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Research Note

Potential of *Lactobacillus casei,* Culture Permeate, and Lactic Acid To Control Microorganisms in Ready-To-Use Vegetables

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ABSTRACT

The effects of various treatments (i.e., the addition of a strain of *Lactobacillus* that produces antimicrobial agents, *Lactobacillus casei* IMPC LC34, its sterile permeate, and 0.5 or 1% lactic acid) on the growth of microorganisms associated with ready-to-use mixed salad vegetables were compared during refrigerated (8°C) storage. The addition of 3% culture permeate to mixed salads reduced the total mesophilic bacteria counts from 6 to 1 log CFU/g, and suppressed coliforms, enterococci, and *Aeromonas hydrophila* after 6 days of storage at 8°C. A similar effect was shown when the *L. casei* culture was inoculated in the vegetables. One percent lactic acid had a bacteriostatic effect on the bacterial groups examined, except for total and fecal coliforms, which were reduced by about 2 and 1 log unit, respectively, while 0.5% lactic acid did not affect the indigenous microflora of the vegetables. The potential of these new hurdles to prevent the growth of spoilage and pathogenic bacteria in ready-to-use salad vegetables is suggested.

Key words: Ready-to-use vegetables, microflora control, antimicrobial agents, lactic acid, *Lactobacillus casei*

The lactic acid bacteria are useful in the production of a variety of foods to improve flavor and palatability, to make novel foods, and as a means of food preservation. The antimicrobial properties of lactic acid bacteria are of great interest to researchers involved in the development of protective starter cultures for foods. In complex environments, indigenous or added lactic acid bacteria which produce inhibitory compounds other than lactic acid may have competitive advantages to suppress undesirable microflora *(13).*

Ready-to-use fresh salad vegetables are perishable products which must be stored under refrigerated conditions and sold within a week *(6).* The microbial population of these minimally processed vegetables is mainly constituted by species of *Enterobacteriaceae* and *Pseudomonas* spp. *(7);* occasionally, psychrotrophic pathogenic bacteria such as *Listeria monocytogenes, Aeromonas hydrophila,* and *Yersinia enterocolitica* have been detected in both chilled salads and in prepacked vegetables *(1–4, 12).* Mesophilic pathogens as *Staphylococcus aureus, Salmonella* spp., *Shigella* spp., and some enterotoxigenic or enteropathogenic strains of *Escherichia coli* can also be present and multiply in vegetables when abuse temperature occurs *(3, 5, 10).* Therefore, contaminated processed fish vegetables might be considered as a vehicle of transmission of potential pathogens and as a possible risk for the health of consumers.

In order to extend the shelf life of these food systems and to ensure their microbiological safety, new "hurdles" must be investigated. Research has indicated that the application of psychrotrophic lactic cultures that produce antimicrobial agents to ready-to-use vegetables was effective in controlling the growth of coliforms, enterococci, and total mesophilic bacteria *(14);* moreover an inhibitory effect on *L. monocytogenes* growth, as well as the suppression of *A. hydrophila, S. typhimurium,* and *S. aureus* was demonstrated *(15),* but the effective agent of such inhibition remained unknown.

The objective of the present study was to evaluate the effect of the addition of a *Lactobacillus casei* culture, its sterile culture permeate, and of lactic acid on the growth of microorganisms associated with ready-to-use salad vegetables during refrigerated storage.

MATERIALS AND METHODS

Inoculum preparation

Antimicrobial agent-producing *L. casei* IMPC LC34 isolated from ready-to-use vegetables and previously characterized *(15)* was used. Stock cultures were maintained at −80°C in MRS broth (Difco Laboratories, Detroit, MI) with the addition of glycerol (10%). To prepare the inoculum suspension, the strain was subcultured overnight in 10 ml of MRS medium at 30°C, centrifuged at 13,000 × *g* for 10 min (Sorvall RC-5C, Sorvall Instruments, Dupont Company, Biomedical Products Division, Wilmington, USA) and the pellet diluted in sterile quarter-strength Ringer solution

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(Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) to reach about 8 log CFU/ml.

Culture permeate preparation

Lactobacillus casei IMPL LC34 was grown overnight in MRS broth and cells were removed by centrifugation (13,000 × *g* for 10 min). Supernatants were adjusted to pH 7 with 2 M NaOH, dialyzed in SpectraPor (Spectrum Medical Instruments, Inc, Houston, USA) dialyzing tubes (molecular mass cutoff 1,000 Da) against water purified with mQ purifier (Millipore Division, Bedford, MA, USA) with several changes, and concentrated 100-fold by a stirred ultrafiltration cell model 8400 (Amicon, Inc, Beverly, MA, USA) using a Diaflo membrane of 10,000 Da molecular mass cutoff (Amicon). The permeate was concentrated 100:1 with 20,000 polyethylene glycol (PEG, Fluka Chemie AG, Buchs, Switzerland), filter-sterilized with a 0.2-μ pore-size filter (Millipore) and stored at 4°C until use.

