RESEARCH LETTER

Antifungal activity of two *Lactobacillus* strains with potential probiotic properties

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Abstract

Aflatoxin (highly toxic and carcinogenic secondary metabolites produced by fingi) contamination is a serious problem worldwide. Modern agriculture and animal production systems need to use high-quality and mycotoxin-free feedstuffs. The use of microorganisms to preserve food has gained importance in recent years due to the demand for reduced use of chemical preservatives by consumers. Lactic acid bacteria are known to produce various antimicrobial compounds that are considered to be important in the biopreservation of food and feed. *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23 are producers of secondary metabolites, such as organic acids, bacteriocins and, in the case of L60, hydrogen peroxide. The antifungal activity of lactobacilli strains was determined by coculture with *Aspergillus* section *Flavi* strains by two qualitative and one quantitative methods. Both L23 and L60 completely inhibited the fungal growth of all aflatoxicogenic strains assayed. Aflatoxin B_1 production was reduced 95.7–99.8% with L60 and 27.5–100% with L23. Statistical analysis of the data revealed the influence of L60 and L23 on growth parameters and aflatoxin B_1 production. These results are important given that these aflatoxicogenic fungi are natural contaminants of feed used for animal production, and could be effectively controlled by *Lactobacillus* L60 and L23 strains with probiotic properties.

Introduction

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Yang & Clausen, 2004). Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are produced naturally on substrates contaminated by aflatoxicogenic *Aspergillus* (Elsanhoty, 2008). AFB_1 is the most abundant aflatoxin, and is considered the most toxic and carcinogenic of the naturally occurring aflatoxins (Koirala *et al.*, 2005; Gratz *et al.*, 2007). Aflatoxin contamination continues to be a serious problem in many parts of the world (Richard & Payne, 2003). It poses a severe threat to both livestock productivity and human health and thus, with contamination causing huge worldwide economic losses each year (Guan *et al.*, 2008). Different physical and chemical methods have been recommended for detoxification of mycotoxin-contaminated food and feed, but only a few have been accepted for practical use (Biernasiak *et al.*, 2006). Alternative control methods are required because of negative public perception about the use of pesticides, development of resistance to fungicides and the high cost associated with production of new chemicals (Laitila *et al.*, 2002; Kotan *et al.*, 2009). Lactic acid bacteria (LAB) are a broad group of gram-positive, catalase-negative, non-sporulating, usually non-motile rods and cocci that utilize carbohydrates fermentatively and form lactic acid as the major end product (Onilude *et al.*, 2005). LAB are widely used in food and feed fermentation, contributing to the hygienic safety, storage stability and attractive sensory properties (Laitila *et al.*, 2002; Savadogo *et al.*, 2006). These bacteria are important in the biopreservation of food and feed, related mainly to the production of antimicrobial compounds, such as organic acids, i.e. lactic and acetic acid, hydrogen peroxide, and other antimicrobial compounds

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as bacteriocins (Messens & De Vuyst, 2002; Prachyakij *et al.*, 2007). There is an increasing interest to find LAB strains that are able to limit fungal growth and consequently mycotoxin production, in this particular case of aflatoxigenic fungi (Yang & Clausen, 2005; Aryantha & Lunggani, 2007; Elsanhoty, 2008).

In previous *in vitro* and *in vivo* experiments with *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23 we showed that these strains have probiotic characteristics (Pascual *et al.*, 2008a, b; Ruiz *et al.*, 2009). Both strains have widespread antimicrobial activity mainly against bacteria and yeast, although there are no studies regarding the antagonistic effect of LAB on filamentous fungi. Therefore, the aim of this study was to evaluate antifungal activity and anti-aflatoxicogenic properties of *L. rhamnosus* strain L60 and *L. fermentum* strain L23 against toxigenic species of *Aspergillus* section *Flavi*.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus rhamnosus L60 and *L. fermentum* L23 strains were obtained from a culture collection of the Bacteriology Laboratory at the National University of Río Cuarto, Córdoba, Argentina. These human strains were selected from among 100 strains of *Lactobacillus* on the basis of their probiotic characteristics and bacteriocinogenic ability. The purity of the strains was confirmed by Gram staining. Strains were identified by standard biochemical tests (Holt, 1994), carbohydrate fermentation profile (Nigatu *et al.*, 2000) and using the API 50 CHL system (BioMérieux, Marcy l'Etoile, France). The identification of *L. rhamnosus* L60 and *L. fermentum* L23 was confirmed by 16S rRNA gene sequence analysis, and the sequences of these strains were registered in the GenBank database system (http://www.ncbi.nlm.nih.gov/sites/entrez) under accession numbers EF495247 (1402 bp) and GQ455406 (1523 bp), respectively. Both strains were grown in De Man Rogosa Sharpe (MRS) agar (Rogosa & Sharpe, 1963) at 37 °C, under a 5% CO_2 atmosphere for 24 h. They were stored at –80 °C in MRS broth containing 30% (v/v) glycerol.

