In situ control of food spoilage fungus using *Lactobacillus acidophilus* NCDC 291

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Revised: 12 July 2011 / Accepted: 1 August 2011 / Published online: 13 August 2011
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Abstract A challenge for food industry today is to produce minimally processed food, without use of chemical preservatives and little compromise on nutritional status. *Lactobacillus acidophilus* NCDC 291 can be directly added to food where it enhances shelf life by competing with other microflora (both bacterial and fungal) for food and also by production of antimicrobial metabolites as bacteriocins. Comprehensive studies have demonstrated the *in vitro* activity of bacteriocins. However their role in preventing fresh food spoilage needs more elucidation. The present study was conducted to evaluate the efficacy of the whole cells of this organism as biopreservative agent against fungi. Four most commonly occurring spoilage fungi were isolated and were identified as *Fusarium*, *Alternaria*, *Penicillium* and *Aspergillus*. Growth of all of them was inhibited in *in vitro* studies, (approximately 33–43% decrease in mycelial dry weight basis between test and control). *In situ* biopreservation of Indian cheese and raw poultry meat was attempted and the colony count of *Alternaria* was significantly ($p < 0.05$, Bonferroni Holm) reduced in presence of *L. acidophilus*. Dip and Keep approach of preservation for *Mangifera* and *Momordica* were carried out in which microbial spoilage was not observed up to 6 days.

Keywords *Lactobacillus acidophilus* · Biopreservation · Spoilage fungi · *Mangifera* · *Momordica* · Hurdle technology

Introduction

With ever increasing population in the world especially India food security has become a burning issue. Whatever grain and fruit/vegetable is produced needs to be made available to the masses through proper post-harvest practices. Uncertain weather is affecting food production and hence it is imperative to prevent wastage and to utilize all the food produced. United Nations University's World Hunger Programme observes that the problem of post harvest food conservation is not getting due attention. They recommended that the concern about the post harvest losses should be one of the three sub-programmes of the World Hunger Programme. India, ranks among the top three nations in fruit and vegetable production. She also produces 232 million tonnes of food grains to meet the food security of the nation (Anon 2011). Even though the Green Revolution has made India self-sufficient, but a lot of food goes waste because of inefficient storage facilities (for grains) and lack of infrastructure on-farm and off-farm for fruits and vegetables such as refrigerated transport and cold storage. Food losses in the world are high- nearly 20% for non-grain crops, fish, perishables etc., and 10% for grain crops. In India alone 30% to 40% of vegetables and fruits produced rot which amounts to about Rs 500,000 million per annum as per the observation of Mangaraj and Singh (2008). Furthermore being a country of tropical and subtropical climate the temperature is an additional cause of the losses. In US post harvest losses are between 2 and 25% depending on the produce.

There is a dire need to produce fresh-tasting, nutrient rich, easy to prepare minimally processed food with little or no chemical preservatives. With more women working nowadays, the trend towards labor saving, convenient, ready to eat (RTE) food is on rise. Also adverse health

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effects from the chemical additives like nitrites, sulphites, etc. in the food, have attracted consumers towards natural and fresher foods with no chemical preservatives. This has stimulated research interest in finding natural but effective preservatives i.e. use of biopreservatives which is very crucial and timely.

Biopreservation refers to the extension of the shelf life and improvement of safety of food using microorganisms and/or their metabolites (Ross et al. 2002). Lactic acid bacteria LAB comprises mainly of four genera namely, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. They are routinely used as starter cultures or as adjuncts in majority of the fermented foods especially in the dairy food products. LAB produce an array of antimicrobial substances and proteinaceous factors such as bacteriocins. These aid in enhancing shelf-life and quality up-keep of foods.

Lactobacillus acidophilus is a Gram positive, rod-shaped, non-spore forming, homo-fermentative (produces lactic acid via glycolytic pathway) bacterium that is normal inhabitant of our gastrointestinal and genitourinary tracts (Klaenhammer et al. 2008). *Lactobacillus acidophilus* when added to food products as a preservative enhances their shelf-life and it acts as a probiotic as well (Gomes and Malcata 1999). It can be used for both *in situ* and *ex situ* food preservation. *In situ* preservation is more economical as it does not include the cost of purification of bacteriocins. Bacteriocin yield is significantly lower in synthetic medium as compared to natural medium and it requires a 3 step purification which is not cost effective when carried out on an industrial scale. Hence, in the present study, use of microorganism as a vehicle for food preservation has been focused on.

