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Application of bacteriocins from *Enterococcus hirae* on butterhead lettuce seeds inoculated with *Escherichia coli* O157:H7

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Abstract

Several outbreaks of *Escherichia coli* O157:H7 infections have been associated with contaminated seeds and sprouts. Effective procedures to control pathogenic bacteria on seeds destined for sprout production should be developed to ensure an acceptable microbiological quality. In this study, the use of bacteriocins produced by *Enterococcus hirae* (Eh9) was analyzed as a potential method to control the endogenous microbial populations of lettuce seeds and avoid pathogen contamination on seeds destined for sprout production. Seeds decontamination with Eh9 cell-free supernatant (CFS) achieved a significative fungiostatic effect without affecting germination. CFS treatment did not exert a biocontrol effect on *E. coli* endogenous; however, a significant bacteriocin biocontrol was reached on lettuce seeds inoculated with exogenous *E. coli* during refrigerated storage (7 days). Germination percentage and potential yield were not affected by the treatments. A better understanding of the behavior of pathogens in preharvest environments will assure the delivery of safe produce to the consumer.

Introduction

Recently, seed sprouts have become popular due to their nutritional value and low cost compared to other fresh products. However, there have been several outbreaks of foodborne illness caused by seeds and sprouts contaminated with *Escherichia coli, Salmonella*, and other enteric pathogens (Beuchat and Scouten, 2002; Waje and Kwon, 2007). Most of the outbreaks in alfalfa, radish, mung bean, and broccoli sprouts are related to contamination with *Escherichia coli* O157:H7. Therefore, initial microbial load should be controlled with antimicrobial treatments to minimize microbial contamination in the sprouts prior to consumption (Akbas and Olmez, 2007; Jin and Lee, 2007).

Several methods have been used to reduce the microbial load on seeds and sprouts, including exposure to ionizing irradiation (Bari *et al*., 2005; Saroj *et al.*, 2006), washing with chlorine (Winthrop *et al*., 2003), sodium hypochlorite (Beuchat *et al.,* 1998), and ozone (Wade *et al*., 2003). Although some of these methods can reduce the pathogenic bacteria, none of them can entirely eliminate pathogens in seeds and sprouts. If pathogenic bacteria are present on the seed, they are likely to grow to significant population levels in the finished sprouts. Therefore, effective decontamination procedures should be developed to eliminate pathogenic bacteria on seeds destined for sprout production, to ensure an acceptable level of microbiological safety. Thus, seed decontamination is an important tool for lettuce, since pollution and early contamination promotes the internalization and anchorage of the pathogens, diminishing the postharvest disinfection efficiency (Warriner *et al.*, 2003).

Lactic acid bacteria (LAB) bacteriocins have proven to be active against a broad spectrum of food spoilage and foodborne bacteria (Audisio *et al*., 2001; Audisio *et al*., 2005; Franz *et al*., 2007; Ibarguren, *et al.*, 2010; Ponce *et al*., 2008). Hence, the use of LAB and/or their natural products for food preservation (biopreservation) appears to be a promising alternative for reducing the growth of pathogens (Cleveland *et al*., 2001; Deegan *et al*., 2006; Galvez *et al*., 2007; Sobrino-Lopez and Martin-Belloso, 2008). Furthermore, this biopreservation strategy is compatible with organic production and is

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an important area of agrifood research.

The ideal biocontrol product for use on sprout seeds and sprouts, would contain non pathogenic microorganisms that are genetically stable, easily cultured and formulated using low cost substrate and materials, is easily applied to seeds, and is highly effective against several human pathogens. LAB are attractive candidates for commercial biological control agents due to their common occurrence on sprout surfaces (Patterson and Woodburn, 1980; Cai *et al.*, 1997), their ability to produce multiple antimicrobial agents, including bacteriocins, hydrogen peroxide, and organic acids *in vitro*, their extensive use in the food industry for fermentation and the lack of known pathogenicity (Breidt and Fleming, 1997).

