Control of *Escherichia coli* O157:H7 in corn silage with or without various inoculants: Efficacy and mode of action

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ABSTRACT

This study aimed to evaluate the effectiveness of 3 commercial bacterial inoculants at controlling *Escherichia coli* O157:H7 in corn silages during ensiling and feedout phases of silage production. A second objective was to determine whether the inoculants exhibited and transferred antibacterial activity against *E. coli* O157:H7 to the silages. Chopped corn forage was ensiled after treatment with the following: distilled water (control); 5×10^5 cfu/g of *E. coli* O157:H7 (EC); EC and 1×10^6 cfu/g of *Pediococcus pentosaceus* and *Propionibacterium freudenreichii* (EC+BII); EC and 1×10^6 cfu/g of *Lactobacillus buchneri* (EC+LB); and EC and 1×10^6 cfu/g of *L. buchneri* and *P. pentosaceus* (EC+B500). Each treatment was ensiled in triplicate in mini silos for 3, 7, 31, and 82 d and analyzed for pH and *E. coli* O157:H7 counts. Samples from d 82 were also analyzed for volatile fatty acids, lactate, and aerobic stability. Antibacterial activity of inoculants and silages was determined by the Kirby-Bauer disc diffusion test. The pH of silages from all treatments decreased below 4 within 3 d of ensiling and remained low until d 82. Therefore, *E. coli* O157:H7 was not detected in silages after any of the ensiling durations. Applying inoculants containing *L. buchneri* resulted in less lactate, more acetate, and greater aerobic stability compared with the control. Applying EC+BII containing *P. freudenreichii* did not increase propionate or aerobic stability. Subsamples of d 82 silages were reinoculated with 1×10^5 cfu/g of *E. coli* O157:H7 either immediately after silo opening on d 82 or after 144 h of aerobic exposure (d 88), and *E. coli* were enumerated 24 h later. All silages reinoculated with the pathogen on d 82 had similar, low pH values (<4) and no *E. coli* were detected 24 h later. Control, EC, and EC+BII silages reinoculated with the pathogen after 144 h of aerobic exposure had relatively greater pH values (4.71, 5.67, and 6.03, respectively) and *E. coli* counts (2.87, 6.73, and 6.87 log

cfu/g, respectively) 24 h later, whereas those treated with *L. buchneri* had low pH values (<4) and undetectable (EC+B500) or 10-fold lower (1.97, cfu/g; EC+LB) *E. coli* counts. All pure cultures of commercial bacterial inoculants exhibited antibacterial activity independent of pH against *E. coli* O157:H7, but the pH-independent activity did not persist in the treated silages, suggesting that *E. coli* elimination from silages was mediated by pH reduction.

Key words: *Escherichia coli* O157:H7, bacterial inoculant, silage, antibacterial activity

INTRODUCTION

Escherichia coli O157:H7, a Shiga toxin-producing gram-negative bacteria, has emerged as an important cause of food borne disease since it was first isolated in 1982 (Riley et al., 1983). Cattle are the main reservoir of *E. coli* O157:H7 and the pathogen may be present in feces, milk, and feed of dairy cows (Chapman et al., 1997; Mechie et al., 1997; Lynn et al., 1998). Corn silage is one of the most widely used components of dairy cow rations and it can be contaminated with *E. coli* O157:H7 via manure or irrigation water (Weinberg et al., 2004). *Escherichia coli* O157:H7 was eliminated from ensiled, contaminated wheat and corn forage when the pH decreased below 5.0 (Chen et al., 2005). Elimination of the pathogen has been hastened by treatment with a bacterial inoculant that accelerated the pH decrease (Bach et al., 2002). Weinberg et al. (2004) also confirmed that ensiling is effective in eliminating *E. coli* spp. in contaminated forages but noted that these bacteria may develop in spoiled silages, which often have high pH levels. To date, no studies have examined whether bacterial inoculants that increase aerobic stability and maintain low pH after aerobic exposure such as *Lactobacillus buchneri* (Filya, 2003; Adesogan et al., 2003, 2004; Huisden et al., 2009) can also prevent the growth of *E. coli* on aerobically exposed silages.