Material and methods

The microbial culture *Lactobacillus acidophilus* NCDC 291 was obtained from National Dairy Research Institute, Karnal, India and was maintained on recommended MRS medium. Standard methods for microbiological examination of foods were followed for the present study (Brackett and Splittstoesser 2001).

Isolation and identification of spoilage fungi

Mango (*Mangifera indica*) and bitter gourd (*Momordica charantia*) were chosen for the isolation of spoilage fungi as they were abundantly available during the time period of study and also because they are averagely priced and hence accessible to large population. Visibly spoilt samples of above mentioned foods were procured and immediately transported to the laboratory for microbiological isolation. Weighed 1 g of the sample, macerated, suitably diluted and plated it on Rose Bengal Chloramphenicol Agar. It was

then incubated at 30 °C for 72 h. Subsequent subculturing was carried out for obtaining pure cultures. The isolates were identified using the services of Plant Pathology Laboratory, Department of Plant Pathology, Punjab Agricultural University, Ludhiana, Punjab, India.

In vitro antifungal effects

In order to study the antifungal effects, PDB (Potato Dextrose broth) was inoculated with approximately 10^7 spores/ml of the each fungal isolate individually and also with 1 ml (corresponding to McFarland Standard 2) of metabolically active bacteriocin producing culture (*L. acidophilus* NCDC 291). After incubation for 72 h at 30 °C the growth of the fungi was ascertained by wet weight and dry weight recordings (Neilson et al. 1997; Roy et al. 1996). A control without bacteriocin producer was also run. Percentage decrease in dry weight was recorded.

Dip and Keep approach

Spoilage isolate II i.e. *Alternaria alternata* was used in *in situ* studies because it was the most abundantly encountered and is a common spoilage organism associated with food chosen for the present study. It is responsible for postharvest decay in mango (Prusky et al. 2006).

On one set of three fresh mangoes (*Mangifera indica*) a spore suspension containing 10^7 spores/ml was applied. Subsequently a metabolically active broth of *Lactobacillus acidophilus* NCDC 291 (McFarland Standard 2) was smeared on to it. One set of test and control (without *L. acidophilus* smear) was incubated at ambient temperature and another set was incubated at refrigeration temperature. Three sets of control and test each were run simultaneously. The presence/absence of microbial spoilage and textural changes (in terms of firmness) were subjectively evaluated every 24 h for the time period of 6 days. Similar procedure was followed for biopreservation of bitter gourd (*Momordica charantia*).

In situ antifungal effects

Weighed 5 g of Indian cheese and cut it into small cubes of equal size and inoculated with 1 ml of spore suspension containing 10^7 spores/ml of spoilage isolate II followed by addition of bacteriocin producer @ 2% (v/w). It was again kept undisturbed for 10 min and then incubated at 10 °C. SPC was recorded every 24 h for a period of 6 days (Roy et al. 1996). Similar protocol was followed for raw poultry meat.

Statistical analysis

For all the experiments each treatment was done in triplicates and the entire experiment repeated twice. The

results are mean of three concordant readings and significance was determined at $p < 0.05$ (Bonferroni Holm).

Results and discussion

Demand for healthy foods as fresh fruits and fruits/vegetable has increased over the last few years mainly because people have become health conscious and want 'minimally processed foods' containing low amount of preservatives. Consumption of such foods prevents lifestyle disorders and also degenerative diseases (Rico et al. 2007). Fresh produce has a dry outer coat/skin which has very low water activity and hence discourages microbial growth but when it gets injured during pre-harvest, during harvest, in post-harvest handling, transportation and storage then the produce becomes vulnerable to microbial growth. It becomes contaminated with pathogenic and spoilage microorganisms which increases the risk for food borne diseases and also mycotoxins in food (Diaz-Cinco et al. 2005). Microbial flora associated with fresh food depends on various factors as pH, water activity, nutrient content, etc. but can be broadly grouped in to three groups- 1) natural microflora associated with the fresh produce 2) plant pathogens 3) air contaminants.