In this study, the application of bacteriocins produced by Enterococcus hirae strain (Eh9) was analyzed as a potential method to control endogenous microbial populations and to avoid contamination with foodborne pathogens on butter head lettuce seeds destined for sprout production. The use of bacteriocins as a decontamination agent of lettuce seeds, has never been investigated before. Taking into account recent outbreaks associated with the consumption of this type of product, the search for new and more environmentally friendly disinfection techniques that guarantee the safety of sprouted seeds is a very interesting topic of research.

Materials and Methods

Bacteriocin solutions preparation

For culture supernatant preparation, *Enterococcus hirae* Eh9 (producing bacteriocin strain) was grown in MRS broth for 24 h at 30°C. Cell–free supernatant (CFS) was obtained by centrifuging at 10,000 g for 20 min at 10°C. To rule out the microbial inhibition due to LAB metabolism acidification, supernatants were adjusted to pH 6.5 with concentrated NaOH (8 mol L^{-1}) and filtered through pore filter (0.22 µm pore size; Millex GS, Millipore, France). CFS was used to evaluate its antagonistic properties against the native microbial populations on lettuce seeds and against *E. coli* O157:H7, ATCC 43895. CFS presented an activity of 134 UA mL^{-1} using *E. coli* O157:H7, ATCC 43895 as indicator strain (Ponce *et al*., 2008).

Inoculated lettuce seeds preparation

E. coli O157:H7, ATCC 43895 (American Type Culture Collection) was used as inoculating strain. Stock culture was maintained on Tryptic Soy Broth (TSB, Britania) containing 40% glycerol (w/v) at −25°C until use. Frozen culture was inoculated into TSB tubes (9 mL) and incubated for 24 h at 35°C. Two subsequent transfers to TSB tubes were performed every 24 h to ensure maximal activity of the strain before seeds' inoculation.

Inoculation was performed based on Lang *et al*. (2000) with minor modifications. Active *E. coli* O157:H7, ATCC 43895 culture was centrifuged at 8000 g for 12 min. The obtained pellet was washed twice with 25 mL of 1% peptonated water and then resuspended in another 25 mL of peptonated water. The resuspended culture was added to 100 mL of peptonated water. Butterhead lettuce seeds (Vilmorin, France) were immersed in the inoculum solution (5 g seed/20 mL inoculum solution) and the suspension was mixed in an orbital shaker (1000 ST, Zhejiang, China) at 100 rpm and 20°C for 10 min. Inoculated seeds were filtered and dried with sterile Whatman filter paper #42 in a laminar flow hood (20°C for 48 h) before being packed in sterile Petri dishes and stored at 2°C for 7 days. Microbial population counts on seeds were performed to assess the inoculation levels in the lettuce seeds (104 CFU g^{-1}).

Seeds' treatment with bacteriocin solution

Imbibition was carried out following the methodology proposed by Wierzbicka and Obidzinska (1998) with modifications. That is, 0.5 g of lettuce seeds (inoculated and non-inoculated) were weighed (0.001 g precision) and then immersed in 30 mL of different imbibition solutions: distilled water (as a control sample) and LAB cell-free supernatant (CFS). Afterwards, the different solution-seed systems were stirred in an orbital shaker (1000 ST, Zhejiang, China) at 100 rpm for 10 min. Treated seeds were filtered through sterile filter paper and dried for 2 min. This was conducted under sterile conditions.

Germination assay

Immediately after seed imbibition with CFS and distilled water (control samples), lettuce seeds were sown in plastic trays (18 x 14 cm^2) with two layers of Whatman filter paper #42, adequately moistened with 20 mL of sterile distilled water. Then trays were covered with plastic foil to prevent dehydration and incubated in a germination chamber (20-22°C and 8 h photoperiod) for 7 days. Percentage of seed germination was determined according to Barassi *et al.* (2006) by counting germinated seeds at day 7. Only those seedlings without defects were considered as germinated. In order to determine absence of defects, each tray was thoroughly inspected with a magnifying glass while germinated seedlings were counted. Each treatment was performed in duplicate and three independent runs were carried out.

After sprouting (day 7) seedlings were measured and weighed to estimate the effect of the treatments in the potential yield. Results are presented in mm/seedling and g/50 seeds, respectively.

