

## ABSTRACT

REINA, LAURA D. Biocontrol of *Listeria monocytogenes* in minimally processed brined refrigerated cucumbers (Under the direction of Henry Fleming and Fred Breidt)

The purpose of the research has been to develop a biocontrol model system for the inhibition of the food-borne bacterial pathogen *Listeria monocytogenes* in minimally-processed fruits and vegetables in order to improve the safety of these products. One application of this model was to evaluate the potential use of a bacteriocin-producer *Lactococcus lactis* or bacteriocin-nonproducer *Lactobacillus curvatus* as a biocontrol bacterium at refrigeration and at abuse temperatures (18°C) in the food system. The research involved the characterization of the microflora growing at refrigeration temperatures in cucumbers held in air and in brine, characterization of the microflora responsible for spoilage of the product at refrigeration and at abuse temperatures in cucumbers held in brine, and the potential use of the microflora (lactic acid bacteria) responsible for acidic spoilage of the product as biocontrol agents in this type of products. Isolates were tested for bacteriocin production against other lactic acid bacteria, Gram-negative spoilage microorganisms, and 5 strains of *Listeria monocytogenes*. The results showed that *Listeria* varied in sensitivity to the bacteriocin-like substance produced by some of the isolates, being strains serotype 1/2a and 1/2b more resistant than serotype 4b. Considerations were also given to the role of the natural microflora present in the cucumber, salt concentration, and the antibacterial properties of spices and garlic that can interfere with the biocontrol bacterium. The combination of salt and garlic was more inhibitory for the biocontrol bacterium and for the pathogen than garlic or salt

alone; pickling spices did not affect either the biocontrol or the pathogen. To avoid interference of the natural microflora present in cucumbers, a blanching step was included in the product preparation; sterile aquaresin garlic and irradiated pickle spices were used for seasoning. The shelf life of the product was extended with this approach, and allowed the enumeration of the biocontrol bacterium and the pathogen added in the product. The use of *Lactococcus lactis* in the product was limited due to its low salt tolerance, cold temperatures and low pH. However, survival in the product was enhanced by previous treatment of the strain with salt and glycine betaine at 20°C, enabling *L. lactis* to inhibit *Listeria* at abuse temperatures. The use of nisin in the product allowed the survival of *L. lactis* in unblanched cucumbers due to inhibition of the natural microflora, but natural nisin-resistant LAB (lactic acid bacteria) were able to grow and inhibit the *Lactococcus* by reduction of the pH. *Listeria* was able to survive at 5°C, but did not grow in the product. It grew at 18°C when the biocontrol agent or the natural LAB were not present. The *Listeria* death rate was faster in the product containing a bacteriocin-producer biocontrol bacterium than when the nonproducer was present. The rapid decline of *Listeria* was likely due to a synergistic effect of the nisin produced by *Lactococcus*, competitive inhibition, and lowering of pH. A salt, cold tolerant biocontrol bacterium able to produce a bacteriocin would be the ideal biocontrol agent for this type of products. Results from this study showed the potential of *Lactobacillus curvatus* as the biocontrol bacterium for minimally processed brined refrigerated cucumbers. It was able to grow slowly at 5°C in cucumber juice containing 4% salt, and it was able to grow in the product. However, being able to produce bacteriocin would be a plus. Results from this study demonstrated that brining fruits and vegetables with a blanching step in the

process and the use of sterile garlic oil and irradiated spices, and the addition of a biocontrol bacterium will ensure the safety of this product. Refrigeration and sanitation will still be required. The use of several hurdles is advised due to the ability of *Listeria* to withstand the stress conditions present in the environment.

**BIOCONTROL OF *Listeria monocytogenes* IN MINIMALLY PROCESSED  
BRINED REFRIGERATED CUCUMBERS**

by  
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## Introduction

The marketing of minimally processed fruits and vegetables (MPFV) continues to rise, and has generated concerns about their safety. This is coupled with an increase in population groups at higher risk for food-borne infections, such as the elderly, as well as cancer and AIDS patients. Fruits and vegetables are commonly contaminated with bacteria; the types will be influenced by the environment where they are grown. They can be contaminated with human pathogens, and they have been involved in food-borne outbreaks. Methods currently used to decontaminate fruits and vegetables have been shown to decrease the number of bacteria, without eliminating their presence. Therefore, it is important to minimize their growth under storage conditions. Methods of preservation rely on refrigeration and modified atmosphere packaging. Under these conditions, the spoilage microflora is inhibited, but psychrotrophic and microaerophilic bacteria are not. *Listeria monocytogenes*, a psychrotrophic pathogen, has been found in fruits and vegetables. It has a high mortality rate of 20-30% for humans, compared to other pathogens. This challenges the food industry to add more hurdles without changing the fresh-like characteristics often demanded by consumers of these products. The use of lactic acid bacteria (LAB) as another hurdle against growth of pathogens has been suggested because they have been consumed by humans for centuries, and consumers see them as natural. Natural LAB are present in low numbers in fruits and vegetables (~100-10,000 CFU/g), but under the proper conditions they can outgrow other types of microorganisms. However, it would be risky to rely only on the natural flora to ensure the safety of MPFV.

Refrigerated pickles include a type of product prepared with fresh cucumbers, washed, covered with brine, garlic and spices, and refrigerated. They have a short shelf life of approximately 3 weeks. Hence, refrigeration and sanitation are the only barriers against the growth of pathogens. A deli-type pickle product was chosen as a model system for this study. It is considered minimally processed because of its characteristics: no preservatives added, relatively high pH (5.5), no heat treatment, and need for refrigeration from production to consumption. *L. monocytogenes* was the pathogen chosen because it can grow at refrigeration temperatures and can overcome several hurdles present in the food, such as high salt and low pH. It has been demonstrated that *Listeria* can grow in the product at different temperatures, when the LAB are not present or present in low numbers. *Listeria innocua* had been isolated from the hydrocooling water of 2 different pickling companies, indicating that there is a possibility for *Listeria* to contaminate the product at any point during processing. In addition, the product is prepared with cucumbers grown locally, and, depending on the season, with cucumbers coming from Mexico. The latter are grown in trellises, and they have very low number of bacteria, including LAB. Thus, there is a need to ensure the safety of this product through selective spoilage by LAB.

The objectives of this research are:

- To determine the bacteria responsible for spoilage of this product at refrigeration and at abuse temperatures.

- To isolate natural LAB as potential biocontrol agents for minimally processed brined refrigerated cucumbers.
- To use one of these isolates or *Lactococcus lactis lactis* as a biocontrol agent of *Listeria monocytogenes* in minimally processed brined refrigerated cucumbers.

**CHAPTER I**  
**Literature Review**

## Introduction

The marketing of minimally processed fruits and vegetables (MPFV) continues to rise due to consumers' awareness of the health benefits of their consumption and the global trade agreement, which allows countries to export their produce, making available a variety of fruits and vegetables during the entire year at competitive prices. However, as concerns about fruit and vegetable consumption and their relation to food-borne disease outbreaks continue to increase, the food industry needs to ensure that it is providing the consumer with a high quality product which has an extended shelf life, and which is also safe.

Fruits and vegetables are usually contaminated with bacteria; the types will be influenced by the environment where they are grown. Surface decontamination of produce only reduces the microbial load, but does not eliminate it. More effective methods for produce decontamination are under development. Blanching and irradiation are the methods that achieve more bacterial reduction, but caution is needed to avoid quality loss of the product or to create an environment suitable for growth of pathogens due to the lack of competing microflora.

The methods of preservation of MPFV rely on refrigeration and Modified Atmosphere Packaging (MAP). Quality of MPFV is retained due to the inhibition of spoilage microorganisms and other chemical and physiological changes occurring in the produce. However, pathogenic bacteria may survive and even grow under these conditions; such is the case with *Listeria monocytogenes*. *L. monocytogenes* has been found in fruits and

vegetables, and it has been linked to human listeriosis cases. This challenges the food industry to add more hurdles to MPFV, without changing the fresh-like characteristics of these products.

The use of lactic acid bacteria (LAB) as another hurdle for growth of pathogens has been suggested because they have been consumed by humans for centuries and consumers see them as natural. However, there is still a need to determine how these biocontrol agents are going to behave in the real product. Biocontrol agents should be chosen according to the specific fruit or vegetable system. The LAB must not shorten the shelf life of MPFV, but they must grow faster than the pathogen at abuse temperatures.

The objectives of this chapter are:

- To present a literature review justifying the view that listeriosis is a potential safety hazard in MPFV.
- To describe the methods of surface decontamination in use by the food industry during preparation of MPFV.
- To describe and point out the characteristics of Minimally Processed Brined Refrigerated Cucumbers, which make them vulnerable to *Listeria monocytogenes* growth.

## **Listeriosis linked to consumption of fruit and vegetables**

Listeriosis is a serious disease of the bloodstream and central nervous system caused by eating food contaminated with the bacterium *Listeria monocytogenes*, a motile Gram-positive coccobacillus which has been isolated from soil, sewage, and several other environmental sources. *Listeria's* presence on plant materials is likely due to contamination from decaying vegetation, animal feces, soil, surface, river, and canal waters, or effluents from sewage treatment operations (Beuchat, 1996). It is capable of growing at temperatures ranging from -1 to 45 °C, in high salt and acid foods. Its ability to adapt to these environments suggests that *Listeria* is able to assimilate information about its environment, and process that information quickly in order to adapt to changing conditions (Hill *et al.*, 2002).

Surveys generally have shown that between 1 and 9% of the human population carries *L. monocytogenes* in feces, with most individuals remaining asymptomatic (Elliot and Elmer, 1991). However, changes in food processing practices, the development of powerful immunosuppressive drugs, and the HIV (Human Immunodeficiency Virus) epidemic have allowed the recognition of the disease as an important food safety issue because of its high mortality rate (20-30%). Listeriosis remains an uncommon infection, affecting mostly infants, the elderly, pregnant women and immunocompromised individuals. The chance of developing the disease depends on the degree of contamination, the virulence of the *L. monocytogenes* strains, and the susceptibility of the

individual exposed to *Listeria*. Local conditions created by the competitive flora, as well as the non specific intestinal mechanisms of defense determine the outcome (Hof, 2001).

While the infectious dose for either healthy or at risk individuals has not been determined, detailed analysis of outbreak data indicated that the pathogen reached  $10^4$  CFU/g in the food prior to consumption (Szabo *et al.*, 2000). In the USA there is zero tolerance of *L. monocytogenes* in ready-to-eat food. However, other countries (European countries and Canada) consider this approach unrealistic because *L. monocytogenes* can be found in the environment in low numbers. These countries have up to 100 CFU/g tolerance of *L. monocytogenes* in ready-to-eat food with threshold dependent on the food type (Norrung, 2000).

Human listeriosis has been linked to the consumption of fruits and vegetables, and *L. monocytogenes* has been isolated from cucumbers, cabbage, lettuce, celery, tomatoes, and other vegetables (Table 1, Beuchat, 2002). *L. monocytogenes* has been found in 0.6% of sprouts or sliced vegetables in numbers higher than 100 CFU/g, and in 34.6% of the samples, with numbers between 10-100 CFU/g (Norwing, 1999). It was also detected in 2-5% samples of minimally processed lettuce (Szabo *et al.*, 2000), and in 0.6% of samples of mushrooms and strawberries (Johanessen *et al.*, 2002). Given enough time and appropriate methodology for detection, *L. monocytogenes* can be found in or on almost all types of fruits and vegetables sampled. Hence, *L. monocytogenes* may pose a safety hazard in fruits and vegetables when they are stored under conditions that will

permit *Listeria* growth, and when the microbial background which usually compete for nutrient is reduced (Beuchat, 1996).

In Minimally Processed Fruits and Vegetables (MPFV) stored under Modified Atmosphere Packaging (MAP) the growth of *Pseudomonas* and other spoilage microorganisms was inhibited, giving more chance for *Listeria* to grow due to nutrient availability provided by the enzymes secreted (pectinases) by *Pseudomonas* (Bennik *et al.*, 2000). When other bacteria were present, *Listeria* did not grow. *Pseudomonas syringae* inoculated into wounds of apples prevented *E. coli* O157H7 from growing (Janisiewicz *et al.*, 1999). It has been demonstrated that *Listeria* can grow in any type of fruit or vegetable at different temperatures when these were inoculated with *L. monocytogenes*, as long as the background microflora had been eliminated or inhibited from growing (Bennik *et al.*, 2000).

Evidence for transmission of listeriosis through foods of plant origin probably dates back to 1922, when investigators in Iceland described a listeriosis-like illness in silage-fed animals. A case in Connecticut linked listeriosis to consumption of unwashed strawberries and/or blueberries (Ryser and Marth, 1991). An outbreak of listeriosis in Boston hospitals was due to the consumption of celery, lettuce and tomatoes (Ho *et al.*, 1986). Cabbage was also implicated in another outbreak in 1981. Cole slaw was the vehicle of serotype 4b, and epidemiological studies found that the cabbage was grown in a field where raw sheep manure was used as a fertilizer (Schlech *et al.*, 1983). Other

cases were shown to be caused by mushrooms, alfalfa tablets, and lettuce (Ryser and Marth, 1991).