Fungal strain and culture conditions

A total of 137 *Aspergillus* section *Flavi* strains were recovered from brewer's grains destined for pig feed in Argentina. Strains producing high levels of AFB_1 were deposited in the collection centre of the National University of Río Cuarto, Córdoba, Argentina (RC) (*A. flavus* RC2053, RC2054, RC2055, RC2056, RC2057, RC2058, RC2059, RC2060, RC2061, *A. parasiticus* RC2062). All isolates were used in qualitative experiments and only the most potent AFB_1 producers were used in growth studies (*A. flavus* RC2053, RC2054, RC2055, RC2056). The isolates were maintained at 4 °C on malt extract agar (MEA) slants and at −80 °C in 15% glycerol.

Inhibition of *A. flavus* by lactobacilli *in vitro*

The effect of lactobacilli strains on *A. flavus* strains was detected by two qualitative methods:

(1) *Lactobacillus rhamnosus* L60 and *L. fermentum* L23 strains were assayed for inhibition of 10 *A. flavus* strains. The agar overlay method was used with some modifications (Magnusson & Schnürer, 2001). MRS agar plates on which *L. rhamnosus* L60 and *L. fermentum* L23 were inoculated in 2-cm-wide lines each and incubated at 37 °C under a 5% CO_2 atmosphere for 48 h. After the incubation period, the plates were overlaid with a soft agar (75% by weight agar) preparation of MEA containing 9.5×10^2 fungal spores mL^{-1}, determined by counting on a Neubauer haemocytometer. The plates were incubated aerobically at 25 °C for 5 days. The zones of inhibition of *Aspergillus* were estimated using a semiquantitative scale: (−), lack of *Aspergillus* growth inhibition over *Lactobacillus* culture; (+/−), minimal inhibition of *Aspergillus* growth over *Lactobacillus* culture; (+), partial inhibition of *Aspergillus* growth over *Lactobacillus* culture; (++), total inhibition of *Aspergillus* growth over *Lactobacillus* culture. Plates containing only the fungal spore inoculums (without *Lactobacillus* strains) were used as a control.

(2) *Lactobacillus* L60 and L23 strains were seeded until covering one-third of the surface of MRS agar plates and incubated in optimal conditions at 37 °C for 48 h. An MEA agar plug with *A. flavus* was placed on the centre of the free surface of these MRS agar plates and incubated aerobically at 25 °C for 5 days in the dark.

Growth parameters

Lactobacillus rhamnosus L60 and *L. fermentum* L23 suspensions were prepared in MRS broth (bioMérieux) (Rogosa & Sharpe, 1963) and adjusted to 0.5 of the McFarland scale, corresponding to final concentration of 1.5×10^8 CFU mL^{-1}. An aliquot of 1 mL from each lactobacillus suspension was placed into sterile Petri dishes. MRS agar (bioMérieux) (Rogosa & Sharpe, 1963) was poured into Petri dishes and stirred to homogenize the content. The plates were inoculated in the centre with a suspension of fungal spores from 7-day-old cultures on MEA in semisolid agar. The plates were incubated at 25 °C and the colony radius was measured daily. 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 tested fungus. The radial growth rate (mm day^{-1}) was subsequently calculated by linear regression of the linear phase of growth and the time at which the line intercepted the *x*-axis was used to calculate the lag phase.