The inhibitory activity of sterile culture permeate and retentate was tested against the following target microorganisms belonging to the collection of the Microbiology Institute, Piacenza, Italy: *A. hydrophila* 2, *L. monocytogenes* EG, *S. aureus* SA4, *Citrobacter freundii* CF2, *Enterobacter cloacae* CF3 and *E. coli* CT4. All the target organisms were grown aerobically in brain heart infusion broth (Oxoid) or nutrient broth (Oxoid) at 30°C. The well-diffusion agar procedure was employed as previously described *(11)*. Three replicates were tested per treatment and means and standard deviations were calculated.

Vegetable preparation and treatments

Commercial packs of ready-to-use mixed salad vegetables, containing carrots, endive, garden rocket, and green chicory, packed under air in polyethylene trays wrapped with polypropylene film, were procured from local suppliers one day after packaging. On arrival to the laboratory, the packs of mixed salads were opened, mixed, and subdivided into five 200-g batches. Each batch was sprayed, using a spray nozzle (International PBI S.p.A., Milano, Italy) with 6 ml of the following treatments: (a) *L. casei* IMPC LC34 suspension to reach approximately 6 log CFU/g; (b) *L. casei* IMPC LC34 culture permeate; (c) and (d) 0.5 and 1% lactic acid (Merck-Schuchardt, Munchen; 10% sterile stock solution), respectively; (e) sterile quarter-strength Ringer solution (Oxoid), as negative control. The differently treated batches were subdivided into two 100-g aliquots, packed in polyethylene trays, wrapped with polyethylene film (two packs for each treatment) and stored at 8°C for 6 days. The procedure was repeated three times in different periods (from March to June 1996).

Microbiological analyses

Vegetables were sampled for microbiological analysis at time 0 (day of inoculation) and at 2-day intervals up to 6 days of storage at 8°C. Ten grams of salad were aseptically removed from each sample and placed into sterile stomacher bags, combined with 90 ml of quarter-strength Ringer solution (Oxoid) and homogenized for 2 min with a Stomacher Lab-Blender 400 (Seward Medical, London, UK). Appropri-

Table 1. *Inhibitory activity of* Lactobacillus casei *IMPC LC34 culture permeate and retentate against food-borne pathogens and coliform bacteria*

	Diameter of inhibition zone (mm), mean ± SD (n = 3)			
	Retentate		Permeate	
Target organisms	8°C	37°C	8°C	37°C
Aeromonas hydrophila 2	—[a]	—	17 ± 0.5	20 ±4.1
Listeria monocytogenes EG	—	—	17 ± 1.0	21 ± 2.5
Staphylococcus aureus SA4	—	—	19 ± 2.45	23 ± 1.25
Citrobacter freundii CF2	—	—	—	15 ± 0.47
Enterobacter cloacae CF3	—	—	18 ± 2.05	17 ± 1.7
Escherichia coli CT4	—	—	22 ± 0.94	21 ± 1.88

[a]No inhibition.