Most cases of listeriosis were caused by serotype 4b, but other serotypes have been found in fruits and vegetables, and the presence of 4b was not the leading serotype among food isolates. This may be due to other serotypes being better adapted to food and the food processing plant, or that the transmission route could be other than contaminated food; or there could be an underestimation of serotype 4b in food due to sensitivity of this serotype to the selective enrichment procedures; or that strains of serotype 4b are more virulent to humans (Kathariou, 2002).

Studies done in relation to the ability of *L. monocytogenes* to attach to the surfaces of food or food-processing equipment demonstrated that serotype 1/2c is able to attach better to stainless steel surfaces than serotypes 4b and 1/2a (Norwood and Gilmour, 1999). However, other investigators did not find any relation between serotype and the ability of the bacteria to attach to surfaces (Kalmokoff *et al.*, 2001). Among different pathogens studied, *L. monocytogenes* 4b was the least attached to the surface of cucumber fruit during washing in water contaminated with the bacteria. Attachment was dependent on time of contact, water temperature, and surface characteristic of the fruit (Reina *et al.*, 2002). *L. monocytogenes* is expected to form biofilms on fruit and vegetable surfaces, but no information is available in the literature. Colonization of fruits and vegetables, and post-harvest contact surfaces by spoilage and non-spoilage microorganisms can provide a protective environment for *Listeria*, reducing sanitizers'

effectiveness. *L. monocytogenes* in a multi-species biofilm was unaffected by treatment with 500 ppm chlorine (Beuchat, 2002).

Methods to clean food processing surfaces and to decontaminate fruits and vegetable surfaces should be designed to kill the bacteria and not to allow it to survive and become more resistant, and perhaps more pathogenic.

Understanding the response of *Listeria* to sequential applications of sub-lethal stresses is imperative to avoid inducing adaptive responses, which may allow the pathogen to survive and persist during storage (Hill *et al.*, 2002). Strains can vary markedly in their response to low pH and high salt environments; cheese isolates tended to be more pH-tolerant than meat isolates. It seems that cheese isolates were already pre-adapted to a low pH, more so than meat or fish isolates (Faleiro *et al.*, 2002). Osmoadaptation induced osmotolerance in some strains and induced a protective effect against acid shock (Faleiro *et al.*, 2002). Identification of acid tolerance, osmotolerance, or sensing systems may provide targets for inactivation strategies and methods of preservation in the future (Hill *et al.*, 2002).

**Table 1. Prevalence of *Listeria monocytogenes* in fresh produce in different countries**

| <b>Produce</b>      | <b>Country</b>   | <b>% of incidence</b> |
|---------------------|------------------|-----------------------|
| <b>Bean sprouts</b> | <b>Malaysia</b>  | <b>85</b>             |
| <b>Cabbage</b>      | <b>Canada</b>    | <b>2.2</b>            |
|                     | <b>Sri Lanka</b> | <b>33</b>             |
|                     | <b>USA</b>       | <b>1.1</b>            |
| <b>Cucumber</b>     | <b>Malaysia</b>  | <b>80</b>             |
|                     | <b>Pakistan</b>  | <b>6.7</b>            |
|                     | <b>USA</b>       | <b>2.2</b>            |
| <b>Eggplant</b>     | <b>USA</b>       | <b>2.2</b>            |
| <b>Lettuce</b>      | <b>Malaysia</b>  | <b>22.7</b>           |
|                     | <b>Sri Lanka</b> | <b>50</b>             |

Adapted from Beuchat, 2002

## **Minimally processed fruits and vegetables**

The consumers' demand for minimally processed fruits and vegetables (MPFV) has increased rapidly in the last years because of their convenience and fresh-like quality, their high nutritional value and their lack of preservatives. Global trade in fruits and vegetables, and changing horticultural practices have enabled year-round supply in the markets of industrialized countries. Minimally processed fruits and vegetables include pre-washed, pre-cut salad items, chopped crudités, sprouted seeds, grated vegetables, prepared fruit salads or combinations involving peeling and cutting of the product before packaging and cold storage (Jayas and Jeyamkondan, 2002).

The quality of MPFV is determined by factors such as texture, color, taste and flavor. During the storage period of MPFV, quality is influenced by two major processes: physiological degradation due to enzymatic activity of the plant tissue itself (such as respiration), and microbiological degradation due to the presence of different microorganisms on the plant. The latter are not removed from the tissue during processing because of the minimal handling of the produce, such as shredding and washing (Jacxsens *et al.*, 2002). Most of these products are generally consumed without further processing.

The plant tissues in fresh-cut produce are still alive and deriving energy, primarily through the process of respiration. Respiration involves the consumption, using atmospheric oxygen ( $O_2$ ), of carbohydrates and organic acids, and the consequent production of metabolic energy, heat, carbon dioxide ( $CO_2$ ) and moisture vapor. Different fruits and vegetables, and even different varieties of a given fruit or vegetable will vary in

their respiration rates. Those that have high respiration rates (such as asparagus, mushrooms, cucumbers and broccoli) tend to be the most perishable, while those with low respiration rates (such as nuts, onions and potatoes) tend to be the least perishable. Furthermore, when fruits or vegetables are cut, sliced, shredded or otherwise processed, their respiration rates increase. This is probably due to the increased surface area exposed to the atmosphere after cutting that allows oxygen to diffuse into the interior cells more rapidly, and to the increased metabolic activity of injured cells. The best way to reduce respiratory metabolism, and thus conserve the plants' stores of carbohydrate, acids and moisture, is to reduce the temperature. Most produce will maintain its best quality at temperatures near 0°C, except some produce of tropical origin, which should be kept at 10-13°C to avoid chilling injury (Zagory, 1998). Having achieved optimal temperature control, Modified Atmosphere Packaging (MAP) can be used to further reduce respiration rate, loss of moisture, production of metabolic heat, yellowing, browning, decay, and sensitivity to ethylene.

MAP and subsequent storage at refrigeration temperature has been developed to prolong the shelf life of MPFV. MAP and Controlled Atmosphere Storage (CAS) are terms used interchangeably, but they are different. MAP is the enclosure of food products in gas-barrier materials in which the gaseous environment is modified dynamically, depending on the respiration rate of the produce and the permeability of film surrounding the produce, whether initiated by the natural respiration of the product itself (passive) or by introducing gases by artificial means (active). In CAS the gas atmosphere is continuously controlled throughout the storage period (Jayas and Jeyamkondan, 2002). Passive MAP is

regularly applied to MPFVs sealed within bags and over those wrapped in semi-permeable films, harnessing the natural respiratory activity of the living tissue to naturally modify atmospheric conditions (Thomas and O'Beirne, 2000).

The effects of MAP are based on the often observed slow plant respiration in low O<sub>2</sub> environments. There is about 21% O<sub>2</sub> in air. As the concentration of O<sub>2</sub> inside the package falls below about 10%, respiration starts to slow down. This suppression of respiration continues until O<sub>2</sub> reaches about 2-4% for most produce. If O<sub>2</sub> gets lower than 2-4% (depending on product and temperature), fermentative metabolism replaces normal aerobic metabolism and off-flavors, off-odors and undesirable volatiles are produced (Devlieghere *et al.*, 2000). Similarly, as CO<sub>2</sub> increases above the 0.03% found in air, a suppression of respiration results for some commodities. Reduced O<sub>2</sub> and elevated CO<sub>2</sub> together can reduce respiration more than either alone. In addition, elevated CO<sub>2</sub> suppresses plant tissue sensitivity to the effects of the ripening hormone ethylene. For those products that tolerate high concentrations of CO<sub>2</sub>, suppression of the growth of many bacteria and fungi results at >10% CO<sub>2</sub>.

The ratio of the gases used is important from the quality and from the safety stand points. Exposure to elevated CO<sub>2</sub> levels (10-20%) may result in the suppression of various metabolic processes. Thus, it is important to establish the ideal ratios of gases to maintain quality. However, concern about the potential growth of pathogenic bacteria, which could survive or even grow at refrigeration temperatures remains the limiting factor for a wider

use of the method. Therefore, the recommended percentage of O<sub>2</sub> in a modified atmosphere for fruits and vegetables for both safety and quality falls between 1 and 5%.

One major concern in the use of MAP in the food industry is the presence and growth of *Clostridium botulinum*. It is generally believed that with the use of permeable films, spoilage will occur before toxin production is an issue. Botulinum toxin was detected in broccoli packaged in bags with oxygen transmission rate (OTR) of 3 and stored at 13°C for 21 days, and in those with OTR of 4 and 3 and stored at 21°C for 10 days. All toxic samples were visibly spoiled (Hao *et al.*, 1999). However, MAP of produce should always incorporate packaging materials that will not lead to an anaerobic package environment when the product is stored at the intended temperature.

Packaging experiments using sensitive fruits and vegetables, with low respiration rates, under high O<sub>2</sub> and CO<sub>2</sub> mixtures (e.g. 70-100% O<sub>2</sub> and 5-30% CO<sub>2</sub>) showed mostly beneficial results. This so called “oxygen shock” or “gas shock” treatment has been found to be particularly effective at inhibiting enzymatic browning, preventing anaerobic fermentation reactions, and inhibiting aerobic and anaerobic microbial growth. However, gas mixtures with high O<sub>2</sub> levels are very reactive (Devlieghere *et al.*, 2000).

It is almost impossible to ensure appropriate refrigeration temperatures to maintain safety and quality during transit, distribution and retail display of MPFV (Orsat *et al.*, 2001). It has been found that temperatures in many refrigerators at retail market stores are above the minimum recommended to maintain quality or safety of the product. Survival, but not growth of *Salmonella enteritidis* and *L. monocytogenes* was shown in shredded carrots and lettuce stored at 4°C under MAP (Kakiomenou *et al.*, 1998). *Staphylococcus aureus*

and *S. typhi* could grow in cucumber and watermelon at room temperature (32°C); carrot retarded their growth, while pineapple did not support their growth. The latter could be attributed to the low pH (3.9) of pineapple (Visnathan and Kaur, 2001). *Aeromonas hydrophila* could survive and grow in asparagus, broccoli, and cauliflower stored under CAS (Berang *et al.*, 1989). These studies demonstrate the potential for pathogens to grow in MPFVs given sufficient time at an appropriate temperature (abuse temperature). Thus, there is a need to add more hurdles against growth of pathogenic microorganisms.

Examples are edible coatings containing a bacteriocin or a biocontrol agent from which a bacteriocin will be released. Edible coatings are thin layers of material over the produce that can reduce moisture loss, lower respiration rate, restrict the entrance of oxygen and carry additives and antimicrobials. Sucrose polyesters of fatty acids and the sodium salt of carboxymethylcellulose, carrageenan and chitosan coatings are commercially available. Active packaging includes various gas absorbents and emitters that can affect respiration rate, microbial activity and hormone activity. Active packaging based on potassium permanganate and activated alumina has been used in diced onions, showing promising results from the quality standpoint. These gas absorbents removed ethylene and reduced the levels of sulphur volatiles and CO<sub>2</sub>. However, further studies are needed to determine the effect on the microflora associated with the produce (Ahvenainen, 1996). Bioactive food packaging using immobilized lactacin 3147 and nisin have been developed. Adsorption of lactacin to the plastic film was unsuccessful, but nisin adsorbed very well, and the antimicrobial effect was noticed in the reduction of *L. innocua* by 2 logs and *S. aureus* by 1.5 logs in cheese (Scannell *et al.*, 2002).

Previous packaging technologies had to merely create a modified atmosphere while avoiding anaerobic conditions. Current packages are often expected to serve also as vehicles for attractive graphics, re-closeable seals, anti-fog coatings, crinkly texture, high transparency and strength. However, package attributes alone are not sufficient to ensure safety and quality of fresh-cut or minimally processed produce. Proper handling of the raw materials prior to packaging and of the package during distribution and marketing are essential if the package is to deliver the desired value.

## Surface Decontamination of Fruits and Vegetables

MPFV are thoroughly washed and often dipped in antimicrobial solutions during processing. Washing is done with water alone or by adding chlorine (100 ppm) or citric or ascorbic acid (1%) to the washing water (Francis *et al.*, 1999). Minimal processing also includes trimming, scrapping, slicing/dicing, shredding/chopping, moisture removal by centrifugation, packaging (MAP, 2-5% O<sub>2</sub>, 3-10% CO<sub>2</sub>), and storage at refrigeration temperature (2-5°C). These processes have been shown to spread indigenous microflora and increase surface area of the produce for bacterial growth (Edgar and Aidoo, 2001).