Many lactic acid and propionic acid bacteria produce substances with antibacterial activity such as peroxides and bacteriocins (Jack et al., 1995; Meile et al., 1999). Gollop et al. (2005) reported that antibacterial activity independent of pH was present in many lactic acid

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bacteria inoculants but that the antibacterial activity was not imparted to some of the silages treated with the bacteria. Little is known about the antibacterial activity of bacterial silage inoculants against *E. coli* O157:H7.

The first objective of this study was to evaluate the effectiveness of bacterial inoculants containing homofermentative bacteria, heterofermentative bacteria, or both types of bacteria at controlling *E. coli* O157:H7 in corn silage during the anaerobic and aerobic stages of silage production. A second objective was to examine whether pH-independent antibacterial activity against *E. coli* O157:H7 existed in the inoculants and persisted in inoculated silages.

MATERIALS AND METHODS

Harvesting, Inoculation, and Ensiling

The experiment was conducted at the Department of Animal Sciences, University of Florida (Gainesville) from October 2007 to July 2008. Corn forage was harvested at the 50% milk line stage (30% DM) with a forage harvester (Claas Jaguar 900, Claas of America LLC, Columbus, IN) adjusted to achieve a 19-mm theoretical length of cut. The following treatments were applied in triplicate to the forage: distilled water (control); 5×10^5 cfu/g of *E. coli* O157:H7 (ATCC 43894; **EC**); EC and 1×10^6 cfu/g of *Pediococcus pentosaceus* 12455 and *Propionibacterium freudenreichii* (**EC+BII**); EC and 1×10^6 cfu/g of *Lactobacillus buchneri* 40788 (**EC+LB**); and EC and 1×10^6 cfu/g of *L. buchneri* 40788 and *P. pentosaceus* 12455 (**EC+B500**). The *E. coli* O157:H7 was supplied by ABC Research Corporation (Gainesville, FL), and bacteria in the EC+BII, EC+LB, and EC+B500 treatments were from commercial inoculants (Biotol Plus II, *Buchneri* 40788, and *Buchneri* 500, respectively; Lallemand Animal Nutrition, Milwaukee, WI). The *E. coli* O157:H7 was grown in trypticase soy broth (Becton Dickinson Co., Franklin Lakes, NJ) for 24 h at 37°C. Bacteria were sedimented by centrifugation ($3,000 \times g$ for 10 min), washed 3 times, and resuspended in buffered peptone water (**BPW**; pH 7.2; Oxoid, Basingstoke, UK). Cells were adjusted to an optical density of 0.5 at 640 nm (Jasco V-530 Spectrophotometer, Jasco Inc., Easton, MD) to achieve 1×10^8 cfu/mL in the suspension (Bach et al., 2002). The population of *E. coli* in the suspension was verified by spread plating on sorbitol MacConkey agar (**SMAC**; Oxoid) supplemented with 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite (**CT**, Oxoid) to yield CT-SMAC. Suspensions of the pathogen were diluted in distilled water and applied to achieve 5×10^5 cfu/g at ensiling. The commercial inoculants were suspended in

distilled water and applied at the rate of 3 mL/kg of fresh forage to achieve 1×10^6 cfu/g in the forage, and the same amount of distilled water was applied to the control. The number of bacteria in the commercial inoculants was previously determined by serial dilutions in BPW, spread plating in de Man, Rogosa, Sharpe (**MRS**) agar (Difco, Detroit, MI), and incubating for 24 h at 35°C.

The forage for each treatment (25 kg) was spread onto a plastic sheet and the treatment was applied using a manual sprayer. Afterward, the 4 ends of the plastic sheet were drawn together and the forage was tumbled for 3 min to ensure even distribution of the inoculum. Approximately 1.5 kg of forage from each treatment was manually compacted into a 14 × 21-cm, thick-walled plastic bag. Each treatment was prepared in triplicate and ensiled for 3, 7, or 31 d at 20°C to give a total of 45 bag silos. Samples from these bags were used to determine changes in *E. coli* O157:H7 counts and pH over time. In addition, 4.5 kg of forage from each treatment were manually compacted in triplicate into 20-L mini silos lined with thick-walled plastic bags and ensiled for 82 d at 20°C at an approximate density of 500 kg/m³. On d 82, these mini silos were opened and their contents were mixed thoroughly and representatively subsampled for analysis of pH, lactate, VFA, aerobic stability, and *E. coli* O157:H7, yeast, and mold enumeration. Additional samples were also taken for reinoculation with *E. coli*.