Extraction and quantification of AFB_1

At the end of the incubation period, three agar plugs (1 × 1 cm) were removed from the plates of each lactobacillus treatment from different points of the colony and extracted with 1 mL of chloroform. The mixture was centrifuged at 4000 ***g*** for 10 min. The solutions were filtered and evaporated to dryness. Quantification of aflatoxin was performed by HPLC according to the methodology proposed by Trucksess *et al.* (1994). The extract was redissolved with 200 μL mobile phase and was derivatized with 700 μL of a mixture of trifluoroacetic acid/acetic acid/water (20 : 10 : 70, v/v). Chromatographic separations were performed on a reversed-phase column (Silica Gel, 150 × 4.6 mm i.d., 5-μm particle size; Varian, Inc., Palo Alto, CA). Acetonitrile/water/methanol (17 : 66 : 17 v/v) was used as mobile phase at a flow rate of 1.5 mL min^{-1}. Fluorescence of aflatoxin derivatives was recorded at λ 360 nm excitation and λ 460 nm emission. Calibration curves were constructed using different concentrations of AFB_1 (Sigma, St. Louis, MO; purity > 99%) standard solutions. Aflatoxin was quantified by correlating sample peak areas with those of standard solutions. The detection limit of the analytical method was 0.4 ng g^{-1}. The recovery of the toxin from MRS agar was 89.2 ± 9.7%.

Statistical analysis

All analyses were carried out in triplicate and the results are presented as mean values. Data were analysed by analysis of variance (ANOVA) using the software INFOSTAT versión 2011 (InfoStat Group, FCA, National University of Córdoba, Argentina). The results were considered to be statistically different at $P < 0.05$. Tukey's test was used for comparing treatment means.

Results

Lactobacillus rhamnosus L60 and *L. fermentum* L23 were able to inhibit the growth and AFB_1 production by *Aspergillus* section *Flavi* species *in vitro*. Table 1 shows the inhibition of growth of 10 *Aspergillus* section *Flavi* strains by *L. rhamnosus* L60 and *L. fermentum* L23 via the agar overlay method. Compared with control, both strains showed highest inhibition of fungal growth. *Lactobacillus rhamnosus* L60 was able to reduce the growth of all *Aspergillus* section *Flavi* strains assayed whereas *L. fermentum* L23 inhibited the growth of 90% of fungal strains. Six toxigenic *Aspergillus* strains (60%) were totally inhibited by either lactobacilli strain. *Lactobacillus fermentum* L23 did not show inhibitory activity on *A. flavus* strain RC 2061. Other results showed that L60 and L23 were able to inhibit the sporulation and reduce esclerotia production on fungal strains compared with controls in both methodologies used. The agar block technique produced similar results on *Aspergillus* strains by both lactobacilli (Fig. 1).

Table 1. Growth inhibition by *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23 on *Aspergillus* section *Flavi* strains

	Lactobacillus strain	
Aspergillus section *Flavi* strain	*L. rhamnosus* L60	*L. fermentum* L23
RC2053	++	++
RC2054	++	++
RC2055	+	++
RC2056	++	++
RC2057	++	+
RC2058	+	+/−
RC2059	+	++
RC2060	++	++
RC2061	+	−
RC2062	++	+

−, no growth inhibition of *Aspergillus* over *Lactobacillus* culture; +/−, minimal inhibition of *Aspergillus* growth over *Lactobacillus* culture; +, partial inhibition of *Aspergillus* growth over *Lactobacillus* culture; ++, total inhibition of *Aspergillus* growth over *Lactobacillus* culture.