The use of sanitizers in washing fruits and vegetables is important to prevent bacterial buildup in the washing water, which is usually recycled, and to avoid cross contamination of the incoming produce. Chlorinated water from the washing bath on a line processing chicory salads was found to contain 10<sup>3</sup> CFU/mL of bacteria, being a source for contamination (Francis *et al.*, 1999). *L. monocytogenes*, *S. aureus*, and *S. typhimurium* contained in the washing water were able to attach onto the surface of cucumber fruit at different temperatures. Attachment was related to contact time with the contaminated water, temperature, and the type of microorganism (Reina *et al.*, 2002). Chlorine dioxide (ClO<sub>2</sub>) was able to keep bacterial loads low in hydrocooling water, but it did not have effect on the microflora from the cucumbers (Reina *et al.*, 1995). However, ClO<sub>2</sub> gas at 21°C was effective in inactivating *L. monocytogenes* on apple surfaces, being more effective on the pulp skin and less effective on those bacteria attached to the calyx or stem cavity (Du *et al.*, 2002).

The numbers and types of microorganisms found on fresh produce are highly variable. Mesophilic bacteria are around  $10^3$ - $10^9$  CFU/g in raw vegetables after harvest (Zagory, 1999), depending on the produce and the growing conditions. Effective washing and decontamination is difficult because attached or entrapped bacteria are difficult to remove (Seymour *et al.*, 2002). Washing procedures generally result in a one or two logs reduction in microbial counts. The efficacy of various disinfectants and sanitizing methods varies greatly, depending on the fruit and vegetable surface, and the type of microorganism, physiological stage and environmental stress conditions (Beuchat, 1998). Sanitizer efficacy is also limited by bacterial adherence, inaccessibility, biofilm formation and internalization (Sapers *et al.*, 2001). Epiphytic bacteria may be more accessible to chemical treatment than pathogens that may be hidden within plant tissues or protected by damaged tissues (Zagory, 1999).

Heat treatment of fruits and vegetables can be used to reduce bacterial populations on fruit and vegetable surfaces. While sufficient heat should be used to destroy microorganisms, care should be exercised to not overheat to avoid quality loss (crispness) of the fruit or vegetable. It is recommended to cool immediately after heating (Etchells and Ohmers, 1941). Blanching has been used prior to freezing fruits and vegetables to preserve quality. The beneficial effect of heat treatments on the quality of different produce is well documented. Blanching vegetables in boiling water or steam for a short period of time is recommended for almost all vegetables to slow down or to stop the action of the enzymes which are active during storage, causing off-colors, off-flavors and toughening. However, at this temperature there will be destruction of texture, flavor, color and nutritional quality. Wounding during the preparation of fresh-cut fruits and

vegetables induces the synthesis of enzymes of phenylpropanoid metabolism, the synthesis and accumulation of phenolic compounds and subsequent tissue browning. These effects can be reduced by mild heat treatment of fruits and vegetables (Loayza-Velarde *et al.*, 2002). Blanching also cleanses the surface of dirt and bacteria, brightens the color, and helps retard vitamin loss. It is vital to quality to follow the recommended blanching time for each vegetable. After blanching, the produce needs to be cooled quickly and thoroughly to stop the cooking process. Low temperature of blanching (50-80°C) can reduce microbial counts by 3 logs (Breidt *et al.*, 2000, Edgar and Aidoo, 2001, Mayer-Miebach, 2003), but the fruits and vegetables will still keep their fresh characteristics of color and texture. Blanching of sliced carrots, spring onions and baby sweet corn reduced the numbers of *Enterobacteriaceae* to a greater degree than *Pseudomonas spp.* or yeasts. *Pseudomonas spp.* were found to be more numerous than other groups in all the vegetable samples, raw or blanched (Edgar and Aidoo, 2001). Heat treatment at 60°C for 30 seconds reduced the microflora of soybean sprouts and watercress by 2 log cycles. Washing alone did not yield any reduction in the microbial load (Park *et al.*, 1998). Vapor heat treatment of table grapes (52.5-58°C) for 10-30 minutes did not affect the quality of the grapes (Lydakakis and Aked, 2002). Heat treatment of strawberries at 50°C for 3 hours in an oven was shown to be effective in reducing initial bacterial counts, but did not affect the mold counts. However, there was no difference compared to the control when other parameters of quality were measured (Vicente *et al.*, 2002). Hot water brushing treatment (60°C) for 20 seconds of nectarines and peaches did not cause surface damage to the fruit or impair quality (Karabulut *et al.*, 2002).

In the fresh juice market, heat treatment has been applied to citrus fruit as part of the FDA-mandated 5-log reduction standard. Data on surface-heat treatment of apples show all surface-borne vegetative microbial cells can be destroyed by simple heat treatment. However, internal microbial populations are largely unaffected by such treatment (Keller, 2002), especially bacteria attached to the calyx and stem. Hot treatment of inoculated cantaloupe in combination with 5% hydrogen peroxide gave 4 logs reduction at 70-80°C (Sapers *et al.*, 2001).

Gamma radiation or cold pasteurization has been gaining acceptance as another method to reduce microbial load of fruits and vegetables. Gamma radiation has been used to decontaminate cucumber fruit. Studies done on the effect of radiation on the natural microflora suggested that bacterial spores are more resistant than non-sporeforming bacteria. No organisms were recovered after treatment with high doses of radiation (3.0 megareps), but cucumbers lost 60% of their initial firmness (Etchells *et al.*, 1961).

Ultrasound has been suggested for decontamination of fruits and vegetables. Ultrasonic fields consist of waves of high amplitude, which form cavitation bubbles. The growth, collapse and oscillation of these bubbles generate the mechanical energy which has a cleaning action on surfaces. However, applications on fruit and vegetable surfaces gave more bacterial reduction when used in combination with chlorine. Attached or entrapped bacteria were more accessible to chlorine when lettuce was treated with ultrasound. The cost of the technology may prevent its use by the food industry (Seymour *et al.*, 2002).

Reduction of the natural microflora could be risky if further hurdles are not applied to the produce, especially if the produce is contaminated with a pathogen after processing.

Safety concerns of MPFVs are the role of the background microflora and the pathogens present in raw fruits and vegetables. Pathogens may be more resistant to the decontamination procedures than the indigenous microflora. By removing this group of microorganisms, a natural barrier for pathogenic growth, which acts by competition, is also removed. As a result, disinfection may provide conditions which favor survival/growth of the pathogen (Francis *et al.*, 1999). Mild heat treatment of lettuce (20-50°C) for 90 seconds facilitated the growth of *L. monocytogenes* during refrigeration storage, which could be due to the reduction of the natural microflora (Li *et al.*, 2002). The natural microflora of lettuce competing with *L. innocua* was shown to belong to the *Enterobacter* species (Francis and O'Beirne, 1998). LAB (lactic acid bacteria) are present in very low numbers in fruits and vegetables, and they can reach very high numbers in MPFVs under certain storage conditions, where they may grow faster than other aerobic spoilage microorganisms (Kelly *et al.*, 1996). Several surveys of MPFV have shown that LAB were the microflora predominating at the end of the shelf life of these commodities.

## Biocontrol in Minimally Processed Fruits and Vegetables

Refrigeration is the main preservation technique used in combination with MAP to ensure quality and safety of MPFV. Fruits and vegetables carry a natural non-pathogenic microflora. However, during growth, harvest, transportation and further processing and handling, the produce can be contaminated with pathogens from human, animal or environmental sources. Fresh produce has been implicated in a number of outbreaks caused by bacteria, viruses, and parasites (De Roever, 1998). The microflora of MPFV consists of epiphytic microorganisms that can cause spoilage, Gram-negative bacteria, such as *Pseudomonas* or *Enterobacter*, Gram-positive bacteria, such as Lactic Acid Bacteria (LAB), and some yeasts and molds. Depending on the conditions under which the produce is stored, and the characteristics inherent to the plant tissue, such as respiration rate, nutrient availability, and pH, the development and success of one or another group will be different (Jacxsens *et al.*, 1999). More sugar-rich vegetables, such as bell peppers, undergo microbial fermentation due to the growth of LAB or yeasts, whereas others, such as leafy vegetables, develop soft rot symptoms due to proliferation of pectinolytic Gram-negative bacteria (Jacsexens *et al.*, 2002; Bagamboula *et al.*, 2002).

Storing MPFV under MAP will influence the type of microflora that will dominate. It has been shown that *Pseudomonas* and *Enterobacteriaceae* will be inhibited by at CO<sub>2</sub> concentrations suitable for MAP, thus extending shelf life (Bennik *et al.*, 2000). However, other psychrotrophic bacteria able to grow under those conditions, such as *L. monocytogenes* or *Aeromonas caviae*, will not be inhibited, and the product may have good quality, but it may be unsafe for human consumption. MAP conditions used to store

chicory endive to preserve product quality retarded the growth of spoilage microorganisms, but did not inhibit the growth of *L. monocytogenes* (Bennik *et al.*, 1996). It is also known that *L. monocytogenes* is more prone to grow on MPFV with a reduced microflora, such as chicory, and less capable of growing in vegetables with a high microbial load, like mungbean sprouts. *L. monocytogenes* was able to grow in a deli-type pickle product stored at 5°C when LAB were in low numbers. Growth was inhibited by the natural LAB or by the biocontrol added into the product (Romick, 1994). *L. monocytogenes* proliferated rapidly on sprouting alfalfa seeds (Palmai and Buchanan, 2002). Storage temperature and type of fruit or vegetable will influence growth of the pathogen. It was shown that bell peppers stored at 2, 4, 7, and 10 °C did not support the growth of the pathogens *L. monocytogenes* or *A. caviae*. However, both pathogens grew in cucumber slices and mixed lettuce stored at temperatures higher than 4°C (Jacsexens *et al.*, 2002). To control the growth of pathogens in MPFV under refrigeration or at abuse temperatures, more hurdles need to be added without changing the concept of MPFV.

Biopreservation of MPFV has been the focus of increased interest because of the implication of preservation by biological barriers, which include substances of plant origin, such as essential oils that have antibacterial and antioxidant properties and add flavor to the food. Some examples of biopreservative agents are: enzymes able to inactivate bacterial cells, and thus preserve the product (lysozyme from eggs, lactoperoxidase and lactoferrin from milk), and metabolites produced by food-grade microorganisms, such as bacteria and yeasts. The concept of microbial antagonism is well known, and refers to the inhibition of microorganisms by the competition for nutrients or by the production of microbial metabolites (Amézquita and Brashears, 2002). Biocontrol

has been applied in fruits and vegetables to avoid post-harvest losses, as an alternative to pesticides and fungicides. These biocontrol agents include yeasts and bacteria. Nutrient competition in wounds of apples appeared to be the principal mode of action, nitrogen being the limiting factor. Commercial biocontrols are available in the market, including a yeast-based product, and bacteria-based product (Vero *et al.*, 2002).

The same principle could be achieved by the addition of bacteriocin-producing LAB, bacteriocins produced by LAB, or simply, by adding LAB that will eliminate the pathogen by competitive exclusion or microbial interference in MPFV. The term “biocontrol” implies a natural means of preservation. LAB are able to produce a number of inhibitors in addition to bacteriocins, such as organic acids, diacetyl, hydrogen peroxide, enzymes, and lytic agents (Breidt and Fleming, 1997). LAB are considered generally recognized as safe (GRAS), and have been used for a long time in fermented food. The challenge is to use LAB as biocontrol agents in non-fermented foods.

Despite a significant increase in the effort to develop protective cultures as biocontrol agents in MPFV in recent years, until now, studies have been performed mostly at a laboratory scale, and rarely in MPFV. This could be due to the fact that many questions about the in situ functionality of such strains remain unsolved (Leroy *et al.*, 2001). The use of LAB in vegetable broths has been shown to inhibit the growth of different pathogens at different temperatures. Results were different when the LAB were applied to the real vegetable product (Bennik *et al.*, 1999; Palmai and Buchanan, 2002). *L. monocytogenes* was inhibited by *L. lactis* during sprouting of alfalfa seeds, but this inhibition was lower than the one obtained in a sterile broth model system of sprouts.

This may be the result of inhibitory activity associated with the microflora of the sprouting seeds (Palmai and Buchanan, 2002). Microbial background is not eliminated from the fruits or vegetables during washing, and sometimes can increase during processing. Ideally, the reduction of the initial microflora will be an asset to allow the biocontrol to succeed. However, the only way to achieve this is by heating the produce without altering the organoleptic characteristics, or by cold pasteurization (irradiation).

Some uses of biocontrols in other food systems have been successful, such as in sausage and cheese to control *L. monocytogenes*. This may be due to the heat treatment applied to milk before manufacturing, reducing the microbial load, and to the nutrient-rich environment found in milk and meat products, immediately available for the biocontrol agent. Blanching fruits and vegetables could be used as an approach to reduce the competing microflora, allowing the use of a biocontrol agent as another hurdle for pathogen growth. Blanching cucumbers for 3 minutes at 77°C were successfully fermented in the absence of salt, when they were inoculated with *Lactobacillus plantarum* (Fleming *et al.*, 1994).