Aerobic Stability

Approximately 1.3 kg of d 82 silage from each treatment replicate was transferred to a plastic bag within an open-top foam container. Silage boxes were covered with 2 layers of cheese cloth to avoid dehydration and stored at 20°C for 144 h. Silage temperature was monitored with thermocouples (Campbell Scientific Inc., Logan, UT) placed in the geometrical center of the silage mass in each box. Thermocouples were connected to a data logger (CR 1000 data logger; Campbell Scientific Inc.) that recorded silage temperatures at 30-min intervals. Aerobic stability was defined as the number of hours the temperature in the silages remained stable before rising more than 2°C above room temperature.

Survival of *E. coli* O157:H7 in Silages Exposed to Aerobic Conditions

This aspect of the experiment determined whether treatment with the commercial inoculants at ensiling would prevent the growth of *E. coli* O157:H7 in silages contaminated with the pathogen after silos were opened. Approximately 1 kg of silages sampled at silo opening

and those aerated for 144 h in the aerobic stability assay were transferred into separate plastic bags and reinoculated with 1×10^5 cfu/g of *E. coli* O157:H7. Silage pH and *E. coli* counts were determined 24 h after reinoculation.

Laboratory Analyses

Silage extracts for enumeration of *E. coli*, analysis of VFA, pH, and determination of antibacterial activity in the silages were prepared by blending 15 g of silage and 135 mL of distilled water in a stomacher (UL Lab-Blender 400, Seward Laboratory, London, UK) for 2 min. For VFA determination, the extracts were filtered through 2 layers of cheese cloth and frozen (-10°C) after addition of 3 mL of 50% sulfuric acid per 100 mL of extract. The extracts for enumeration of yeasts and molds were prepared by blending 25 g of silage with 225 mL of distilled water for 2 min in the stomacher.

Microbial Enumeration. For enumeration of *E. coli* O157:H7 in the suspensions prepared to inoculate silages and in silage extracts, serial 10-fold dilutions were prepared in BPW followed by spread plating on CT-SMAC agar. Plates were incubated for 24 h at 35°C . Colonies on CT-SMAC were screened by API-20E System (BioMerieux Inc., Hazelwood, MO) and confirmed as *E. coli* O157:H7 by latex agglutination using O157 and H7 antisera (Difco). The number of bacteria in suspensions of commercial inoculants was determined by serial dilutions in BPW and spread plating on MRS agar. Plates were incubated for 24 h at 35°C . For yeast and mold enumeration, silage extracts were serially diluted in BPW and spread plated on Rose Bengal agar (Oxoid) supplemented with 1.0 mL/L dichloran 0.2% in ethanol (Ultra Scientific, Kingstown, RI) and 0.1 g/L of chloramphenicol (Fisher Biotech, Fair Lawn, NJ). Plates were incubated for 5 d at 27°C . For all microbial counts, a range of dilutions was prepared in duplicate and plates yielding 30 to 300 colonies were counted.

Chemical Analysis. The DM content in the initial forage and silages was determined by drying samples in a forced-air oven at 55°C for 48 h. The pH of silage extracts was measured using a digital pH meter (Accumet AB15, Fisher Scientific). Lactate and VFA concentrations were analyzed by HPLC as described by Muck and Dickerson (1988).

Antibacterial Activity. Antibacterial activity against *E. coli* O157:H7 in pure cultures of the inoculants and d 82 silage extracts was determined by the Kirby-Bauer disc diffusion test. Petri plates containing 1% of the *E. coli* O157:H7 inoculum (10^8 cfu/mL) were prepared on CT-SMAC agar. Bacteria in the commercial inoculants were grown in MRS broth at 35°C for 24 h. Cell-free supernatants were prepared by centrifuging

the cultures at $3,000 \times g$ for 10 min at 5°C . The pH of the supernatants was adjusted to 5.0 with 2 N NaOH. Silage extracts were similarly centrifuged and the cell-free supernatants were adjusted to pH 5. Two paper discs (6-mm diameter; cat. no. 231039, Becton Dickinson Co.) were immersed in the supernatants for 15 s using flame-sterilized forceps and then placed on the surface of CT-SMAC agar plates containing the indicator microorganism. Plates were prepared in duplicate and incubated for 24 h at 35°C . Zones of inhibition around the paper discs were measured with a ruler.