Molds of serious concern vis-à-vis food safety and food quality are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. *Penicillium expansum* and *Botrytis cinerea* can cause significant losses and gain entry into the plant tissue through wounds or cuts. Other causal agents of post-harvest spoilage and diseases of the fresh produce include *Geotrichum*, *Colletotrichum*, *Mucor*, *Rhizopus*. Mycotoxins are secondary metabolites produced by these fungi. These toxins cause various diseases like mycotoxicosis i.e. interference with normal liver and renal function in man and other short term and long term adverse effects. *Alternaria alternata* is associated with spoilage of commercially important fresh produce as fruits and vegetables (Stinson et al. 1980). *Alternaria* species produce numerous toxic secondary metabolites such as tenuazonic acid, alternariol, alternuene, alternariol methyl ether and alter-toxin I, II III (King and Schade 1984). They exhibit toxic effects on cultures of bacterial, plant, animal and also human cells (Stack and Prival 1986; Yekeler et al. 2001).

Various food processing techniques like use of heat, use of low temperature, chemical preservatives, modified atmosphere, etc. are used extensively to prevent microbial food spoilage but each method has its own drawbacks (Davidson 2001). Hence discovery and application of natural substances has become very important. LAB are widely used in fermentations and are known to be probiotic and also preservative in nature. LAB are associated with most of the foods and are a prominent member of plant

microflora. They have been granted GRAS (generally regarded as safe) status which makes them an ideal candidate for biopreservation. They provide preservative benefit by competing for nutrition and also by the production of antimicrobial metabolites as bacteriocins, fatty acids, cyclic dipeptides and organic acids which are antibacterial and also antifungal. Antifungal activities of different lactobacilli have been studied by Lavermicocca et al. 2000; De Muynck et al. 2004a, b; Yang and Clausen 2005. Magnussen and Schnurer (2001) elucidated the antifungal attribute of *L. coryniformis* subsp. *coryniformis* strain Si3. Antifungal metabolites produced by *L. brevis* O2 were observed by Patra et al. 2010.

Lactobacillus acidophilus is an important member of LAB family and is a principle probiotic organism. In addition to this it also produces certain antimicrobial substances such as acids, hydrogen peroxide, fatty acids, dipeptides, bacteriocins etc. which can be exploited as a biopreservatives (Ogunbanwo and Okanlawon 2008). *L. acidophilus* NCDC 291 is a known bacteriocin producer. Its antimicrobial spectrum is mainly directed towards food borne bacterial pathogens. In the present investigation an attempt was made to ascertain its efficacy against fungi which are a major cause of microbial food spoilage.

Three approaches are commonly used in the application of bacteriocins for biopreservation of food: (i) inoculation of food with LAB which then produces bacteriocin in the product but subject to the physiological and environmental conditions prevalent (ii) addition of purified or semi-purified bacteriocins as food preservatives, (iii) use of a product previously fermented with bacteriocin producing strain as an ingredient in food (Schillinger et al. 1996). The first approach was exploited in the present study.

In the present investigation four fungal isolates were obtained after plating samples of visibly spoiled produce and they were identified as isolate I: *Fusarium* sp., isolate II: *Alternaria alternata*, isolate III: *Penicillium* sp., isolate IV: *Aspergillus flavus*.

L. acidophilus inhibited the growth of all the four fungal isolates i.e. *Fusarium*, *Alternaria alternata*, *Penicillium* sp., and *Aspergillus flavus* *in vitro*. Growth was inhibited to the maximum extent in *Fusarium* where 43.05% reduction in mycelium on dry weight basis was observed. Growth of *Alternaria alternata*, *Penicillium* sp., and *Aspergillus flavus* was inhibited by 36%, 33.23% and 38.4%, respectively. *L. acidophilus* is capable of providing good protection from food spoilage agents. Wet weight observations (data not shown) were in concurrence with dry weight observations. Inhibition of all of them *in vitro* from approximately 33–43% on mycelial dry weight basis was recorded. Karunaratne et al. (1990) reported that cells of *L. acidophilus* grown in a semi-synthetic medium inhibited the growth of *A. flavus*, whereas cell free cultures had no effect on fungal growth.

Batish et al. (1990) also reported production of antifungal substance by LAB. De Muynck et al. (2004a, b) found *Lactobacillus* to be a very promising agent having antifungal activity. A mixture of *Lactobacillus* species from a commercial silage inoculums reduced mold growth and inhibits aflatoxin production by *Aspergillus flavus* subsp. *parasiticus* (Gourama and Bullerman 1995).