At the same time, it is crucial to determine the conditions under which the protective culture will be exposed; for example: high salt concentration, lack of nutrients, and low temperatures. Current studies have focused on isolating strains of LAB that grow rapidly at refrigeration temperatures to inhibit the growth of psychrotrophic pathogens, but this will not be practical from the quality standpoint, as growth of LAB in the product will cause spoilage (Amézquita and Brashears, 2002). Biocontrol strategies for MPFV may include isolation of potential biocontrol LAB from the refrigerated product; reduction in

the total microflora in the vegetable product by mild heat treatments; addition of a bacteriocin-producing biocontrol culture; and storage of the product under refrigeration (Breidt and Fleming, 1997). The product shelf life will be dictated by the biocontrol culture, which will grow faster under abuse conditions, preventing the growth of the pathogen (Ahvenainen, 2000). The selection of the biocontrol agent needs to take into consideration the conditions under which the product is processed and stored, the ingredients, and the potential pathogen because bacteriocins have narrow spectra of activity. Food composition and structure can affect the rate of growth of the biocontrol microorganism, the rate of production of inhibitory substances, and their biological activity (Rodgers, 2001). Using biocontrol in MPFV could enhance safety, but not necessarily increase the shelf life. The advantage of using biocontrol cultures is that both the increase in safety risks and activation of the biocontrol are temperature-dependent (Rodgers *et al.*, 2002). In mayonnaise-based potato salads with pH values of 5.5-6.0 that were exposed to ambient temperature for a week, the biocontrol agent (*L. lactis* or *Leuconostoc mesenteroides*) greatly reduced the hygienic risk, but did not extend the shelf life.

The ecological adaptation of added LAB is an important factor for their effectiveness as biocontrol cultures, and their isolation from the same vegetables on which they will be further used as inhibitory agents is crucial (Vescovo *et al.*, 1996, Gomez *et al.*, 2002). Biocontrol agents should be adapted to the food to which they are going to be applied. The criteria for selection of biocontrol microorganisms will be based on the type of fruit or vegetable, the presence of salt or sugar, minimum temperature for growth, rate of acidification at refrigeration temperature, rapid growth, acid formation, bacteriocin

production at abuse temperatures, and the potential pathogens at refrigeration or abuse temperatures. At the same time, biocontrols could be used in combination with bacteriocins or bacteriophages against the potential pathogens to avoid development of resistance to the bacteriocins used.

Bacteriocins can be defined as a group of potent antimicrobial peptides or proteins primarily active against closely related organisms. For most bacteriocins the antimicrobial effect seems to be bactericidal. Bacteriocins produced by LAB are a relatively heterogeneous group of ribosomally synthesized small proteins, which are readily degraded after ingestion. Thus, they can be considered safe for human consumption. They have been detected in all investigated LAB, including lactobacilli, pediococci, leuconostocs, and enterococci. However, the best known bacteriocin is nisin (Klaenhammer, 1993). Bacteriocins differ in their spectra of activity, biochemical characteristics, and genetic determinants. Most bacteriocins are small (3-10 KDa), have a high isoelectric point, and contain both hydrophobic and hydrophilic domains.

Nisin produced by *Lactococcus lactis* is the best-studied bacteriocin, and the only one having a GRAS application in the U.S. and in 50 other countries. It is active against a broad spectrum of Gram-positive bacteria, including *L. monocytogenes* and other food pathogens. *Escherichia coli* and other Gram-negative bacteria are only affected when their outer membranes are weakened or disrupted. It has dual activity against spore-forming bacteria: it inhibits the outgrowth of spores and kills cells in the vegetative states. It has found a special application in the preservation of late-blowing of cheese by inhibiting the outgrowth of *Clostridium* spores (Daeschel, 1989).

Bacteriocins can be classified into 4 groups: Class I, unusual aminoacids dehydroalanine and dehydrobutyrine, and they are called lantibiotics. Examples are: nisin, lactacin 481, lactococcin, lactocin S, and carnocin. Class II, small heat-stable proteins. They are divided into 3 groups: *Listeria* active peptides, poration complexes consisting of two proteinaceous peptides, and small, heat-stable, and non-modified bacteriocins translated with *sec*-dependent leaders. Class III, large heat-labile proteins. Class IV, complex bacteriocins, composed of protein plus one or more chemical moieties (carbohydrate, lipid) (Klanhaemmer, 1993).

Resistance to bacteriocins is an issue. The use of bacteriocins in combination with other hurdles may reduce the development of resistance by the pathogens. Relying only on bacteriocin-producing LAB or bacteriocins has positive and negative implications, because the pathogens can be killed, but they can also develop resistance, and the bacteriocins can be degraded or inactivated at the pH of the food system.

In studies done in broth with nisin, *Enterococcus faecium* and *L. lactis* showed complete inactivation of *L. monocytogenes*. The amount of nisin required was less than when nisin was used alone to achieve the same effect. The added cultures were able to suppress proliferation of the *Listeria* survivors to nisin at 10°C. Tofu prepared with nisin and the two LAB showed that smaller amounts of nisin were required to inhibit *Listeria* in the product (Schillinger *et al.*, 2001). It has been shown that three bacteriocins are needed to avoid developing of resistance by *Listeria* species in a meat system. When only one bacteriocin was used, *Listeria* died at the beginning, but started growing again after 24 hours (Vignolo *et al.*, 2000). However, another study using nisin and pediocin together

showed that development of resistance was 10-100 fold higher than expected when using one bacteriocin alone (Gravesen *et al.*, 2002). *L. monocytogenes* was shown to develop resistance to nisin in a frequency of  $1 \times 10^{-6}$ - $1 \times 10^{-8}$  (Harris *et al.*, 1992). Resistance was due to physiological changes in the target cell membrane. A more rigid membrane, usually having a lower C15:C17 ratio, results in increased tolerance to nisin (Mazzota *et al.*, 1997). However, resistance of *L. monocytogenes* to pediocin PA-1 showed increased expression of gene fragments that code for  $\beta$ -glucoside-specific phosphoenolpyruvate dependent phosphotransferase systems (PTS), but the exact mechanism is not known. Resistance to class IIa bacteriocins may be expected to arise at a frequency of  $10^{-6}$ , irrespective of the prevailing conditions; one strain of *Listeria* was able to develop resistance to nisin and pediocin at a frequency of  $10^{-6}$  (Gravesen *et al.*, 2002). Whether resistance is genetically encoded or an adaptation, there is contradictory data regarding cross-resistance when bacteriocins from different classes are used (Cleveland *et al.*, 2001). In addition, nisin is the only bacteriocin allowed to be used in some food systems, and commercial applications are limited due to its high cost (Rodgers, 2001).

## **Minimally Processed Brined Refrigerated Vegetables and Fruits**

Brining is an old method used to preserve bulky or whole vegetables for extended periods of time. It is usually accompanied by fermentation that is characterized by chemical changes induced by microbial activity. The salt has a selective action on the natural microflora, resulting in fermentation developed as the consequence of the growth of one or more surviving salt tolerant groups. Salt can be added in two ways: dry or solid salt is added directly to vegetables, or the produce is covered with brine, which is a solution of salt and water (Etchells *et al.*, 1943). Brine or salt draws water from vegetables, decreasing the salt concentration in the brine itself. The salt used in brine production should be free of flow agents (anticaking agents, silicon dioxide) and iodine to avoid producing a turbid brine and darkening of fruits and vegetables.

During the pickling process, brine strength is usually raised gradually; otherwise, acid development would be inhibited and the vegetables would become soft. Both the concentration of salt in the brine and the salting technique affect the quality of the finished product. Salt reduces the competition from undesirable microorganisms, which encourages lactic acid fermentation. Salt and acid concentrations not only control the growth of microorganisms, but also influence enzyme activity. The temperature also helps determine the rate of acid production and the kinds of bacteria involved in it. The preserving effect of the brine is due to the action of salt and acid production by fermentation. If higher concentrations (>12%) of salt are used, there is no fermentation (Etchells *et al.*, 1943). In the brines of relatively low salinity (1.5-2%) there was a rapid

development of brine acidity; the opposite happened in brines of high salinity: acid formation occurred at increasingly slower rates (Jones, 1940).

Refrigerated pickles have gained popularity over the past few decades (approximately 20 percent of all pickles sales) because they undergo minimal processing, keeping their crispness and freshness. Refrigerated pickles may or may not be fermented before refrigeration; they are not pasteurized. They may or may not be acidified and have preservatives added, and are held at room temperature for a few days, then refrigerated to slow down the fermentation. Refrigerated pickles are available in many varieties, including kosher dills, genuine dills, half-sour, overnight, and sweet pickles, and are available whole or cut into halves, spears, slices, chips or relish, or are sliced lengthwise for sandwiches (Etchells *et al.*, 1976).

A type of non-fermented, non-acidified, no-preservatives-added pickle has to be refrigerated during its whole storage life. This type of product represents 5% of the refrigerated pickles in the retail store (Fleming, 2003). The first step in producing refrigerated pickles is choosing high quality fresh cucumbers. They are graded, washed and packed within hours after they are picked. Cucumbers are chosen for their size, firmness, color, texture and flavor. They are put into jars, covered with a seasoned solution (4% brine, equilibrated to 2%) and vacuum sealed. It may contain some vinegar (acetic acid), depending on the product, and may include spices such as fresh garlic cloves (crushed) and dill. The filled jars are immediately refrigerated (Etchells *et al.*, 1976). They are held in refrigeration for a few weeks, so the cucumbers can absorb the

seasonings. This curing step is important in developing the perfect refrigerated pickle. Also, it is the step that officially changes the cucumber to a pickle.

Refrigeration gives these pickles a distinct, fresh, raw cucumber flavor. The production and storage of this item under refrigeration prevents the pickles from spoiling. The pickles are ready for shipping when the concentration of salt within the pickle equals the concentration of the brine. The finished pickles have a distinct, fresh flavor (very much like a deli pickle), are very crisp, and maintain their fresh green cucumber color desired by an important segment of consumers.

These “perfect-for-munching” pickles are shipped under refrigeration and stocked in the refrigerated section of the supermarket. After purchase, the pickles must be stored in the refrigerator before and after the jars are opened. They are a perishable product, and will spoil if mishandled. In addition, it is recommended that the pickles be used by the expiration date on the jar or within one month after opening the jar, whichever comes first. The salt content is 2% after equilibration, and the pH is usually above 4. In a survey of acidified versus non acidified refrigerated pickles done in 4 metropolitan areas, it was found that the pHs vary between 3.81 and 5.27 (Table 2) (Fleming *et al.*, 1992, unpublished data).

Because these pickles are preserved by refrigeration, they have a relatively short shelf life. The shelf life of pickle products is dictated primarily by consumer acceptance of the product. Appearance, texture and flavor are the main traits that influence acceptance or

rejection. Shelf life is determined by the development of turbidity, usually due to the growth of LAB, increase of acid concentration, change of fresh green cucumber color to yellowish-straw (Etchells and Thompson, 1974). Depending where or when turbidity develops, the product will be disposed by the consumer or processed into another product by the processor, such as relish. However, due to recent information about the ability of survival and growth of pathogenic microorganisms, it is important to ensure that turbidity of the product is due to the growth of LAB. The number of LAB present in cucumbers is very low, and varies depending on the harvesting season and maturity of the fruit (Etchells *et al.*, 1961). However, under appropriate conditions of salt concentration and temperature, the LAB will predominate in the brined vegetable. Four stages can be differentiated in vegetable fermentations, each characterized by different groups of microorganisms: Initiation (various Gram-positive and Gram-negative bacteria), Primary fermentation (LAB and yeast), Secondary fermentation (yeasts), Post fermentation (surface growth of oxidative yeasts, molds and bacteria (Fleming, 1982). Hence, it is important to determine what kind of microorganisms are growing at the initiation step of fermentation and at refrigeration temperatures. Most vegetable fermentations occur at 18-25°C, and LAB are classified as mesophilic micororganisms.

Safety concerns in this type of product are the growth of psychrotrophic pathogens at refrigeration temperatures and the growth of mesophilic pathogens at abuse temperatures. The growth of *L. monocytogenes* in this product has been demonstrated at 5, 10, 18°C (Romick, 1994), and it was able to grow in cucumber juice containing 2% salt at different temperatures. However, its growth was inhibited by the natural LAB growing in the

product, or by an added culture as biocontrol. The use of *Lactobacillus plantarum* as biocontrol in this type of product was successful, but the shelf life of the product was shortened by the growth of the biocontrol at refrigeration temperatures.

After the product leaves the plant, there is no control of the temperature in the refrigeration truck, in the supermarket shelves, nor in the consumer's hands. Thus, there is a need to find the ideal biocontrol agent for this type of product. The ideal biocontrol agent could be defined as follows:

- one belonging to the LAB group,
- being a homofermentor to avoid bloater formation and explosion of jars,
- being able to survive the initial harsh environment of 4% salt and low temperatures of storage,
- able to grow slowly, but faster than the pathogen (*L. monocytogenes*) at refrigeration storage temperatures,
- able to grow faster than the pathogen at abuse temperatures,
- able to produce inhibitory substances (bacteriocins) against the pathogen at abuse temperatures.
- the terminal pH for growth of the ideal biocontrol LAB should be around 4 to preserve the fresh green cucumber color and flavor.