Statistical Analysis

The experiment had a completely randomized design with 5 treatments and 3 replicates per treatment. Data were analyzed with the General Linear Model procedure of SAS (SAS Institute Inc., Cary, NC) and a model including the treatment effect. In addition, the model used for analyzing changes in pH and *E. coli* during ensiling included time and treatment $\times$ time. Differences between means were determined using the Tukey test. Significant differences were declared if $P < 0.05$.

RESULTS

Anaerobic Phase

The pH of all silages decreased to values below 4 within 3 d of ensiling and remained low at all subsequent ensiling durations (Figure 1). The pH at final silo opening (d 82) was greater ($P < 0.05$) in silages treated with commercial inoculants (3.72) than in control and EC silages (3.52). *Escherichia coli* was not detected in silages after any of the ensiling durations.

Silages treated with inoculants containing *L. buchneri* (EC+LB and EC+B500) had lower lactate and greater acetate concentrations than other treatments (Table 1). The EC+LB silage had less ($P < 0.05$) DM than control, EC, and EC+BII silages. Propionate and butyrate concentrations were low or undetectable in all silages. Applying the inoculant containing propionic bacteria (EC+BII) did not result in greater propionic acid concentration compared with other treatments.

Aerobic Phase

Yeasts and molds were not detected in silages treated with *L. buchneri* inoculants but they were present in other silages. Treatment with *L. buchneri* inoculants improved aerobic stability by at least 115% compared with the control, but treatment with EC or EC+BII did not affect aerobic stability (Figure 2).

Escherichia coli O157:H7 was not detected in silages reinoculated with the pathogen at final silo opening (d

Figure 1. Changes in pH in corn forage inoculated with 5×10^5 log cfu/g of *Escherichia coli* O157:H7 (EC) or EC plus bacterial inoculants and ensiled for different durations (BII = *Pediococcus pentosaceus* + *Propionibacterium freudenreichii*; LB = *Lactobacillus buchneri*; B500 = *P. pentosaceus* + *L. buchneri*). Treatment $\times$ day SE and *P*-value for pH = 0.33 and 1.00, respectively; *pH values differed at this ensiling duration.

82) and exposed to the air for 24 h, probably because pH remained below 4 in all silages during that period (mean = 3.78 ± 0.13). However, the pathogen was present in all silages reinoculated 144 h after silo opening except in the silage treated with EC+B500 (Figure 3). One day after reinoculation, control, EC, and EC+BII silages had relatively high pH values (4.71, 5.67, and 6.03, respectively) and *E. coli* counts (2.87, 6.73, and 6.87 log cfu/g, respectively; $P < 0.05$), whereas those treated with *L. buchneri* inoculants had low pH values (<4) and undetectable (EC+B500) or low (1.97, cfu/g; EC+LB) *E. coli* counts. Counts of the pathogen were at least 10-fold less in silages treated with *L. buchneri* inoculants compared with other treatments.

Figure 2. Effect of inoculation with 5×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) or EC and bacterial inoculants at ensiling on aerobic stability of corn silages ensiled for 82 d (BII = *Pediococcus pentosaceus* + *Propionibacterium freudenreichii*; LB = *Lactobacillus buchneri*; B500 = *P. pentosaceus* + *L. buchneri*). Bars with different letters differed ($P < 0.05$); SE = 4.11.

Antibacterial Activity

The pH-corrected cell-free supernatants from pure cultures of each commercial inoculant produced a 2.5-mm zone of inhibition against *E. coli* O157:H7, whereas no zone was evident from that of the control. In addition, no inhibition zone against the pathogen was produced by cell-free supernatants from extracts of d 82 silages treated with these inoculants.