Table 2 shows the effect of lactobacilli strains on lag phase prior to growth of four *Aspergillus* section *Flavi* strains. These fungal strains were selected by their ability to produce higher levels of AFB_1. In relation to the control treatment, a decrease in the lag phase of all fungal strains co-cultured with L60 and L23 was observed

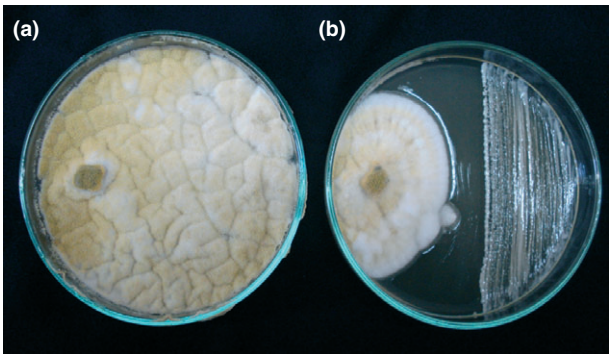

Fig. 1. Growth inhibition of RC2053 strain by *Lactobacillus rhamnosus* L60 (b) and control (a).

Table 2. Effect of *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23 on growth parameters

Aspergillus section *Flavi*	Lag phase (h, mean ± SD)		
	Control	*Lactobacillus* L23	*Lactobacillus* L60
RC2053	26.4 ± 0.6[a]	22.3 ± 0.2[b]	18.4 ± 0.2[c]
RC2054	27.3 ± 1.3[a]	21.3 ± 0.2[a]	9.7 ± 3.9[b]
RC2055	25.9 ± 1.8[a]	21.6 ± 0.7[ab]	16.4 ± 0.4[b]
RC2056	26.5 ± 2.7[a]	20.8 ± 0.3[b]	18.3 ± 0.5[b]

Mean values based on quadruplicate data ± standard deviation (SD). Mean in a line with a letter in common are not significantly different according to Tukey test ($P < 0.05$).

($P < 0.05$). The lag phase ranged between 9.73 and 18.36 h when co-culturing *Aspergillus* strains with L60 and between 20.63 and 22.31 h in presence of L23. The lag phase prior to growth of all fungal strains was significantly ($P < 0.05$) reduced by *L. rhamnosus* L60 compared with *L. fermentum* L23.

In all *Aspergillus* section *Flavi* strains assayed, growth rate decreased significantly ($P < 0.05$) when coculturing with L60 and L23. *Lactobacillus rhamnosus* L60 significantly reduced ($P < 0.05$) the growth rate from 77% to 96%, while *L. fermentum* L23 significantly reduced ($P < 0.05$) the growth rate from 36% to 50%, with respect to control (Fig. 2). The highest reduction of growth rate was observed with both bacterial strains on *A. flavus* RC2054. *Lactobacillus rhamnosus* L60 was most effective in reducing the growth rate on all *Aspergillus* section *Flavi* strains assayed when compared with *L. fermentum* L23. The effect of L60 and L23 on inhibition of AFB_1 production is shown in Fig. 3. In general, AFB_1 production exhibited a similar pattern to growth rate,

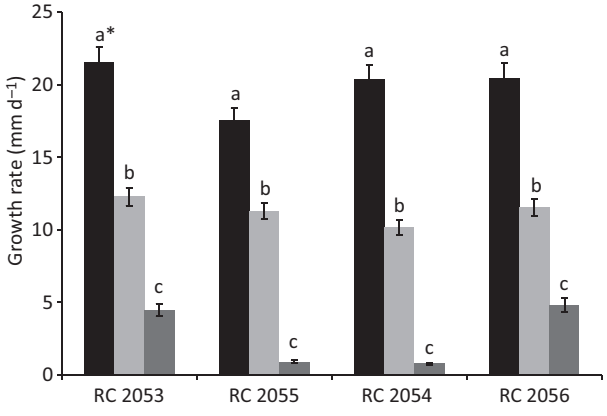

Fig. 2. Effect of lactobacilli strains on rate growth by *Aspergillus* section *Flavi*. ■ Control; ▒ *Lactobacillus fermentum* L23; ▒ *Lactobacillus rhamnosus* L60. Mean values based on quadruplicate data. *Mean with a letter in common are not significantly different according to Tukey's test ($P < 0.05$).