Microbiological studies

For each treatment, a sample of 50 seeds was homogenized in 10 mL of phosphate-buffered solution (pH=7.2) and vigorously mixed for 60 s. Aliquots of each supernatant were used for microbial determinations by duplicate. Microbial population counts were performed with the following culture media and incubation conditions (Ponce *et al*., 2008): mesophilic aerobic bacteria (MES) on Plate Count Agar (PCA) incubated at 30 ± 1°C for 48 ± 3 h; *Lactobacillus* spp. (LAB) on MRS agar (*Lactobacillus* agar) incubated in anaerobic jars with Anaerocult C (Merck) at 30±1°C for 5 days; yeast and molds (YM) counted in Yeast–Glucose–Chloranphenicol (YGC) medium at 25°C for 5 days; and *Enterobacteriaceae* and total coliforms (TC) in Mac Conkey agar with the addition of superficial virgin layer incubated at 30–32°C for 24 h. *E. coli* colonies (EC) were performed on the surface of Eosin Methylene Blue (EMB) agar (a selective culture medium for Gram-negative bacteria) and incubated at 37±1°C for 24 ± 2 h (Leininger *et al.*, 2001).

Treatment effects on the seeds microbial load were analyzed immediately after imbibition (4 h after treatments, day 0), at day 2 for short-term effect, and after sprouting (day 7). Results are presented as the mean of three independent runs, expressed as log CFU g^{-1} seeds.

Statistical analysis

Data were analyzed using SAS, software version 8.0 (SAS Institute Inc., 1999). PROC GLM (General Linear Model Procedure) was used for the analysis of variance (ANOVA). PROC UNIVARIATE was used to validate ANOVA assumptions. Results reported in this paper are lsmean values (least square mean, means estimators by the method of least squares) along with their standard error (Kuehl, 2001). When significant differences were found with the GLM procedure, the Tukey–Kramer multiple comparison test was carried out ($P < 0.05$). For germination assays, where results correspond to proportions instead of normally distributed data, PROC RANK was used to group the proportions in order to select the treatment with higher germination rate.

Results and Discussion

Microbial populations evolution

At day 0 (Figure 1), microbial counts obtained for untreated seeds (control samples) were 2.16±0.45 and 2.66±0.53 log CFU g^{-1} for MES and YM, respectively. Lactic Acid Bacteria (LAB) counts were lower than the detection level (< 10 CFU g^{-1}). After 24 h of enrichment in Brain and Heart Infusion (BHI), total coliform (TC) microbial counts were 2.31±0.39 log CFU g^{-1}, while *E. coli* (EC) counts were less than 1 log CFU g^{-1}.

MES microbial counts between 3 and 5 log CFU g^{-1} have been reported for alfalfa, onion, and mung bean seeds (Andrews *et al.*, 1982; Prokopowich and Blank, 1991). Piernas and Guiraud (1997) reported MES counts of 7 log CFU g^{-1} for rice seeds. In the present study, lower microbial counts were observed for lettuce seeds; however, these microbial cells can grow exponentially. In fact, imbibed seeds can increase their microbial counts 10-fold overnight (Prokopowich and Blank, 1991). Sprouts germinated in sterile containers had reached MES counts of 9.2×10^8 CFU g^{-1}, which proves that microbial growth during sprout production can occur even if controlled sanitary conditions are used (Splittstoesser *et al*., 1983). This could be even more dangerous if pathogenic cells are present. In artificially inoculated seeds, Bacillus cereus increased from 10^3 to 10^7 CFU g^{-1} during sprouting (Harmon *et al*., 1987). *E. coli* O157:H7 inoculated into radish seeds increased between 4 and 5 log during sprouting (Harmon *et al*., 1987). This justifies the need for initial decontamination treatments that are able to reduce or fully eliminate the initial microbial load and the potential pathogenic bacteria present in lettuce seeds before sprouting.

Figure 1A shows the evolution of mesophilic population (MES) in bacteriocin- treated, inoculated and control seeds during sprouting. Mesophilic microorganisms give an estimate of total viable populations and are indicative of the endogenous microorganisms present in the material. Seeds treated with LAB cell-free supernatant (CFS) and seeds inoculated with *E. coli* presented higher microbial counts with respect to control samples in a range of 1.40 to 2.43 log CFU g^{-1}. The seed treatment with CFS could facilitate the release of nutrients which together with high water activity and pH close to neutrality, generates favorable conditions for bacterial growth.