DISCUSSION

The fact that *E. coli* O157:H7 was undetected in silages during the anaerobic phase is most probably attributable to the inhibitory low pH resulting from formation of fermentation acids during ensilage. A bacterial inoculant that accelerated the rate of lactate accumulation and the resulting pH decrease reduced (7 vs. 15 d) the period required for elimination of *E. coli* O157:H7 from barley silage contaminated with the pathogen (Bach et al., 2002). In this study, as in others on corn and grass silage (Byrne et al., 2002; Chen et al., 2005), *E. coli* was eliminated during ensiling even in the absence of inoculation because a low pH was rapidly achieved.

Inoculants containing *L. buchneri* have improved the aerobic stability of different silages (Driehuis et al., 2001; Weinberg et al., 2002; Kleinschmit et al., 2005). In this study, inoculation with *L. buchneri* inoculants increased the aerobic stability of corn silage by at least 115%. This effect is attributable to the inhibitory effect of acetate produced by *L. buchneri* on spoilage-causing fungi (Driehuis et al., 2001; Ranjit et al., 2002). Yeasts are controlled by acetic acid levels above 5.6

Table 1. Dry matter, organic acids, and yeast and mold values of corn forage inoculated with *Escherichia coli* O157:H7 (EC) alone or EC and commercial bacterial inoculants and ensiled for 82 d

Attribute	Treatment ¹					SE
	Control	EC	EC + BII	EC + LB	EC + B500	
DM (%)	26.3 ^{ab}	26.6 ^{ab}	27.1 ^a	25.0 ^c	25.8 ^{bc}	0.34
Lactate (% of DM)	2.69 ^a	2.10 ^a	2.12 ^a	0.48 ^b	1.10 ^b	0.31
Acetate (% of DM)	2.42 ^c	1.73 ^c	1.98 ^c	4.77 ^a	3.81 ^b	0.35
Propionate (% of DM)	0.26	0.00	0.00	0.00	0.01	0.2
Butyrate (% of DM)	0.21	0.00	0.00	0.00	0.00	0.11
Yeasts and molds (log cfu/g)	2.13 ^{ab}	5.67 ^a	6.30 ^a	0.00 ^b	0.00 ^b	1.7

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Control = distilled water; EC = 5×10^5 cfu/g of *Escherichia coli* O157:H7; BII = 1×10^6 cfu/g of *Pediococcus pentosaceus* and *Propionibacterium freudenreichii*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; B500 = 1×10^6 cfu/g of *Pediococcus pentosaceus* and *Lactobacillus buchneri*.

g/L of culture medium (Woolford, 1975). As in this study, inoculation with *L. buchneri* typically results in acetate concentrations ranging from 36 to 50 g/kg of DM (Driehuis et al., 2001; Taylor et al., 2002).

Propionic acid is the most effective antimycotic agent among short-chain fatty acids at pH 5 or lower (Woolford, 1975); therefore, inoculants containing propionic acid bacteria have been tested for their ability to improve aerobic stability. Merry and Davies (1999) indicated that such bacteria have not consistently inhibited the growth of yeasts and molds or improved aerobic stability because they do not grow well when ensiling conditions are conducive to a rapid decrease in pH. Previous studies showed that inoculation with *P. freudenreichii* (1×10^4 cfu/g) with or without homolac-

tic bacteria did not affect propionic acid concentration, yeast and mold counts, or aerobic stability of maize and barley silages (Ranjit et al., 2002; Taylor et al., 2002). Likewise, treatment with the inoculant containing *P. pentosaceus* and *P. freudenreichii* did not affect these measures in this study.

Although antibacterial activity against *E. coli* was evident in cultures of the commercial inoculants, it was not detected in silages treated with the inoculants. Gollop et al. (2005) also showed that pure cultures of different strains of *L. plantarum*, *Enterococcus faecium*, and *L. buchneri* inhibited the growth of *Micrococcus luteus* and *Pseudomonas aeruginosa*, but only some of the extracts of silages treated with these bacteria had antibacterial activity independent of pH. Antibacterial

Figure 3. Effect of reinoculation of corn silages with 1×10^6 cfu/g of *Escherichia coli* O157:H7 (EC) 144 h after silo opening (d 82) on pH and *E. coli* counts (log cfu/g) of silages treated with EC or EC and bacterial inoculants at ensiling (BII = *Pediococcus pentosaceus* + *Propionibacterium freudenreichii*; LB = *Lactobacillus buchneri*; B500 = *P. pentosaceus* + *L. buchneri*). Similarly shaded bars with different letters differed ($P < 0.05$); SE values for pH and *E. coli* data were 0.41 and 1.04, respectively.

activity is dependent on several factors, including water activity, pH, temperature, and VFA concentration, and it varied with crop species and crop moisture concentration in the study of Gollop et al. (2005). The antibacterial activity of inoculant bacteria can be exploited to enhance forage preservation and food safety; therefore, future work should determine the source of such activity in silage inoculants and devise methods to ensure its persistence against spoilage-causing or pathogenic microorganisms in silages.