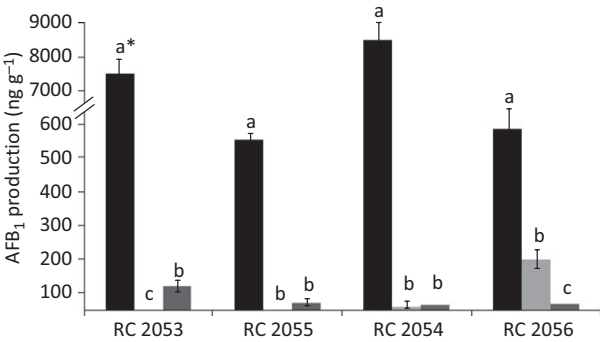

Fig. 3. Effect of lactobacilli strains on the production of AFB_1. ■ Control; ▒ *Lactobacillus fermentum* L23; ▒ *Lactobacillus rhamnosus* L60. Mean values based on quadruplicate data. *Mean with a letter in common are not significantly different according to Tukey's test ($P < 0.05$).

when the fungal strains were cocultured with L60 and L23. The presence of L60 and L23 did not stimulate the production of AFB_1 in any of the *Aspergillus* section *Flavi* strains assayed. *Lactobacillus fermentum* L23 was able to inhibit AFB_1 production of *A. flavus* RC2053 and *A. flavus* RC2055. *Aspergillus* section *Flavi* strains showed a significant reduction ($P < 0.05$) in AFB_1 production when grown in the presence of L60 and L23, with decreased production of the toxin between 96% and 99% and 73% and 99%, respectively. Toxin production of *Aspergillus* section *Flavi* was significantly reduced ($P < 0.05$) by both lactobacilli strains assayed compared with control.

Discussion

The *Lactobacillus* strains used were previously characterized by Pascual *et al.* (2008a, b) and Ruiz *et al.* (2009) as presenting probiotic properties: colonization, self-aggregation, adherence to epithelial cells and coaggregation with bacterial pathogens. *Lactobacillus rhamnosus* L60 and *L. fermentum* L23 are producers of secondary active metabolites, such as organic acids, bacteriocins and, in the case of *L. rhamnosus* L60, hydrogen peroxide. Bacteriocin production was previously characterized and the substance was purified (Pascual *et al.*, 2008a, b). The two strains showed a wide spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, some being human and animal pathogens. The present study shows the potential of *L. rhamnosus* L60 and *L. fermentum* L23 in control of *Aspergillus* section *Flavi* growth and AFB_1 production *in vitro*. Biopreservation, the use of microorganisms to preserve food and feed stuffs, has been gaining increasing interest due to consumers' demand for reduced use of chemical preservatives (Prema *et al.*, 2010). As LAB are 'generally recognized as safe' organisms (Hoque *et al.*,

2010), they could have useful application in the prevention of fungal contamination in raw materials, food and feed, and in reducing the health hazards associated with mycotoxins.