At day 2 of germination, the control sample incremented about 5.48 log respect to its initial count. On the other hand, the increases observed for samples treated with CFS, both inoculated and

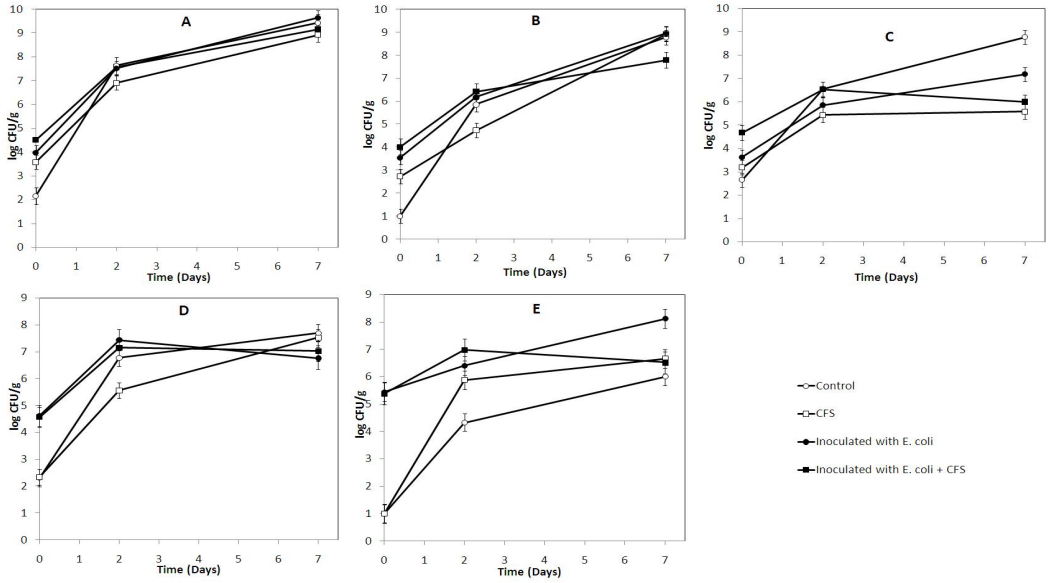

Figure 1. Effects of bacteriocin application on microbial populations of butterhead lettuce seeds (inoculated and non-inoculated with *E. coli* O157:H7). A: total mesophilic bacteria; B: lactic acid bacteria; C: yeast and molds; D: total coliforms; E: *Escherichia coli.* Data is shown as lsmean ± standard deviations. CFS: cell-free supernatant

non-inoculated, were between 3.5 and 4.5 log. After 7 days of germination, no significant differences between treatments were observed for mesophilic counts. The microbial growth rate from day 2 to day 7 was approximately the same in every sample. This naturally occurring population may have rapidly increased during germination and sprouting stages which are characterized by high moisture and a temperature range of 21 to 25°C (Taormina and Beuchat, 1999).

Figure 1B shows the evolution of lactic acid bacteria (LAB) population. In the control sample, the initial value of LAB population was lower than 1 log CFU g^{-1}, while in samples treated with CFS the initial values was 2.7 log CFU g^{-1}. Lettuce seeds inoculated with *E. coli* and seeds inoculated with *E. coli* plus CFS treatment showed values between 3.5 and 4 log CFU g^{-1}, approximately.

At day 2 of germination, control sample incremented LAB counts 5 log respect to its initial counts, while lettuce seeds treated with CFS showed an increase of 2.01 log CFU g^{-1}. On the other hand, LAB counts on seeds inoculated with *E. coli* and treated with supernatant incremented in a range of 2.0–2.5 log. At day 7 the sample inoculated with *E. coli* and treated with CFS was the only one significantly different from the control, reaching values of 7.79 log CFU g^{-1}. The rest of the samples had no significant differences from control, with values in the range of 8.72–8.95 log.

