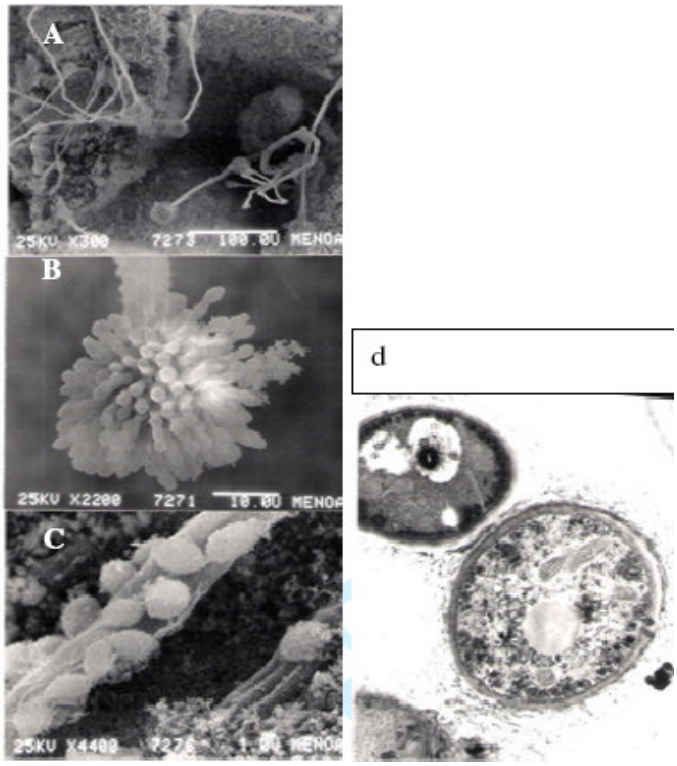

Figure 5. Scanning electron micrographs of *A .nomius* VSC23 after inhibition by *L. fermentum* 27A.

a) Hyphae and head of *Aspergillus* (300x), b) Head of *A. nomius* (2200x) and; c) Spores (4400x). Transmission electron micrograph of *A. nomius* VSC23 cells after inhibition by *L. fermentum* 27A (d).

DISCUSSION

Nearly 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (37). In addition, poultry for human consumption is generally fed on cereals or their products (31). There was a predominance of *Aspergillus* species during storage period of coffee beans (21).

Therefore growth of the fungus in cereal crops could affect humans not only after consumption of infected cereals, but also after chicken consumption. In addition, the presence of toxigenic moulds represents a potential risk of mycotoxin contamination and considering the worldwide increased use of herbal products as alternative medicine (8).

Inhibition of mycotoxigenic fungi is necessary in order to avoid toxin formation in food and feed. According to our results, natural control of the microflora could be realized by beneficial microorganisms. The number of publications on antifungal LAB is still low (34). LAB produce a variety of antimicrobial compounds. Various mechanisms have been suggested to be responsible for the inhibitory effects of the bacteria on fungal growth, such as nutritional competition, secondary metabolites, pH or combinations of these mechanisms (20). Mycotoxins could also be removed by adhesion of LAB to cell wall components (1, 11). *Lactobacillus acidophilus* and *Bifidobacterium animalis* strains are able to detoxify the mycotoxins. Both species can be used for the production of probiotic fermented foods, therefore

our findings may contribute to the development of strategies for the detoxification of contaminated plant derived products with these toxins by use of LAB (23).

Other authors have suggested that aflatoxin biosynthesis was inhibited by LAB but that the bacteria were not efficient enough to remove aflatoxin from the medium (13, 38). Our studies confirm previous studies demonstrating the inhibitory activity by LAB against a mycotoxin-producing fungus.

L. rhamnosus O236 isolated from sheep milk and selected for its technological properties showed highest fungal inhibition of the microorganisms assayed.

The inhibitory activity of lactobacilli against moulds could be due to different factors. Consecutive inoculation of mixed cultures showed higher inhibition of fungal growth than simultaneous inoculation under the same assay conditions. Our results are in agreement with those previously observed by El-Gendy and Marth (18). These authors studied simultaneous and consecutive inoculation of *L. casei* ATCC 393 and *Aspergillus parasiticus* NRRL 2999 (inoculation of *L. casei* three days prior to inoculation of the fungus) in LAPT broth for 10 days at 28°C and they found that fungal weight was less after consecutive inoculation (i.e. more inhibition). Our studies also showed that inhibition of *A. nomius* VSC after consecutive inoculation was significantly higher than after simultaneous inoculation. An important detail is that contrary to other authors (15) the current study used the same culture medium for simultaneous and consecutive growth in order to resemble natural conditions.

Temperature and incubation period are essential factors that modulate LAB growth and significantly affect the amounts of antifungal metabolites produced by LAB (3). The studies carried out by Sathe *et al.* (33) demonstrated that antifungal activity of *Lb. plantarum* CUK501 was maximal at 30°C, when the culture was at the end of its logarithmic phase. In concordance, we found higher antifungal activity of LAB at higher temperature. Batish *et al*. (5), observed that the antifungal activity of a *L. acidophilus* strain was maximal at 30°C after 48 h incubation, whereas increasing the incubation period resulted in a lower antifungal activity. These ‘‘anti-mycotoxinogenic” metabolites could also be produced during LAB growth (16)

Lactobacillus spp. has also been used as probiotic agents to modify the gastrointestinal environment in order to prevent the overgrowth of pathogens. Murry *et al*. (29) reported the effects of *L. salivarius* and *L. plantarum* on the growth of *E. coli*, *Salmonella enterica* ser. *typhimurium*, and *Clostridia perfringens* in poultry feed media. Other authors indicated that some lactic acid bacteria were able to reduce rot diameters caused by *Penicillium expansum* by 10-50% (39). *Lactococcus cremoris* (22) was reported to control mycotoxinogenic mould growth. *Lb. plantarum* strains VTTE-78076 and VTTE-79098 have also been described as being active against different plant pathogenic, toxigenic and gushing-active Fusarium fungi (27).

It is well known that *S. cerevisiae* grows better when surface-inoculated and consequently, low inhibition of *A. nomius* may be partially due to limited growth of the yeast in poured plates.

Fermentation is one of the oldest forms of food processing and preservation in the world going back as many as 7000 years in Babylon (6). The use of LAB in fermentation or ensilage processes is an ancient method that nowadays could be considered as an organic or natural procedure for feed elaboration. Such as was reported previously, however, despite of relatively high abundance of LAB in food and vegetables, only low percentage of these bacteria had inhibitory property (39). For these reasons, the inhibitory activity towards moulds could be considered a characteristic for the selection of LAB used as starter cultures in grain ensiling of animal food in order to prevent avian fungal infection.

The use of antifungal LAB instead of chemical preservatives would enable the food industry to produce organic food without addition of chemical substances. In addition to the already known excellent properties of LAB they could enhance the nutritional value and prolong conservation of food.

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