**Table 2. pH measurements of refrigerated pickles by type and location**

| <b>Location</b>    | <b>Acidified<br/>pH</b> | <b>Non Acidified<br/>pH</b> |
|--------------------|-------------------------|-----------------------------|
| <b>Chicago</b>     | <b>4.26</b>             | <b>5.27</b>                 |
| <b>Los Angeles</b> | <b>3.94</b>             | <b>3.81</b>                 |
| <b>Miami</b>       | <b>4.05</b>             | <b>4.41</b>                 |
| <b>New York</b>    | <b>3.95</b>             | <b>4.67</b>                 |

Adapted from Fleming *et al.*, 1992 (unpublished data).

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## **CHAPTER II**

### **Microbial Safety of Minimally Processed Brined Refrigerated Cucumbers**

## Abstract

Microbial changes in pickling cucumbers held in air or brine and stored at 5°C were determined to assess the potential safety and quality of minimally-processed, brined, refrigerated cucumbers designed to resemble a deli-type pickle product. Bacterial counts were higher in cucumbers stored in air than those stored in brine after 5 days of storage ( $P < 0.001$ ). *Aeromonas spp* counts remained the same, and *Listeria spp*, when present, increased 10 fold for cucumbers held in air. After 5 days, cucumbers stored in air became moldy and obviously spoiled, but cucumbers stored in brine kept their fresh characteristics: green dark color and clarity of the brine. After prolonged storage they underwent fermentation by lactic acid bacteria (LAB). When brined cucumbers were inoculated with *Listeria monocytogenes* at a concentration of  $10^5$  CFU/mL, *Listeria* survived during the first 5 days, but decreased slowly after that, and LAB counts reached  $10^8$  CFU/mL after prolonged storage. The same effect was observed when cucumbers were blanched to reduce initial microbial load, but the shelf life of the brined cucumbers was extended to more than 3 weeks. Bacteria isolated from cucumbers held in air or brine over the period of storage included: *Enterobacter agglomerans*, *Pseudomonas fluorescences*, *Aeromonas hydrophila*, *Pseudomonas sp.*, *Klebsiella ozanae*, and *Citrobacter freundii*. Among the LAB isolated and identified were *Leuconostoc mesenteroides* and *Lactobacillus curvatus*. These results indicate that storage of cucumbers in brine at 5°C can extend the shelf life of fresh cucumbers by slowing down the growth of bacteria and allowing the growth of LAB. This product can be considered as safe as fresh cucumbers.

Key words: food safety, vegetable, psychrotroph

## **Introduction**

The increasing demand for minimally-processed fruits and vegetables (MPFVs) has resulted in efforts to extend the storage life, while ensuring the safety of such products. The number of documented outbreaks of human infections associated with the consumption of raw fruits and vegetables, and unpasteurized fruit juices has increased in the last 10 years (Beuchat, 1999). Changes in agronomic, processing, preservation, packaging, distribution, and marketing technologies on a global scale have enabled the consumer to enjoy high quality fruits and vegetables year round. Some of the same technologies have also introduced an increased risk for human illness associated with pathogenic microorganisms (Beuchat, 2002). Minimal processing technology is based on the hurdle principle, in which microbial inactivation or control is attained by the use of a combination of multiple sublethal processes or mild treatments. The benefit to the consumer is to get a fresh-like product (Hill *et al.*, 2002). Adaptation to stress environments can result in a pathogen becoming better suited for survival and growth, or to become more virulent (Beuchat, 2002). There is a great concern related to the microbial safety of MPFVs stored under modified atmosphere packaging (MAP). MAP is accompanied by refrigeration to extend the shelf life of MPFV. Elevated levels of CO<sub>2</sub> retard the growth of molds and Gram-negative psychrotrophs, leading to a modification of the microflora to slower growing Gram-positive bacteria (Szabo *et al.*, 2000).

Pathogenic growth may be stimulated by the gas composition before spoilage becomes evident. Psychrotrophic pathogens, such as *Aeromonas hydrophila*, *Yersinia*

*enterocolitica*, and *Listeria monocytogenes* are of concern during storage. Mesophilic pathogens are also of concern, even though they cannot grow at refrigeration temperatures (De Roever, 1999). Nevertheless, they can survive in the product and multiply at abuse temperatures. *Salmonella* was shown to survive in watermelon stored at 4°C and grow in tomatoes stored at room temperature (De Roever, 1999). For some pathogens, such as *Shigella*, survival becomes a concern regardless of multiplication due to the low concentration needed to cause infection in humans.

*L. monocytogenes* was able to grow and survive in mushrooms stored under MAP at 4 and 10°C, but was inhibited by *Pseudomonas* when packed in perforated film. A higher growth of *L. monocytogenes* corresponded to a lower growth of *Pseudomonas spp.* (Gonzalez-Fandos et al., 2001). Reliance on microbial competition as one of the barriers that controls *L. monocytogenes* in food depends on the conditions of handling and storage of the produce. Depending on the CO<sub>2</sub> level used, the microflora dominating spoilage in MPFVs is either Gram-negative bacteria belonging to the *Pseudomonas*, *Xanthomonas*, or *Enterobacteriaceae* families; or, Gram-positive bacteria belonging to the lactic acid bacteria (LAB) group. However, studies have shown that the shelf life of MPFVs can be extended by MAP due to the reduction in the growth of spoilage microorganisms. This may affect the interactions between the pathogen and the natural competitive microflora sufficiently to indirectly enhance *L. monocytogenes* growth. *Listeria* can grow in a low O<sub>2</sub> (3%) and high CO<sub>2</sub> atmosphere (21%) (Francis and O'Beirne, 1998). Thus, there is a need to better understand the role of the background microflora on pathogen growth under storage conditions. Some naturally occurring bacteria may have an antagonistic effect on *L. monocytogenes* and other pathogens (Bennik et al., 1999).

Minimally Processed Brined Refrigerated Cucumbers (MPRC) were chosen as a model system of MPFV for this study. They are preserved by refrigeration and have a relatively short shelf life (3 weeks at 5°C). They do not have preservatives, and the salt concentration is 2%. The initial pH is 5.5, which makes them a perishable product (Etchells and Moore, 1976). The shelf life of MPBRC is dictated primarily by consumer acceptance of the product. Appearance, texture and flavor are the main traits that influence acceptance or rejection (Etchells and Thompson, 1974). In MPRC the shelf life is determined by the development of turbidity in the brine, usually due to the growth of LAB, increase of acid concentration (lactic acid), and change of fresh green cucumber color to yellowish-straw.

The objectives of this study were:

- To determine and characterize the microflora responsible for spoilage of pickling cucumbers stored in air or weak brine at 5°C;
- To study the behavior of *L. monocytogenes* inoculated in cucumbers held in brine at 5°C;
- To compare the effect of blanching cucumbers to reduce the initial microbial load on the survival/growth of *L. monocytogenes* inoculated in MPBRC at 5°C.

## Materials and Methods

### Microbial changes in cucumbers stored in air or brine

Pickling cucumbers, size 2B (3.5-3.8 cm in diameter), were washed with tap water at room temperature (20°C), weighed ( $680 \pm 5$  g) and held at 5°C for 5-45 days in air or 2% brine (after equilibration). Cucumbers stored in air ( $200 \pm 5$  g) were blended with 2X sterile saline solution at high speed for 2 minutes, and plated for total aerobes, total *Enterobacteriaceae*, *Pseudomonas*, *Aeromonas*, *Listeria*, and LAB on PCA (Difco, Detroit, MI), VRBG (containing 1% of glucose, Difco, Detroit, MI), PSIA (*Pseudomonas* Isolation Agar, Difco, Detroit, MI), SA (Starch Ampicilline Agar), MMOX (Modified Oxford Agar containing colistin sulfate and moxalactam 20mg/L), MMRS agar (MRS agar containing 0.02% sodium azide), Total Gram-negative bacteria on PCA with crystal violet (0.01%), respectively, and plates were incubated at 30 or 37 °C for mesophilic count and 8°C for psychrotrophic count.

### Blanching

Pickling cucumbers, size 2B, were washed with cold tap water, weighed ( $680 \pm 5$  g) and blanched at 80°C for 15 seconds (Breidt *et al.*, 2000) and placed in 4% cold brine (5°C). Aquaresin garlic (Kalsec, Kalamazoo, MI) (1 g) and 5 g of irradiated pickling spices (13 KGy) (Ba-Tampte Pickle Products, Brooklyn, NY) of undefined composition due to proprietary rights of the company, were added to mimic the product from the supermarket. Spices were irradiated in plastic containers of 1 L capacity by using Co<sup>60</sup> (Nuclear Engineering Department, NC State University, Raleigh, NC).

Controls were washed and stored under the same conditions. Samples of brine were taken at different times of storage for microbial and chemical analyses. Samples of brine were appropriately diluted in sterile saline solution (0.85%) and plated on the same media mentioned above with a spiral plater Model D (Spiral Biotech, Cincinnati, OH).

### **Behavior of *L. monocytogenes* in brined cucumbers**

*L. monocytogenes* (B164) serotype 4b that contains plasmid pGKE conferring erythromycin resistance (Romick, 1994) at a concentration of  $1 \times 10^5$  CFU/mL was inoculated in refrigerated, brined cucumbers. *Listeria* was grown overnight at 37°C in TSG (Trypticase Soy broth with 1% glucose) broth containing 5 µg/mL of erythromycin (em), transferred to 2% cucumber juice containing 5 µg/mL of em; grown overnight at 37°C and inoculated into the brined cucumbers. Cucumbers, size 2B, were washed with tap water and blanched or packed in 46-ounce glass jars to which 4% cold (5°C) sterile brine was added to achieve a 50/50 pack out ratio (weight/volume). Spices and oleoresin garlic were also added. Jars were sealed under vacuum with lids containing septa, and stored at 5°C. Samples of brine were taken at several points for microbial and chemical analyses. Samples were plated on PCA, MMRS, MMOX (5 µg/ mL of erythromycin), and VRBG agar by spiral plater. Plates were incubated at 30 (PCA, MMRS, VRBG) and at 37°C (MMOX). Colonies were counted after 24 and 48 hours.

### **Isolation and Characterization of bacteria**

Representative colonies were randomly selected and re-streaked for isolation on non-selective agar (TSG or MRS). Isolated colonies were further characterized by inoculating

in broth and performing Gram stain (Difco, Detroit, MI), Oxidase test (Difco, Detroit, MI), and catalase test. Bacterial isolates in the broth were analyzed for motility and shape by phase contrast microscopy.

Isolates obtained from the different selective media were identified following the procedure of Enterotube II (Roche, Montclair, NJ) (*Enterobacteriaceae*), *Aeromonas*, *Pseudomonas* were identified by Oxi/Ferm tube II (BBL, Cockeysville, MA), Micro-ID *Listeria* (Organon Teknika, Durham, NC) (*Listeria spp*), and Biolog AN plates (LAB), (Anaerobic microplate, Biolog, Hayward, CA). Representative isolates of LAB were further identified by ITS-PCR with digestion of the PCR product by *RsaI* (Breidt and Fleming, 1996). DNA was extracted following the recommendations of a PROMEGA kit. PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA) and primers G1-16s (5'→3'): GAAGTCTAACAAGG and L2-23S (5'→3'): GGGTTTCCCATTCGGA (Sigma-Genosys, Texas, US) that amplified the intergenic transcribed spacer region.

### **Statistical Analysis**

The analysis of variance was computed by the General Linear Models Procedure of SAS version 8.0 (SAS Inc., Cary, NC).

## Results and Discussion

### Microbial changes in cucumbers stored in air versus brine

Pickling cucumbers used in this study arrived to the lab in good conditions, and the total aerobic counts varied between  $10^3$ - $10^7$  CFU/g, depending on the harvesting season and growing conditions. Cucumbers grown in trellises had very low counts ( $10^3$  CFU/g). When blanched and stored in brine, cucumbers did not show any microbial growth after a year of storage at 5°C, as demonstrated by plate count, pH and turbidity of the brine (data not shown). Blanching reduced the initial microflora by 3 log cycles (Fig. 1). Similar results were reported by Breidt *et al.* (2000). The shelf life of the product was extended from 3 weeks to several months, compared to the unblanched cucumbers, which had a shelf life of 3 weeks (Etchells and Moore, 1976). Shelf life of MPBRC was determined by appearance of turbidity in the brine caused by bacterial growth in the jars; at the same time the fresh cucumber color changed from dark green to yellowish-straw and the pH dropped from 5.5 to 3.5.

The shelf life of the cucumbers stored in air was influenced by the initial bacterial counts; cucumbers coming with initial counts of  $10^7$  CFU/g were spoiled after 5 days of storage at 5°C. They were moldy and soft (data not shown). Cucumbers stored in brine at 5°C had lower counts for psychrotrophic and mesophilic bacteria, compared to those stored in air (Fig. 2a and b). Even though storage of cucumbers in brine at 5°C did not inhibit the growth of bacteria, it extended their shelf life by slowing down bacterial growth and selecting the groups that were going to predominate (Table 1). In this study brined cucumbers underwent lactic acid fermentation. Lactic acid fermentation of vegetables is a

complex microbial process in which a small population of LAB becomes the predominating microflora (Daeschel *et al.*, 1987).