Figure 1C shows the effect of bacteriocin treatments on yeast and molds (YM). Food spoilage by mold and the occurrence of its mycotoxins constitutes a potential health hazard. Development of biological controls should help improve product safety by controlling mycotoxin contamination. Data have actually shown that many LAB can inhibit mold growth and that some of them have the potential to interact with mycotoxins (Dalié *et al.*, 2010).

At day 0, YM counts were significantly different ($P< 0.05$) between samples, with higher values for all samples inoculated with *E. coli* (1 or 2 log higher than control sample). This may be due to the wide dispersion presented by the population of YM in lettuce seeds. The growth evolution was different among samples, especially after 7 days of germination. YM counts in samples treated with CFS showed no significant changes from day 2 to day 7 of germination, showing a fungistatic effect on yeast and mold populations. There is a supernatant effect on one side and a pathogen inoculum effect on the other. In both cases, the resulting effect is that at the end of the germination period, yeast and mold counts on treated samples were lower compared to control. In accordance with our results, there are several reports on antifungal activity of LAB. Okkers *et al*. (1999) found that *L. pentosus* exhibited fungistatic effects against *Candida albicans*. Lavermicocca *et al.* (2000) reported the production of antifungal compounds by a sourdough *L. plantarum* strain. The same was observed for *Lactobacillus coryneformis* (Magnussib and Schnürer, 2001), as well as isolates of *L. plantarum, L. coryneformis, Lactobacillus salivarius, Lactobacillus sake, E.*

hirae, and *Enterococcus durans* from various sources (Magnusson *et al*., 2003). Katrin *et al.* (2002) also found that *L. plantarum* MiLAB 393 produced antifungal substances. Addis *et al.* (2001) reported that LAB inhibited the growth of several yeasts, with *L. plantarum* being active against *S. cerevisiae* and other yeast species.

Figure 1D shows the total coliforms (TC) evolution at different germination times. At day 0, microbial counts were different in non-inoculated and inoculated samples, with approximate values of 2.3 and 4.6 log CFU g^{-1}, respectively. TC counts after two days of germination were similar in all samples, without significant differences among them. These results indicate a rapid colonization of total coliforms in samples that were non-inoculated, and on the other hand a lower colonization rate in the inoculated samples. This trend continued until the end of the germination period, with final values of approximately 6.8–7.7 log CFU g^{-1}.

E. coli *evolution*

Effects of CFS treatment on *E. coli* populations are shown in Figure 1E. Samples artificially inoculated with the pathogen resulted in an initial *E. coli* count of approximately 5.45 log. On the other hand, *E. coli* counts on non-inoculated seeds were lower than the detection level (< 1 log CFU g^{-1}). Treatment with the CFS did not reduce *E. coli* population with respect to the control sample. After 2 days of germination, *E. coli* counts in the control sample increased by approximately 3 log. In non-inoculated samples (both control, and treated with CFS), the *E. coli* counts increased by 4.5 log. This would indicate no biocontrol effect of CFS on endogenous *E. coli* growth. There have been a very limited number of studies on seed sanitization using naturally contaminated rather than artificially inoculated seeds (Stewart *et al*., 2001; Fett, 2002a; Suslow *et al*., 2002). In this sense, the efficacy of bacteriocin-like substances on lettuce seeds for eliminating bacterial human pathogen was questionable. However, *E. coli* counts on samples artificially inoculated with *E. coli* and treated with CFS only increased by 1 log, showing a significant biocontrol effect on exogenous *E. coli*. Numerous chemical treatments in addition to chlorine, as well as several physical treatments, have been tested individually or in combination for eliminating pathogens from artificially inoculated sprout seeds. To date, there are few reports of individual treatments (chemical or physical) capable of eliminating pathogens from artificially inoculated sprout seeds, without significant adverse effects on seed germination or sprout seeds. One consistent finding, is that Salmonella and *E. coli* when inoculated in sprout seeds are not eliminated even by treatments of 16000 to 20000 ppm available chlorine for 10 to 15 minutes (Fett, 2002b). Nandiwada *et al.* (2004) tested the efficacy of colcin Hu194 treatment against *E. coli* O157:H7 strains on alfalfa seeds. A significant reduction of 3 log on *E. coli* strains was found when applied 20-fold higher concentrations of colcin Hu194. This means that Eh9 cell-free supernatant could be concentrated in order to achive higher concentrations of the bacteriocin-like substances, and improve the treatment against *E. coli* O157:H7.