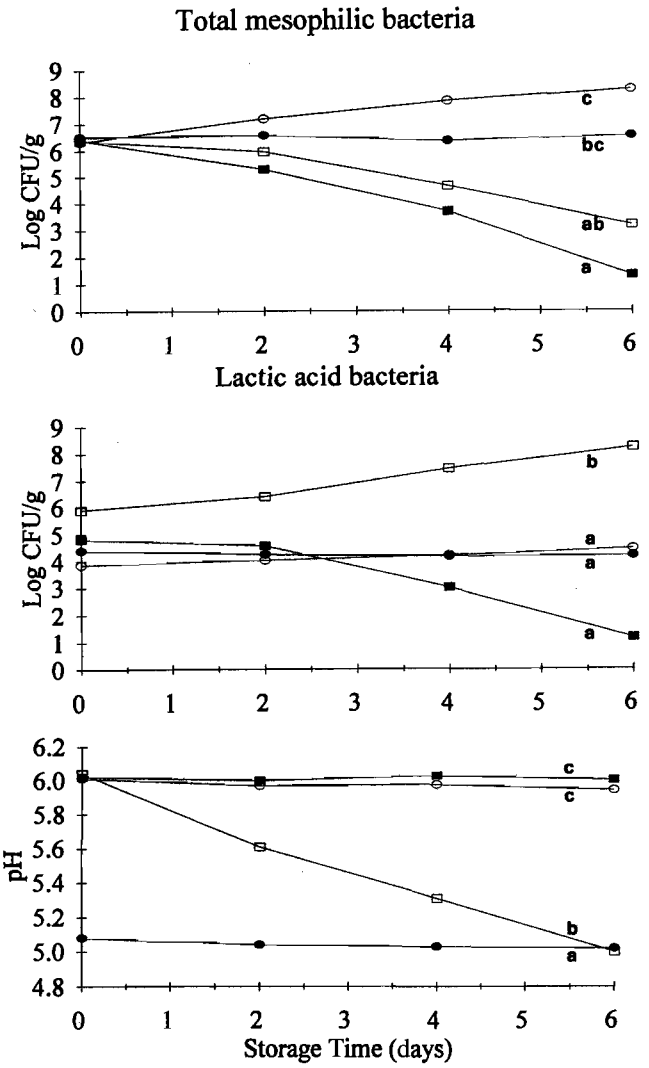

FIGURE 1. *Variations of microorganism counts and pH values in differently treated ready-to-use mixed salad vegetables during storage at 8°C for 6 days. control; inoculated with* Lactobacillus casei *IMPC LC34; treated with* L. casei *IMPC LC34 culture permeate; treated with 1% lactic acid. Each data point represents the mean of three duplicate trials. Treatments associated with different letters are significantly different ($P < 0.05$) according to Duncan's test.*

ate serial dilutions of salad homogenate were plated, in duplicate, on selective media to enumerate the following microorganisms: total mesophilic bacteria in plate count agar (Oxoid) (48 h, 30°C); total and fecal coliforms in violet red bile agar (Oxoid) (24 h, 37 and 44°C, respectively); lactic acid bacteria in MRS agar (Difco) (48 h, 30°C in anaerobiosis); enterococci in Slanetz & Bartley (Oxoid) (24 h, 37°C); *A. hydrophila* in starch-ampicillin agar *(8)* (24 to 36 h, 30°C).

The measurement of pH was performed on the first homogenized dilution of the salad samples during storage with a Crison pH meter model micro pH 2001 (Crison, Alella, Barcelona, Spain).

Sensory evaluation

A sensory evaluation session was conducted by a panel of 10 randomly chosen judges to evaluate the differences among the differently treated mixed salads and controls for flavor, odor, appearance, and texture after 6 days of storage at 8°C. Samples were scored on a hedonic scale of 1 to 9; 9 was considered as excellent, 5 acceptable, 1 poor. A high score indicated presence of typical fresh flavor, odor, and appearance, brilliant color and good firmness.

Statistical analysis

Data were analyzed with two-way analysis of variance followed by Duncan's multiple range test *(9)* for comparison among means.

RESULTS AND DISCUSSION

The effect of various treatments on the growth of microorganisms associated with ready-to-use mixed salad vegetables, stored at 8°C for 6 days, was investigated. A comparison was made among the addition to vegetable samples of antimicrobial agent-producing *L. casei* IMPC LC34 strain, its sterile permeate, and lactic acid at two different concentrations (0.5 and 1%).

Preliminary trials in vitro demonstrated that *L. casei* IMPC LC34 culture permeate displayed inhibitory activity toward food-borne pathogens and coliform bacteria at 8°C and at 37°C (Table 1). All the target organisms tested were inhibited by permeate at the two temperatures considered, except for *C. freundii* at 8°C, while no inhibitory effect was detected using retentate.

Figures 1 and 2 show changes in counts of microorganisms and in pH values of differently treated mixed salads during storage. The culture permeate sprayed on mixed salads to verify its efficacy in a real food system strongly reduced ($P < 0.05$) the total mesophilic bacteria counts from 6 to 1 log CFU/g, whereas total and fecal coliforms, enterococci, and *A. hydrophila* were completely suppressed ($P < 0.05$) after 6 days of refrigerated storage.

The *Lactobacillus casei* culture inoculated in vegetables at a level of 6 log CFU/g was able to grow at 8°C, reaching 8.24 log CFU/g after 6 days. Consequently, the counts of total mesophilic bacteria, coliform groups, enterococci, and