Fenlon and Wilson (2000) reported that *E. coli* O157:H7 numbers in poorly fermented, artificially inoculated silages grew from 10^3 to 10^6 log cfu/g during the first 7 d of ensilage when the pH stayed above 5. Similarly, in this study, *E. coli* counts in samples reinoculated with the pathogen after 144 h of aerobic exposure increased from 10^5 to more than 10^6 cfu/g when the pH was above 5. Weinberg et al. (2004) showed that though low pH achieved during ensilage eliminates *E. coli* from contaminated forages, the pathogen can be found in decaying parts of commercial silages with high pH. In agreement, in this study, *E. coli* was undetected in silages when pH was less than 4 during ensiling. Furthermore, growth of *E. coli* in silages reinoculated with the pathogen after 144 h of aerobic exposure depended on pH. High populations were present in control, EC, and EC+BII silages, which had high pH (>4), whereas they were at least about 10-fold lower in silages treated with *L. buchneri* inoculants, which had pH greater than 4. These results indicate that preventing aerobic deterioration and the attendant pH increase can prevent or minimize the growth of *E. coli* O157:H7 in silages contaminated with the pathogen during the feedout phase.

Maintenance of the low pH achieved at silo opening for 144 h by inoculants containing *L. buchneri* is probably attributable to the inhibitory effect of the acetate produced by *L. buchneri* on yeasts that metabolize lactate and thereby increase the pH in aerobically exposed silages. Our data suggest that *L. buchneri* inoculants can be used to maintain low pH (<4) in silages during the feedout phase and thereby curtail the growth of *E. coli* O157:H7 from extraneous sources that might contaminate the forage.

The *E. coli* strain used in this experiment (ATCC 43894) was reported to have low acid tolerance (Benjamin and Datta, 1995), indicating its susceptibility to inhibition during acidic silage fermentation. However, *E. coli* O157:H7 may acquire acid tolerance, and acid-tolerant strains could conceivably survive the low pH achieved in silages. Nevertheless, all studies we reviewed on the subject indicate that the ensiling process in well-managed silages is efficient at eliminating *E. coli* O157:H7 (Bach et al., 2002; Byrne et al., 2002; Chen et

al., 2005). Future work should examine whether other factors such as bacteriocins, competition with other microorganisms, and high organic acid concentrations contribute to elimination of the pathogen when forages are ensiled.

CONCLUSIONS

This study shows that *E. coli* O157:H7 was eliminated from forages contaminated with 5 log cfu/g of the pathogen when the pH dropped to 4 within 3 d of ensiling. Inoculant treatment did not affect *E. coli* O157:H7 elimination during ensiling but, unlike other treatments, application of *L. buchneri* inoculants eliminated yeast and mold populations, increased aerobic stability, and kept the pH below 4 for the duration of the 144-h aerobic exposure period. Reinoculation of silages with *E. coli* O157:H7 after 144 h of aerobic exposure resulted in relatively high ($P > 0.05$) *E. coli* O157:H7 counts of 2.87, 6.73, and 6.87 cfu/g for the control, EC, and EC+BII silages, respectively, whereas counts in EC+LB silages (1.96 log cfu/g) were low and the pathogen was not detected in EC+B500 silages. This suggests that *L. buchneri* inoculants can be used to curtail the growth of *E. coli* in silages contaminated with the pathogen at the feedout stage. All pure cultures of commercial bacterial inoculants exhibited antibacterial activity independent of pH against *E. coli* O157:H7, but the activity did not persist in the treated silages, suggesting that *E. coli* O157:H7 elimination from the silages was mediated by pH reduction.

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