Biopreservation of fresh fruit mango (*Mangifera indica*) and vegetable bitter melon (*Momordica charantia*) by Dip and Keep Method was performed. In *Mangifera indica* (control) upon incubation at ambient temperature, textural change was observed after 48 h and microbial spoilage after 96 h of incubation. In the corresponding test sample no microbial spoilage was observed upto 6 days, however marginal degree of textural change was seen after 96 h. The trial was performed at refrigeration temperature as well, where, in the control no microbial spoilage occurred till 6 day of run but textural changes were seen after 96 h. In the test sample no textural and no microbial deterioration was seen throughout the duration of this experiment. Identical results were obtained with *Momordica charantia*. Use of whole cell culture in Dip and Keep method significantly delayed the onset of microbial spoilage. It did not cause any deterioration in the textural properties. Similar results were obtained on fresh produce by other investigators in *in vitro* as well as *in situ* studies against bacteria and fungi. Trias et al. (2008) also concluded that LAB have potential to be used as bioprotective agents in ready-to-eat fresh fruit and vegetable products.

Allende et al. (2007) tested bacteriocins as substitutes for chemical disinfection of fresh cut iceberg lettuce. Washing solutions containing bacteriocin inhibited the survival and multiplication of food borne pathogens. Natural microflora associated with the fresh produce was minimally effected. Control of *L. monocytogenes* in fresh alfalfa sprouts, soybean sprouts and green asparagus was tested by Molinos et al. (2005) by dip and keep approach with enterocin AS-48. Count of *Listeria* was reduced by 2 to 2.4 log units. Viable *Listeria* counts were reduced below detection levels at days 1 to 7 for alfalfa and soybean sprouts and green asparagus. Enterocin AS-48 in combination with chemical preservatives were found to be effective in decreasing *Listeria* count.

Nisin alone and in combination with EDTA, Sodium lactate and Potassium sorbate were used to reduce the count of five strains of *Salmonella* in whole cantaloupe and fresh-cut cantaloupe. Washing treatment with Nisin-Sodium lactate-Potassium sorbate was significantly ($p < 0.05$) more effective than the other combinations resulting in reduction of 1.4 cfu/g. Sensory evaluation was also attempted to evaluate the acceptability of these treatments (Ukuku and Fett 2004).

Significant *in situ* biocontrol of *Alternaria alternata* was observed in Indian cheese and raw meat sample (Fig. 1). Indian cheese is an acid food with high water activity. It has a shelf life of approximately 2–3 days at refrigeration temperature. Fungal spoilage due to temperature abuse is commonly observed. No fungal count was observed in test sample for initial 48 h and after 6 days of incubation it attained a count of 1.1 log cfu/g. In the control which was run simultaneously the fungal count was observed within 24 h (0.5 log cfu/g). After 6 days of incubation the fungal count attained was 1.6 log cfu/g. A decrease of 0.5 log cfu/g ($p < 0.05$ (Bonferroni Holm), i.e. 45.4% was achieved with use of *L. acidophilus*.

Inhibition of *L. monocytogenes* has been demonstrated in dairy products as dressed cottage cheese, half and half cream and cheese sauce by pediocin designated as PA-1 produced by *Pediococcus acidilactici*. There was very rapid decrease in the cell count of this pathogen within 24 h of incubation (Pucci et al. 1988). A growth reduction of *L. ivanovii* and *L. monocytogenes* by 1 to 2 log units in cheese was reported by Eppert et al. (1997) by the use of undefined microbial floras derived from surface of ripe cheeses containing linocin producing *Brevibacterium linens*. Growth and survival of *L. monocytogenes* in Cheddar cheese in the presence of *L. lactis* subsp. *lactis* MM217, a starter culture containing pMC 117 coding for pediocin PA-1 was traced by Buyong et al. (1998). *L. monocytogenes* counts decreased to 10^2 cfu/g within 1 week of ripening and then to 10 cfu/g within 3 months whereas in control the count increased to 10^7 cfu/g within 2 weeks of ripening and then gradually decreased to 10^3 cfu/g after 6 months.