It is important to prevent pathogen colonization due to contamination because attachment is a requirement for colonization and subsequent transmission of pathogens via the edible parts of plants. Indeed, once the pathogen is attached, it is very difficult to remove from contaminated fruits and vegetables by washing (Beuchat and Scouten, 2002).

After 7 days of germination, the biocontrol effect of CFS on inoculated samples with *E.coli* was maintained. The results indicate that during this period of germination, in samples inoculated and treated with Eh9 supernatant, *E. coli* counts were not increased; however, in untreated samples, pathogen counts were increased by about 1.7 log. Ponce *et al.* (2012), studying biopreservation effect exerted by LAB on the *E. coli* O157:H7 population on lettuce leaves, determined that there was a bacteriostatic effect of *E. faecium* and *E. hirae.* Microbial competition strategies require time, which in a product such as lettuce could produce rapid deterioration in quality; however, this may be a possibility in lettuce seeds. In the present study, CFS obtained from *E. faecium* (Eh9) cultures was applied crude. More concentrated extracts of Eh9 should be tested to improve the biocontrol effect of *E. coli* without affecting germination and biomass parameters.

Germination assay

Modern crop production systems are required for a high degree of crop establishment (Salter, 1985). The need for high plant densities in the greenhouse and uniform plant stands demand that treatments applied to seeds do not affect these characteristics. The viability of the majority of lettuce seed lots used in commercial farms is high, usually over 98% (Grey *et al*., 1988). Therefore, the application of the supernatant in lettuce seeds should not reduce its germination percentage or affect its biomass parameters. Germination percentage could be related to plant density, while weight and length of the seedlings could be associated with yield and emergence (Grey *et al*., 1988).

Table 1. Effects of baceriocin application on germination percentage and biomass parameters (weight and length) of lettuce seeds (inoculated and non-inoculated with *E. coli* O157:H7) after 7 days of sprouting. Values are lsmeans (least square means) ± standard deviations. CFS: cell-free supernatant

TREATMENT	Germination (%)	Weight (g)	Length (mm)
Control	98.3 ± 1.2	0.0254±0.0021	53± 3
CFS	97.4 ± 0.9	0.0244±0.0042	52± 4
Inoculated	97.4 ± 1.1	0.023±0.0019	51± 3
Inoculated+CFS	98.2 ± 1.2	0.0295±0.0051	55± 4

Results regarding germination and potential yield are shown in Table 1. The treatments applied in the present study did not affect germination percentage or potential yield (length and weight of seedlings). No significant differences ($P > 0.05$) were found in treated seedlings compared to the control sample after 7 days of sprouting in optimal conditions. Therefore, no economic consequences should be expected from the application of the proposed treatment. These are important results for the producer, since horticultural seeds are expensive and high germination percentage and stand are always expected. Moreover, differences in plant size and date of maturity between plants that can result from an extended period of seedling emergency may affect yield per plant, especially in close-space crops such as lettuce (Goñi *et al.*, 2012).

Conclusions

In this study it was possible to apply the cell-free supernatant "in vivo", with the antimicrobial efficiency presented in previous *"in vitro"* studies with the same pathogen. Germination percentage and potential yield were not affected by the treatment. Results indicated no biocontrol effect of cell-free supernatants on endogenous *E. coli* growth. However, samples inoculated with the pathogen and treated with the supernatant showed a slight reduction in *E. coli* counts. Prevention of preharvest contamination is essential for minimizing the risk of illness caused by consuming raw products, because postharvest treatment with sanitizers cannot be relied upon to eliminate pathogens. Further studies are needed for a potential application of the bacteriocin previously isolated, purified and concentrated. A better understanding of the behavior of pathogens in preharvest environments will enhance the likelihood of developing effective strategies and interventions that will assure the delivery of safe produce to the consumer.

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