The present study clearly shows the antifungal effect of *L. rhamnosus* L60 and *L. fermentum* L23 on aflatoxigenic fungal isolates. Nevertheless, *L. rhamnosus* L60 was the most effective strain in inhibiting growth of all *Aspergillus* section *Flavi* strains assayed *in vitro*. Our results agree with those reported by Vanne *et al.* (2000), who assayed the effects of *Lactobacillus casei* on growth and aflatoxin production by *A. parasiticus.* Onilude *et al.* (2005) demonstrated that *Lactobacillus plantarum*, *L. fermentum*, *Lactobacillus brevis* and *Lactococcus* spp. have *in vitro* antifungal effects on aflatoxigenic fungal isolates in similar proportions to those detected in this study. The results obtained in the present study agree with those of other researchers, who assayed *Lactobacillus* species similar to those used in this study but with other LAB strains in the *in vitro* growth control of *Aspergillus* spp. and other fungal strains (Magnusson & Schnürer, 2001; Zara *et al.*, 2003; Kam *et al.*, 2007; Muñoz *et al.*, 2010; Voulgari *et al.*, 2010). The growth rate inhibition by lactobacillus strains on fungal species may be caused by production of secondary metabolites. *Lactobacillus rhamnosus* L60 and *L. fermentum* L23 are producers of organic acids, bacteriocins and, in the case of *L. rhamnosus* L60, hydrogen peroxide (Pascual *et al.*, 2008a, b; Ruiz *et al.*, 2009). The presence of these substances in culture media could inhibit the fungal development of *Aspergillus* section *Flavi* species, as observed in our assays. Lactic and acetic acids are the main products of the fermentation of carbohydrates by LAB. These acids diffuse through the membrane of target organisms in their hydrophobic undissociated form and then reduce cytoplasmic pH, thereby causing loss of viability and cell destruction (Gerez *et al.*, 2009; Dalié *et al.*, 2010). Although there is no clear evidence of the role of protein compounds in the inhibition of mould growth, several authors have reported that some lactic strains produced antifungal metabolites that were sensitive to proteolytic enzymes (Magnusson & Schnürer, 2001; Rouse *et al.*, 2008). On the other hand, the strong inhibitory activity can be attributed to competition between LAB and *Aspergillus* section *Flavi* species in batch conditions. However, the observed reduction of the lag phase is probably due to rapid adaptation of fungal strains to the culture medium but LAB may have advantages over fungi as they are simpler organisms with a faster metabolim. Therefore, bacteria can utilize the original substrate earlier to produce more cell biomass, while fungi develop later after nutrient levels are lower. We have clearly demonstrated here the inhibitory effect of growth of *Aspergillus* section *Flavi* strains by secondary metabolites of LAB. However, future studies will need to determine the optimal concentration of pure organic acid, bacteriocins and hydrogen peroxide that inhibit fungal growth. When fungal isolates were grown in the presence of *Lactobacillus* strains, AFB_1 accumulation showed the same trend as growth rate, with accumulation of this metabolite reduced significantly ($P < 0.05$). None of the LAB strains stimulated AFB_1 accumulation in any of the fungal strains assayed. On the contrary, toxin production of *A. flavus* RC2053 and *A. flavus* RC2055 was totally inhibited by *L. fermentum* L23. It is likely that the low concentration of AFB_1 in the presence of *Lactobacillus* strains could be due to low mycelial biomass formation. Growth inhibition could directly affect AFB_1 production as a result of low synthesis of the enzymes involved. Furthermore, AFB_1 is a secondary metabolite that does not occur during primary growth of fungus, so that growth inhibition may reduce its production. In this study we have showed that there could exist a relationship between fungal growth and AFB_1 production. In fact, these results showed that minimal yields of toxin coincided with minimal mycelial growth. Tukey's test of the data revealed the influence of *L. fermentum* L23 and *L. rhamnosus* L60 on growth parameters (lag phase and growth rate) and AFB_1 production. Our results agree with Zinedine *et al.* (2005), who demonstrated the ability of some strains of LAB to reduce the initial concentration of AFB_1 in MRS broth. Similar observations were made by Aryantha & Lunggani (2007), who observed that *L. plantarum*, *L. fermentum* and *Lactobacillus delbrueckii* significantly inhibited fungal growth of *A. flavus* and AFB_1 production. Dalié *et al.* (2010) established that the main LAB recognized for their ability to limit mycotoxinogenic mould growth belong to the genera *Lactococcus* and *Lactobacillus*, including *L. rhamnosus*, in agreement with our results. These results reflect a strong ability to inhibit growth rate and AFB_1 production by both *Lactobacillus* strains with a wide spectrum of antimicrobial activity and high probiotic potential. This suggest that the use of LAB with antifungal properties instead of chemical preservatives would enable the food and feed industry to produce organic food without chemical additives. In addition to the known excellent properties of *Lactobacillus* strains, they could enhance the nutritional value and prolong the conservation of food. These results are important given that these aflatoxicogenic fungi are natural contaminants of raw materials used for food and feed production, which could be effectively controlled by *L. rhamnosus* L60 and *L. fermentum* L23, both strains having probiotic properties. It is concluded that, under favourable conditions, the two lactobacilli strains not only inhibited aflatoxicogenic fungal growth, but also inhibited AFB_1 biosynthesis. Future studies with *L. rhamnosus* L60 and *L. fermentum* L23 may test

the application of these lactobacilli as biocontrollers of fungal contaminants and also to extend the self life of food and feed stuffs, approaching *in situ* their probiotic properties.

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