Fermentation is one of the oldest methods of preservation; it extends the shelf life of many foods, including cucumbers. However, for MPBRC, fermentation will be undesirable from the consumers' standpoint. Statistical analysis of these data showed a significant difference ( $P \leq 0.001$ ) between the numbers of bacteria present in air versus brined cucumbers. Cucumbers held in brine kept their fresh characteristics related to color and texture compared to those stored in air, which were soft, mushy and moldy. At the end of the shelf life of cucumbers held in air the microflora predominating was the Gram-negative group, which reached almost the same counts as the total aerobes (Table 1).

*Listeria spp*, when present, increased by 10 fold in cucumbers stored in air. Even though *L. monocytogenes* was not found in these cucumbers, *L. innocua* was identified. *L. innocua* was isolated from hydrocooling water from two different processors, indicating that the conditions exist for any *Listeria* to enter the production process of this type of product (Romick, 1994). It has also been shown that *L. monocytogenes* 4b can attach to cucumber surfaces during washing (Reina *et al.*, 2002). This fact may represent a safety concern since *Listeria* can grow at refrigeration temperatures. *Aeromonas spp* remained the same in cucumbers stored in air, and *Pseudomonas spp* grew in cucumbers stored in air or brine. It has been shown that *A. hydrophila* can survive and grow in asparagus, broccoli, and cauliflower stored under CAS (Control Atmosphere Storage) (Berang *et al.*, 1989). *L. monocytogenes* and *A. caviae* grew in cucumber slices and mixed lettuce stored

at temperatures higher than 4°C (Jacxsens *et al.*, 2002). However, in brined cucumbers the counts of *Listeria* were always under the level of detection by spiral plater (<400 CFU/mL). *Aeromonas* counts went down, *Pseudomonas* counts went from undetectable to detectable, but the counts were still low compared to the total *Enterobacteriaceae* counts, which remained constant up to the 15<sup>th</sup> day of storage. LAB started to grow after 8 days of storage, reaching higher counts at 15 days (data not shown). After 40 days of storage at 5°C, the group that predominated was LAB, reaching almost the same number as the Total Aerobic count; the pH was lower than 4, and the total *Enterobacteriaceae* counts went down to levels undetectable by spiral plater (Fig. 2c). During initiation of fermentation, facultative and strictly anaerobic bacteria from the cucumber start growing, but are inhibited by the growth of LAB which become established (Fleming, 1982). The sequence of LAB in vegetable fermentations depends on many factors, such as initial load, growth rates, salt and acid-tolerance and temperature, and it is usually started by *Leuconostoc* and *Streptococci* (Daeschel *et al.*, 1987). In this study *Leuconostoc spp* and *Lactobacillus curvatus* were found when the product showed turbidity due to bacterial growth at 5°C.

#### **Fate of *Listeria monocytogenes* in brined cucumbers**

When *L. monocytogenes* was inoculated into the cucumbers stored in brine, counts of *L. monocytogenes* remained the same during the first 5 days. However, they decreased slowly as the LAB started growing and changing the pH (Fig. 3 and 4). Statistical analyses of the data suggest that the growth of the LAB inhibited the growth of *L. monocytogenes*. It was demonstrated that *Listeria* can grow in MPBRC as long as the

LAB are not present (Romick *et al.*, 1994). Blanching the cucumbers to reduce bacterial load did not affect *Listeria* survival; the same trend was observed when *Listeria* was inoculated in the product made with blanched cucumbers. Counts of *Listeria* went down, when the LAB started growing, meaning that brining cucumbers favored selectively the growth of LAB at 5°C; or both LAB and *Listeria* could grow, but bacteriocins or inhibitory metabolites produced by LAB inhibited *Listeria*.

When *L. monocytogenes* is present in vegetables, it must compete with the diverse microflora, which will be affected by the storage conditions. *E. cloacae* reduced the growth of *L. monocytogenes* in air (Francis and O'Beirne, 1998). However, the growth and inhibitory activity of *E. cloacae* were inversely affected by the CO<sub>2</sub> concentration. *Leuconostoc citreum*, on the other hand, showed better inhibitory activity against *L. monocytogenes* when the CO<sub>2</sub> concentration increased. However, 21% CO<sub>2</sub> inhibited *L. citreum* (Francis and O'Beirne, 1998).

### **Identification of cucumber microflora**

Cucumbers harbored a variety microflora, and the predominating group depended on the time the sample was taken and the storage condition (air versus brine). Under aerobic conditions, the Gram-negative bacteria predominated at the end of the shelf life; in brine, the cucumbers underwent fermentation by LAB. Among the bacteria isolated were *L. curvatus* and *L. mesenteroides* (Table 2). The growth of the LAB prevented the growth of *L. monocytogenes* by lowering the pH (Fig. 4); the counts of *Listeria* went down to undetectable levels (<400 CFU/mL); no colonies were observed on the MMOX agar plates. LAB are known to produce bacteriocins and other metabolites able to prevent

growth of pathogens. The removal of the natural microflora from fruits and vegetables could lead to a situation where, even in adequately refrigerated products, *L. monocytogenes* could reach high levels before the product becomes visually spoiled (De Roeve, 1999). Blanching the cucumbers did not have any effect on the predominating microflora (LAB), even though there was a 2-3 log reduction in the LAB counts compared to the unblanched fruit.

Adding spices and garlic to the cucumbers did not affect the growth of the LAB; they still were the predominating microflora and inhibited *L. monocytogenes* from growing. Garlic and spices are known for their antibacterial properties. However, statistical analysis of the data comparing the growth rate of LAB in cucumbers with or without garlic or spices showed that the ones with garlic and spices had higher growth rates of LAB ( $P < 0.035$ ). It has been shown that LAB grow in many fermented food products that have garlic. These strains grew better when garlic was added as an ingredient in the food, and garlic was the source of fermentable carbohydrates, such as glucose and inulin (Paludan-Muller *et al.*, 2002).

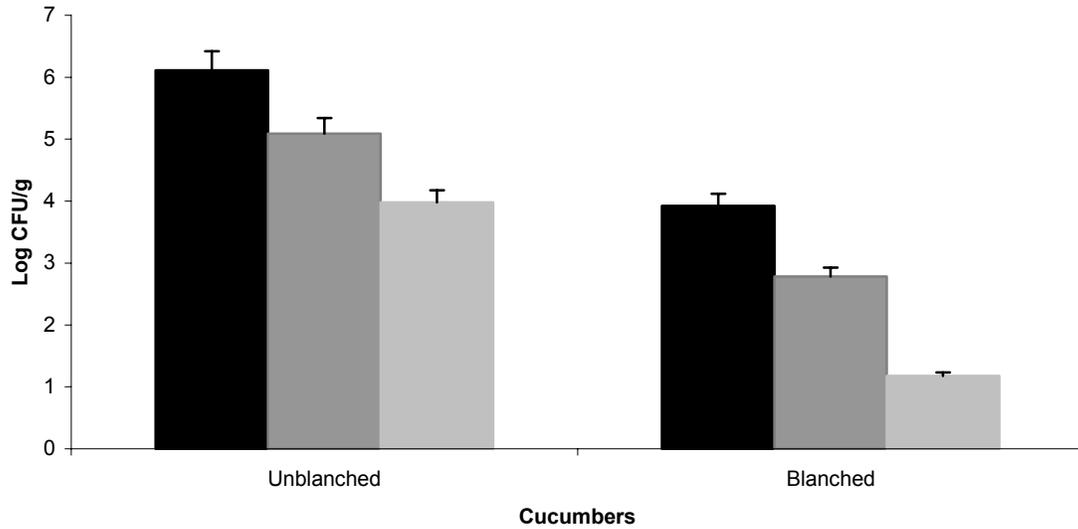
### **Conclusions**

- Compared to MAP storage of minimally processed fruits and vegetables, storing them in brine could be considered safer. Several studies done in fruits and vegetables under MAP demonstrated that *Listeria* can grow better because MAP suppressed the growth of other competitors that are sensitive to CO<sub>2</sub> concentration, such as *Pseudomonas*. Some LAB were also inhibited under high

concentrations of CO<sub>2</sub>. Such is the case of *L. citreum* (Francis and O'Beirne, 1998).

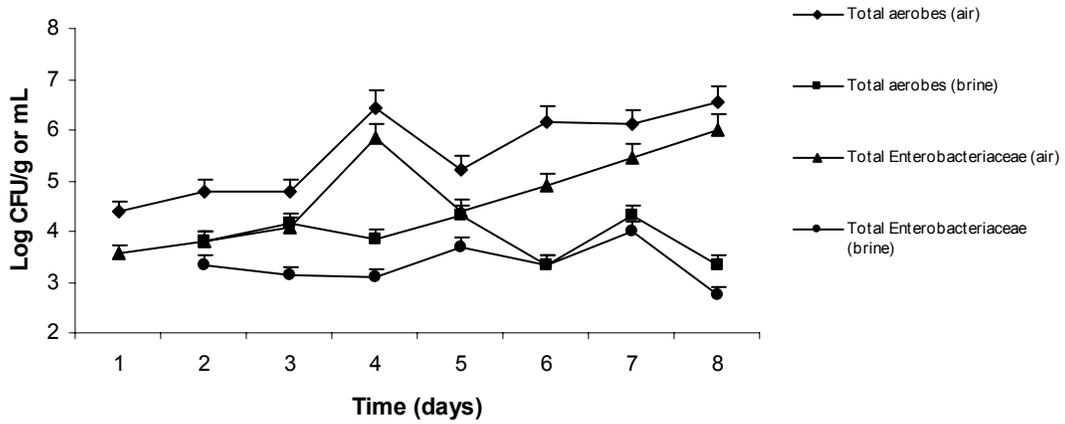
- New approaches to decontaminate fruits and vegetables are being questioned due to the fact that the natural microflora removed may prevent pathogenic growth. However, blanching cucumbers to reduce the microbial load extended the shelf life of the product without jeopardizing its safety. LAB were responsible for turbidity of the product at the end of the shelf life.
- Based on these results, we can conclude that storing cucumbers in brine can select and allow the growth of LAB, which can inhibit the growth of other bacteria, including pathogens, either by competition or by formation of inhibitory metabolites. It would be interesting to survey those strains of LAB growing in the product to know more about the mechanism of inhibition of *L. monocytogenes*.
- Further studies are needed to determine if the fate of *L. monocytogenes* in brined cucumbers is the same in the case of other *Listeria* strains. It is known that the response to different hurdles differs among different strains of *Listeria*.

Figure 1. Effect of blanching on the microflora of pickling cucumbers

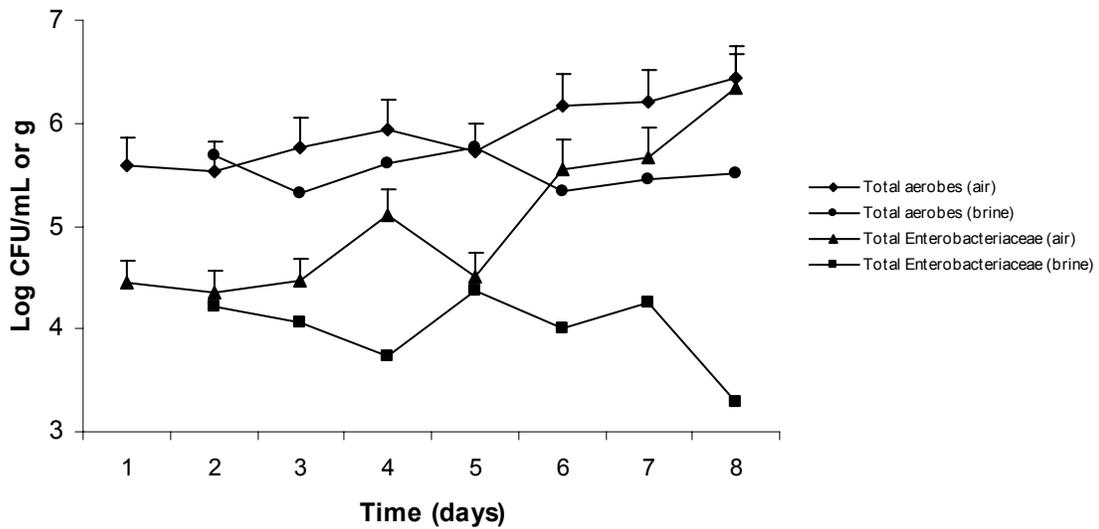


Cucumbers (size 2B) were blanched for 15 seconds at 80°C. ■ Total Aerobes, ■ Total *Enterobacteriaceae*, ■ Total LAB. The numbers are the average of 2 samples. Error bars are the standard deviations.

Fig 2. Microbial counts of fresh and brined pickling cucumbers.



2a. Cucumbers (size 2B). Plates incubated at 8°C for psychrotrophic count. The numbers are the average of 2 samples. Error bars show the standard deviation.



2b. Plates incubated at 30°C for mesophilic count. The numbers are the average of 2 samples. Error bars show the standard deviation.