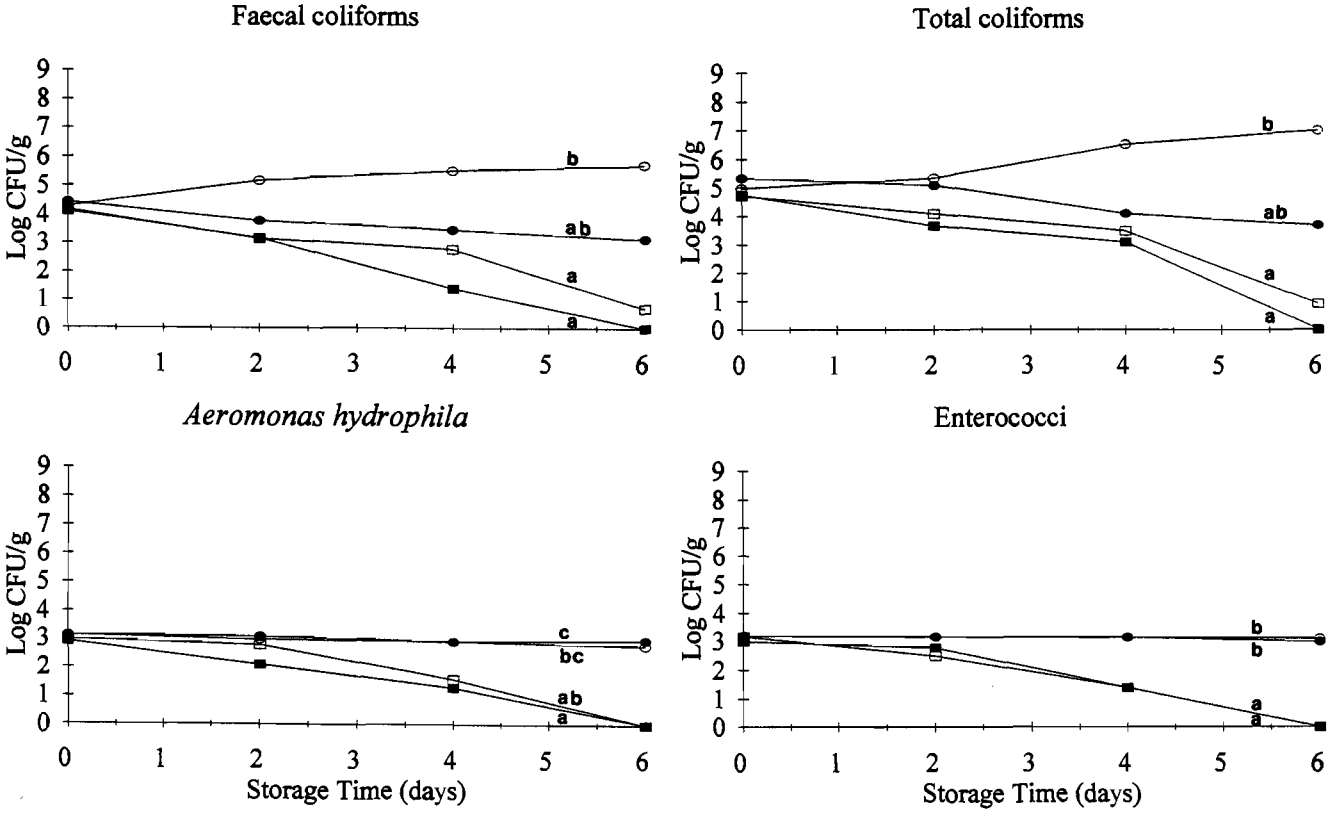

FIGURE 2. *Variations of microorganism counts in differently treated ready-to-use mixed salad vegetables during storage at 8°C for 6 days.* -○- *control,* -□- *inoculated with* Lactobacillus casei *IMPC LC34;* -■- *treated with* L. casei *IMPC LC34 culture permeate;* -●- *treated with 1% lactic acid. Each data point represents the mean of three duplicate trials. Treatments associated with different letters are significantly different* ($P < 0.05$) *according to Duncan's test.*

A. hydrophila markedly decreased ($P < 0.05$) during storage.

The addition of 1% lactic acid caused an immediate pH decline to 5.0 in the mixed salads, a value reached also in the samples inoculated with the *L. casei* strain at the end of refrigerated storage (Fig. 2). One percent lactic acid had a bacteriostatic effect on the bacterial groups examined except for total and fecal coliforms, which were reduced by about 2 and 1 log unit, respectively. The treatment of vegetables with 0.5% lactic acid did not affect the microflora counts, which were similar to counts of control, even if the pH value was lower (5.7) during the entire storage period (data not shown).

No significant difference was detected by a sensory panel in flavor, odor appearance, and texture among mixed salads with different treatments and controls after 6 days of storage.

The results of this study indicate that the inhibitory effect of *L. casei* on the microbial population of mixed salads is due not only to lactic acid, but probably to an active agent, different from hydrogen peroxide and other organic acids, present in its permeate. Further studies are needed to characterize this compound.

The addition of antimicrobial agent-producing strains or the use of culture permeate, combined with refrigerated storage and good hygienic handling practices, could be helpful to control spoilage and pathogenic bacteria in ready-to-use vegetables, so providing a longer shelf life and assuring a safer product for consumption.

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