Raw meat is also classified as low acid food and is highly perishable. It is an excellent medium for microorganisms to grow in. It requires refrigerated storage and

Fig. 1 *In situ* control of *Alternaria alternata* by *Lactobacillus acidophilus* NCDC 291 in (a) Indian cheese and (b) raw meat

transport and has a shelf life of not more than 1 day. In the test sample fungal count of 1.1 log cfu/g was recorded after 24 h which increased to 1.5 log cfu/g after 6 days of incubation. In control this count was 1.5 log cfu/g after 24 h and attained a value of 1.9 log cfu/g after 6 days of incubation. A decrease of 0.4 log cfu/g ($p < 0.05$ (Bonferroni Holm), i.e. 26.7% was achieved.

Compared to dairy products the use of bacteriocins in meat products has met with limited success because of poor solubility, uneven distribution and lack of stability. Also the effective dose required to control meat borne pathogens and spoilage agents is very high and exceeds the acceptable daily intake (Hugas 1998). Therefore, use of whole cell as a biopreservative agent is a possible alternative method which was explored in the present investigation. Most of the studies in meat system are targeted against bacterial pathogens as *L monocytogenes*, *S aureus*, *C perfringens* and *E faecalis*. Nisin is mainly used to inhibit *Clostridia* in meat. It is also effective in inhibiting *Brochothrix thermosphacta* (Cutter and Siragusa 1998) and *Carnobacterium divergens* (Cutter and Siragusa 1994). Application of pediocin to chicken before cooking provides maximum effect against *L monocytogenes* (Goff et al. 1996). Apart from bacterial spoilage agents meat is susceptible to fungal growth as well. The present investigation is a pioneering work on evaluating the efficacy of *L acidophilus* as an antifungal agent in meat model system. It was found to be effective in inhibiting the growth of *A alternata*. Raw poultry meat were well preserved by *L acidophilus*, a decrease of 26.7% log units in raw meat was recorded.

Bacteriocins have exhibited their efficacy in *in vitro* and also in *in situ* studies, in predominantly dairy products (Davies et al. 1997; Ross et al. 1999; Ferreira and Lund 1996). They have potential application in all food systems and are a valuable part of multiple hurdles to manipulate the microflora in food. LAB are an important member of natural microflora associated with fresh produce which makes their physiological adaptation rapid. Bacteriocins being part of hurdle approach work in synergy with other preservative methods. Being probiotic they provide an additional benefit. There is no known toxicity of these bacteria who are part of our daily diet. Application of whole cells as biopreservative agents in non-dairy foods especially fresh produce needs further elucidation. It is promising biopreservative technique which can be used with ease.

Conclusion

Partially purified/purified bacteriocin, a tool in biopreservation, can be used as a food additive or the producer can be incorporated into the food system. Use of viable cells of a bacteriocin producer directly on to the food eliminates the

need to purify the metabolite thereby reducing the cost of production. In the present investigation use of whole cell approach was employed. Growth of four most commonly encountered spoilage fungi namely *Fusarium*, *Alternaria*, *Penicillium*, and *Aspergillus* was observed to be inhibited *in vitro* by *Lactobacillus acidophilus* NCDC 291. *Alternaria* was evaluated *in situ* where its colony count was significantly ($p < 0.05$) reduced in presence of *L. acidophilus*, a decrease of 45.4% in Indian cheese and 26.7% in raw meat was recorded. Surface application on *Mangifera* and *Momordica* delayed texture deterioration by more than 48 h and microbial spoilage was not observed upto 6 days at both ambient and refrigeration temperatures. It is concluded that use of whole cells is a viable option.

Majority of the farmers in the developing world are small scale to marginal scale farmers. Indian farmer produces, harvests and also markets his fresh produce. A LAB based product comprising whole cell and a microbial stabilizer with direct application on fresh food by either dip or spray method would be a useful and timely technology. It will delay onset of microbial spoilage enabling long distance transport and will facilitate import and export besides preventing post harvest losses which are a serious cause of loss of revenue. It can be a boon for developing country like India which accounts for approximately 52% of world mango production and ranks number one. Export of Indian mango to USA has recently begun.

Acknowledgements Organizers of International Association for Food Protection (IAFP) are gratefully acknowledged for selecting this work for presentation at their Annual Meet held at Anaheim, CA, USA in Aug. 2010. Also University Grants Commission (UGC), Council for Scientific and Industrial Research (CSIR) and Department of Biotechnology (DBT) are gratefully acknowledged for granting Partial Travel Support for attending IAFP 2010. Sincere gratitude to the Department of Biotechnology, Punjabi University, Patiala, Punjab, India and Department of Plant Pathology, Punjab Agricultural University, Ludhiana, India.

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