2c. Mesophilic count in brined cucumbers at 5°C. ♦ Total Aerobes ● Total *Enterobacteriaceae*. Dotted line means below the level of detection with spiral plater. The numbers are the average of 3 samples. Error bars are the standard deviations.

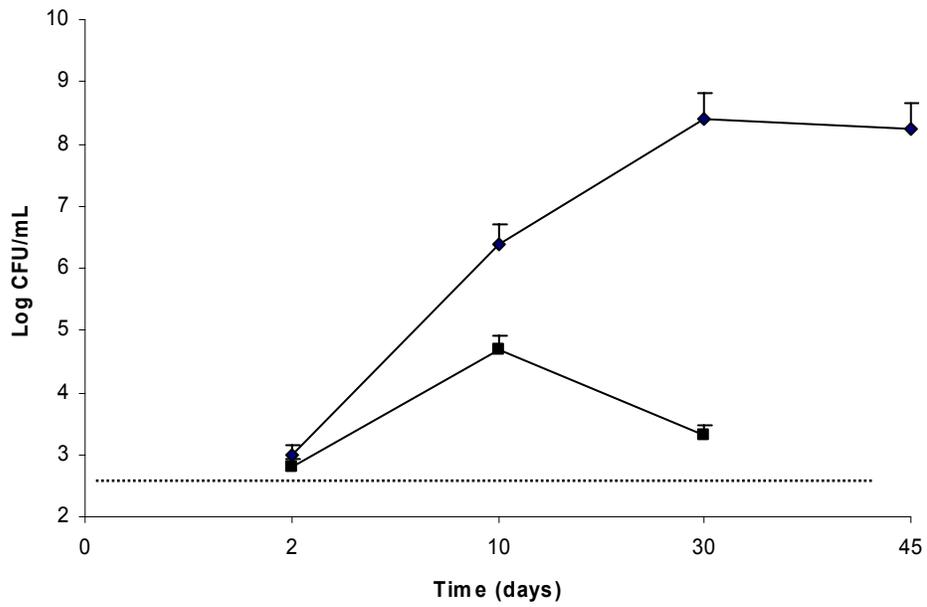
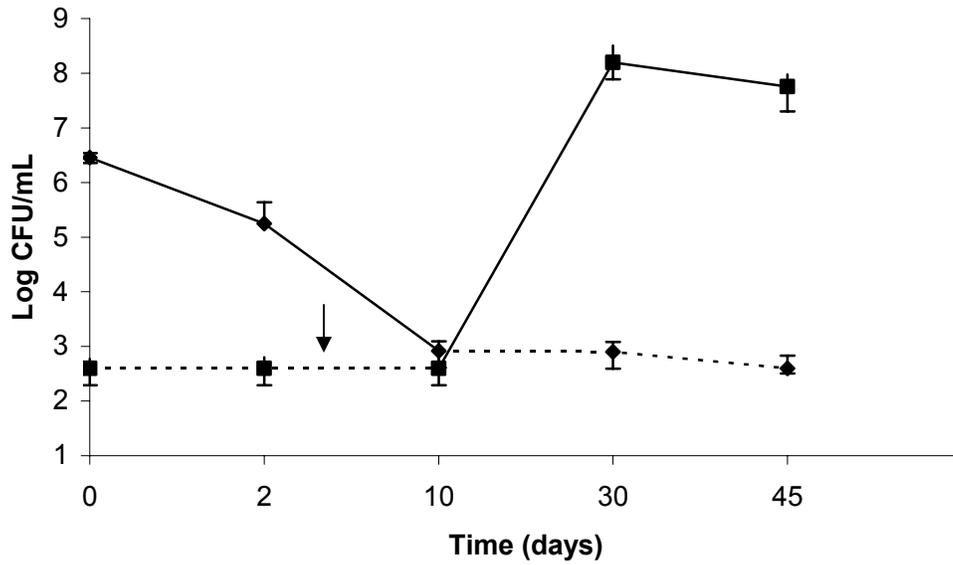
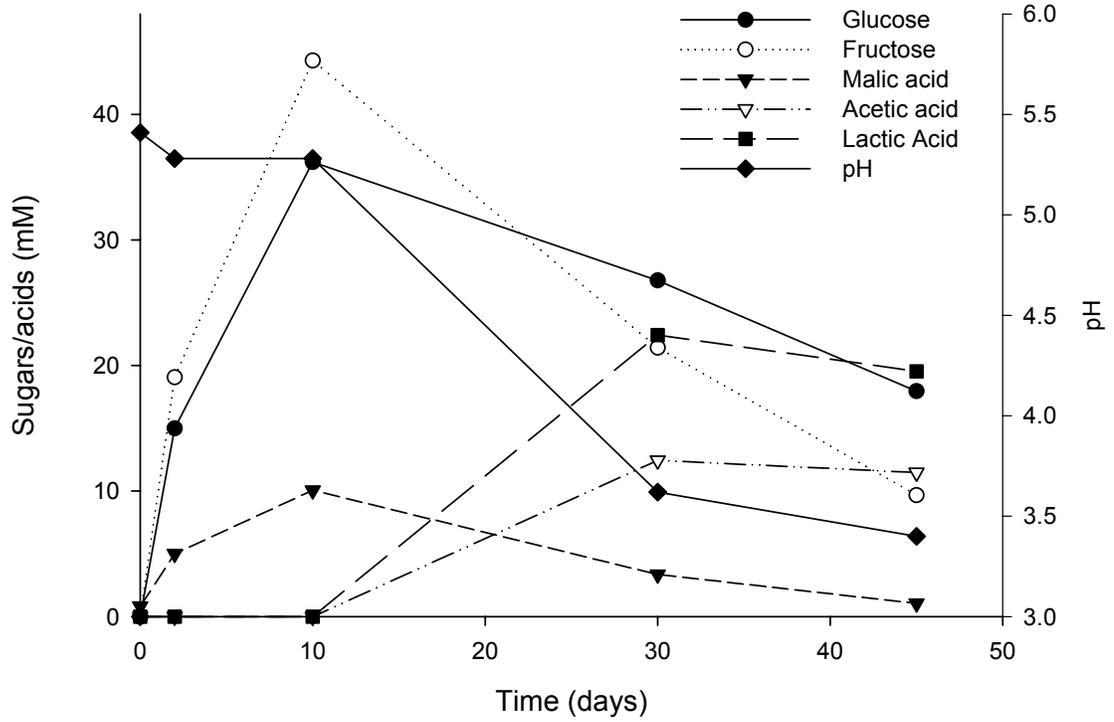


Fig 3. Fate of *Listeria monocytogenes* serotype 4b in minimally processed brined refrigerated cucumbers at 5°C



■ Natural LAB, ♦ *L. monocytogenes* 4b, Cucumbers were washed with tap water. The numbers are the average of 5 samples. (↓) dotted line means below the level of detection by spiral plater. The same pattern was obtained when cucumbers were blanched. Error bars are the standard deviations.

Fig 4. Chemical changes of brined pickling cucumbers at 5°C



Cucumbers were blanched and stored at 5°C. Samples of brine were taken as indicated in the graph.

**Table 1. Microbial counts in fresh and brined cucumbers stored at 5°C**

| Sample                      | Day  | Total Aerobes <sup>1</sup> | Total <i>Enterobacteriaceae</i> <sup>1</sup> | Non <i>Enterobacteriaceae</i> <sup>1</sup> | <i>Aeromonas sps</i> <sup>1</sup> | <i>Listeria sps</i> <sup>1</sup> | LAB <sup>1</sup>  |
|-----------------------------|------|----------------------------|--|--|-----------------------------------|----------------------------------|-------------------|
| Cucumber (air)              | 0    | 5.3                        | 4.3  | 4  | 3.7                               | 3.2                              | <2.6 <sup>3</sup> |
| Cucumber (air) <sup>2</sup> | 5    | 6.7                        | <2.6   | 6.4  | 3.6                               | 4.6                              | <2.6              |
| Brine                       | 0.25 | 4.4                        | 4.3  | 4.1  | <2.6 <sup>3</sup>                 | <2.6 <sup>3</sup>                | <2.6 <sup>3</sup> |
| Brine                       | 8    | 4.1                        | 2.7  | 2.6  | <2.6 <sup>3</sup>                 | <2.6 <sup>3</sup>                | <2.6 <sup>3</sup> |

<sup>1</sup>Numbers are the average of 2 samples, Log CFU/g.

<sup>2</sup>Cucumbers held in air were spoiled at 8<sup>th</sup> day.

<sup>3</sup><2.6 indicates below the level of detection with spiral plater.

**Table 2. Identification of microorganisms in fresh and brined cucumbers stored at 5°C**

| Strain   | Sample | Time <sup>1</sup> (days) | Number of colonies <sup>2</sup> | Method of ID        |
|--|--------|--------------------------|---------------------------------|---------------------|
| <i>A. hydrophila</i> <sup>3</sup>                      | Fresh  | 0, 5                     | 10                              | Oxi/Ferm            |
| <i>E. agglomerans</i> <sup>4</sup>                     | Brine  | 5                        | 5                               | Oxi/Ferm            |
| <i>L. innocua</i> & <i>sp</i> <sup>5</sup>             | Brine  | 3                        | 6                               | Listeria ID         |
| <i>K. ozanae</i> <sup>4</sup>                          | Fresh  | 5                        | 5                               | Enterotube II       |
| <i>Citrobacter freundii</i> <sup>4</sup>               | Fresh  | 5                        | 4                               | Enterotube II       |
| <i>P. fluorescens</i> <sup>6</sup>                     | Brine  | 1, 2, 3                  | 5                               | Oxi/Ferm            |
| <i>Leuconostoc mesenteroides cremoris</i> <sup>7</sup> | Brine  | 8, 15                    | 5                               | Biolog, ITS pattern |
| <i>L. mesenteroides</i> <sup>7</sup>                   | Brine  | 35                       | 5                               | ITS pattern         |
| <i>L. curvatus</i> <sup>7</sup>                        | Brine  | 35                       | 5                               | ITS pattern         |

<sup>1</sup> Time at which colonies were isolated, <sup>2</sup>Number of colonies isolated and identified from countable plates.

<sup>3</sup>SA agar, <sup>4</sup>VRBG, <sup>5</sup>MMOX, <sup>6</sup>SIA, <sup>7</sup>MMRS.

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## **CHAPTER III**

### **Isolation and Selection of Lactic Acid Bacteria as Biocontrol Agents for Minimally Processed Vegetables**

## Abstract

Fresh pickling cucumbers (3.5-3.8 cm in diameter) were blanched at 80°C for 15 seconds, cooled and brined to equalize at 4% NaCl in 1.4 L glass jars. Sterile spices and garlic oil were added to simulate a deli-type pickle product. The product was stored at 5°C for 3 weeks and then transferred to various abuse temperatures (16, 25, 30 °C). A total of 118 lactic acid bacteria (LAB) were isolated from the product. The isolates were Gram stained and biochemically characterized by gas production, and oxidase and catalase reactions. They were also characterized and identified using molecular techniques. They were grouped according to their intergenic transcribed spacer region (ITS)-PCR and *RsaI* restriction endonuclease profiles and further identified to species level by sequencing the variable regions V1 and V2 of the 16S rRNA gene.

The isolates were also tested for bacteriocin-like production against Gram-positive and Gram-negative pathogens and spoilage bacteria. Among the LAB identified were the genera of *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Weissella*, and *Enterococcus*. Five isolates produced bacteriocin-like activity against *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Listeria*. The addition of a bacteriocin-producing LAB or their metabolites, if effective against food pathogens, may improve the safety of minimally processed vegetable products.

Key words: LAB, Minimally-Processed Vegetables, Bacteriocin

## Introduction

Fresh and minimally processed fruits and vegetables (MPFV) have grown in popularity in recent years. Food safety issues have resulted from some of these MPFV, stimulating efforts to ensure the safety of such products. The major safety concern of ready-to-eat foods is the possible outgrowth of psychrotrophic pathogens, such as *Listeria monocytogenes* (Kelly *et al.*, 1998). *L. monocytogenes* and *Aeromonas caviae* were able to grow in cucumber slices and mixed lettuce stored at temperatures higher than 4°C (7, 10, 12°C) under Modified Atmosphere Packaging (MAP) (Jacsexens *et al.*, 2002).

Brined refrigerated cucumbers are included among the MPV products because they undergo minimal processing, keeping their crispness and freshness. Refrigerated pickles may or may not be fermented before refrigeration. They may or may not be acidified and have preservatives added, and held at room temperature for a few days, then refrigerated to slow down the fermentation. Refrigerated pickles are available in many varieties, including kosher dills, genuine dills, half-sour, overnight, and sweet pickles, and are available whole or cut into halves, spears, slices, chips or relish, or are sliced lengthwise for sandwiches (Etchells *et al.*, 1976). Refrigeration gives these pickles a distinct, fresh, raw cucumber flavor. The production and storage of this item under refrigeration prevents the pickles from spoiling. The pickles are ready for shipping when the concentration of salt within the pickle equals the concentration of the brine. The finished pickles have a distinct, fresh flavor (very much like a deli pickle), are very crisp, and maintain their fresh green cucumber color desired by an important segment of consumers.

The product is made with fresh washed cucumbers, spices, garlic and salt to a final concentration of 2%. It has a shelf life of 3 weeks at 5°C, and an initial pH around 5.5. The shelf life of pickle products is dictated primarily by consumer acceptance of the product. Appearance, texture and flavor are the main traits that influence acceptance or rejection (Etchells and Thompson, 1974). Shelf life is determined by the development of turbidity, usually due to the growth of LAB, increase of acid concentration, change of fresh green cucumber color to yellowish-straw. Depending on where or when turbidity develops, the product will be disposed by the consumer or processed into another product, such as relish by the processor. However, due to recent information about the ability of survival and growth of pathogenic microorganisms at refrigeration temperatures, it is important to ensure that turbidity of the product is due to the growth of LAB. The number of LAB present in cucumbers is very low and varies depending on the harvesting season and maturity of the fruit (Etchells *et al.*, 1961). However, under appropriate conditions of salt concentration and temperature the LAB will predominate in the brined vegetable (Fleming, 1982).

Currently, there is increasing interest in bacteriocins as natural food preservatives to ensure safety of MPFV, as they are believed to be safe for human consumption. Humans have been consuming fermented foods for a long time. Moreover, there is strong evidence that these antimicrobials may play an essential role in food fermentation, enabling the producers to selectively and efficiently inhibit part of the competing microflora, which may include several spoilage bacteria and food-borne pathogens (Harris *et al.*, 1992; Leroy *et al.*, 2001).

Nisin is the only bacteriocin approved to be used in selected foods. In the USA, nisin is used to prevent clostridial growth in processed cheeses (Li and O'Sullivan, 2002). Thus, inoculating the product with a bacteriocin-producing organism will not require any previous approval, and the bacteriocin will be produced in situ. Satisfactory performance under optimal laboratory conditions is no guarantee for success when the bacteriocin-producing organisms are applied in the food under typical process conditions (Leroy *et al.*, 2001). Salmon slices inoculated with nisin producer *Lactococcus lactis* as a biocontrol agent for *L. monocytogenes* worked well at abuse temperatures, but failed at 5°C due to the effect of salt and low temperature on the culture (Wessels and Huss, 1996). *L. monocytogenes* was inhibited by *L. lactis* during sprouting of alfalfa seeds, but this inhibition was lower than the one obtained in a sterile broth model system of sprouts. This may be the result of inhibitory activity associated with the microflora of the sprouting seeds (Palmai and Buchanan, 2002). Microbial background is not eliminated from the fruits or vegetables during washing, and sometimes can increase during processing. Careful selection of the strains is therefore indispensable. Since strains have to be perfectly adapted to the food environment in which they are going to be applied, the use of natural food isolates seems most promising.

The objective of this research was:

- To isolate, characterize biochemically and genetically, and select naturally occurring LAB from a model brined cucumber product for potential biocontrol use in minimally processed brined vegetable products to control the growth of *L. monocytogenes* at refrigeration and abuse temperatures.

## Materials and Methods

### Deli-type pickle product

Cucumbers (3.5-3.8 cm in diameter) were washed and blanched for 15 seconds at 80°C (Breidt *et al.*, 2000) and immediately cooled with 4% cold brine (5°C) containing 1 g of sterile garlic oil (Aquaresin garlic, Kalsec, Kalamazoo, MI), and 5 g of irradiated pickling spices (13 KGy). Spices were irradiated in plastic containers of 1 L capacity by using Co<sup>60</sup> (Nuclear Engineering Department, NC State University, Raleigh, NC).

Jars were placed at 5°C for 3 weeks and then transferred to various abuse temperatures (16, 25, 30°C). Brine samples were appropriately diluted in sterile saline solution (0.85%) and plated on MMRS agar (Modified MRS, 0.02% sodium azide) by a Model D spiral plater (Spiral Biotech, Cincinnati OH). Isolated colonies with different morphology were re-streaked on MRS agar (Difco, Detroit, MI).

Samples of blanched and unblanched cucumbers were also taken to enumerate the initial load of LAB. Two hundred grams of cucumbers were blended with 2X sterile saline solution (0.85%) for 2 minutes at high speed. Samples were filtered in a stomacher bag and plated. Three different methods of plating were used to test the best recovery of LAB: spread plating, concentration by filtration using Millipore filter bottles (Millipore Corporation, Bedford, MA) and following the manufacturer's instructions, and pour plating on MMRS agar.

## Bacterial Identification

Isolates growing on MRS agar were tested for gas production from glucose in MRS broth with Durham tubes. Positive for gas was demonstrated by the space inside the tube due to gas production. To confirm if they were hetero or homo-fermentors, the HHD (Homofermentor, Heterofermentor Differential) medium (McDonald *et al.*, 1987); this medium has fructose as a carbon source, and a pH indicator. Heterofermentors will produce less acid than homofermentors, not changing the pH of the medium. However, the homofermentors will decrease the pH, changing the color of the medium to yellow. Catalase reaction was determined by the 3% H<sub>2</sub>O<sub>2</sub> method (Paludan-Muller *et al.*, 1999), cytochrome oxidase by Dry Slide Oxidase strips (Difco, Detroit, MI).

Bacteriocin-like production against selected LAB: *L. plantarum* (LA70), *Leuconostoc mesenteroides* (LA81), *Pediococcus dextranicum* (LA 61), and *L. monocytogenes* strains (serotypes: 4b, 1/2a, 1/2b) (B164, B181, B182, B183, B184), *Pseudomonas aeruginosa* (B14), and *Escherichia coli* (B41).

Isolates giving positive results for LAB were characterized by ribotyping, by using primers designed to amplify the 16S-23S intergenic transcribed spacer region and digestion of the PCR product by *RsaI* (10u/μl) (Promega, Madison WI) as described by Breidt & Fleming, 1996. PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA). Representative isolates from each group were identified by sequencing of the variable regions V1 and V2 of the 16S rRNA gene (Barrangou *et al.*, 2002), the product of PCR was purified by using a Wizard PCR preps DNA purification

kit (Promega). The samples were sequenced commercially (Davis sequencing, Davis CA). Biochemical identification was done by AN Microplate method (Anaerobic Microplate, Biolog, Hayward, CA) and API 50 CHL (bioMerieux, Hazelwood, MI).

Primers used for ITS-PCR:

GAAGTCGTAACAAGG (5'→3'), GGGTTTCCCCATTCGGA (5'→3')

Primers used for 16S-PCR:

AGAGTTTGATCCTGGCTCAG (5'→3'), GTCTCAGTCCCAATGTGGCC (5'→3')

Primers were obtained from Sigma-Genosys (The Woodlands, Texas, USA)

### **Bacteriocin Assay**

Bacteriocin-like production was assayed by the spot test method (Fleming *et al.*, 1975). Isolated colonies were grown overnight in MRS broth at 30°C. The supernatant was obtained by centrifugation, and the pH adjusted to 7 with NaOH. Then, filter sterilized, and 10 µL were spotted onto MRS agar or TSG agar, allowed to dry and overlaid with the test microorganism in soft agar 0.75%, which was prepared by adding 100 µL of a turbid overnight culture into 100 mL agar. The plates were incubated either at 30 or 37 °C and checked for inhibition zones after 24 hours. A nisin producer *L. lactis* (LA218) was included in each test.

### **Test Microorganisms**

*L. monocytogenes* serotype 4b, *L. monocytogenes* serotype 1/2a, *L. monocytogenes* serotype 1/2b were obtained from the culture collection of Sophia Kathariou (NCSU, Raleigh, NC). Bacteria were grown overnight at 37°C in TSG broth.

*L. plantarum*, *L. mesenteroides*, *P. acidilactici*, *P. dextranicum*, and *L. lactis* were obtained from the USDA-ARS unit culture collection (NCSU, Raleigh, NC). Bacteria were grown overnight at 30°C in MRS broth.

*E. coli* K12 and *Pseudomonas aeruginosa* were obtained from the USDA-ARS unit culture collection (NCSU, Raleigh, NC). Bacteria were grown overnight at 37°C in TSG broth (Difco, Detroit, MI).

### **Results and discussion**

LAB were screened from different batches of product during the past 2 years. Even though they were present in the cucumbers in low numbers after blanching (10-100 CFU/g) (Fig. 1), they dominated the fermentation of the product, as demonstrated by turbidity of the brine at refrigeration and at abuse temperatures. Three different methods of plating were tested to see if there was any difference in the counts of LAB. According to these results, concentration of the bacteria by membrane filtration and pour plating gave higher numbers than spread plating in the case of blanched cucumbers.

Counts on MMRS agar were similar to those obtained on PCA, but were sometimes lower. This may be due to the fact that some LAB are sensitive to sodium azide used in MMRS. Some colonies were very small on MMRS, but grew well on MRS alone. Similar results were reported by Fleming *et al.*, 1994. However, we couldn't get rid of this ingredient due to the mixed background microflora present in cucumbers and able to grow on MRS agar. Such is the case with some species of the *Enterobacteriaceae* group.

Similar results were reported by Vaughan *et al.*, 1994, when plating vegetable samples. LAB colonies on MRS agar grew along with non-LAB colonies, described as catalase positive, rod shaped; and other large, mucoid, transparent colonies that were catalase-negative bacteria.

Depending on the source and growing conditions of the cucumbers, turbidity of the brine occurred at 5°C or higher temperatures, or never occurred. This was the case of cucumbers coming from Mexico, which were grown on trellises (never in direct contact with the soil). The microflora of fruits and vegetables are related to agronomic practices, including irrigation water, fertilizers, and soil type in the field (Beuchat, 2002). Change of the microflora depends upon the processing conditions and characteristics of the fruits and vegetables being processed. *Lactobacilli* were the predominant microflora in tomatoes stored under MAP and in air at 4 and 10°C due to the low pH of the fruit. Initially, the microflora consisted of LAB, yeasts, molds and Gram-negative bacteria (Drosinos *et al.*, 2000).

### **Phenotypic Characterization of LAB**

A total of 118 isolates were obtained from MPBRC brine stored at 5, 16, 25, and 30°C. They were identified tentatively as LAB: Gram-positive, oxidase/catalase negative, and non-motile. Rods and cocci, 45.76% homofermentors and 54.24% heterofermentors were found, the majority of the isolates being heterofermentors (Table 1 and 2). This may be due to the low salt concentration used in the brine. According to Etchells (1943), salt concentration will have an impact on the type of microorganisms carrying out the

fermentation: low salt concentrations (1.5-2%) will allow the prevalence of the heterofermentors; higher salt concentrations (6-8%), the growth of the homofermentors. In vegetable fermentations where low salt concentration is used, the *Leuconostoc* start the fermentation, and then the homofermentors will follow. Four stages can be differentiated in vegetable fermentations, each characterized by different groups of microorganisms: Initiation (various Gram-positive and Gram-negative bacteria), primary fermentation (LAB and yeast), secondary fermentation (yeasts), and post fermentation (surface growth of oxidative yeasts, molds and bacteria (Fleming, 1982). The samples of brine in this study were taken as soon as turbidity was noticeable.

Gas production was tested by the Durham tube and confirmed by color change of HHD medium (McDonald *et al.*, 1987). Some of the isolates indicated as heterofermentors by Durham tube gave the opposite reaction by HHD medium (Table 1). This may be due to tiny bubbles present in the Durham tube and reported as positive. However, comparison of gas production by such strains with a known heterofermentor (*Leuconostoc mesenteroides*, LA81) was unquestionable (there was a clearly noticeable presence of gas in the tube). They were classified as homofermentors.

### **Genetic and Biochemical Characterization of LAB**

Bacteria with different fingerprinting, as shown by their ITS pattern, were identified by sequencing of the variable regions (V1 and V2) of the 16S rRNA gene (Table 1). Genotypic characterization of the isolates correlated well with their biochemical profile, with exception of *Weissella* and *Enterococcus*. In the case of *Enterococcus*, no ID could

be found in the BIOLOG or the bioMerieux data base. In the case of *Weissella spp.*, various isolates (16) were identified as *Lactobacillus coprophillus* by API 50 CHL. Some *Lactobacillus* and *Leuconostoc spp.* are reclassified as *Weissella* and *L. coprophillus* is now known as *W. confusa* (Paludan-Muller *et al.*, 1999). Perhaps in the future, identification will be possible when both databases are updated to include *Enterococcus* and *Weissella* in the list of LAB. Another factor could be the need to know the genus in advance in order to choose the correct temperature of incubation. *L. lactis* was not identified as *Lactococcus* at the beginning in the Biolog data base. The temperature recommended by Biolog is 37°C. However, after knowing the genus and species by sequencing the variable region of the 16S RNA gene, it was identified as *L. lactis lactis* with a 100% probability. The only change made was the temperature of incubation from 30°C to 37°C. However, many bacteria have very similar phenotypes but they are genetically different. Thus, biochemical profiles for identification can be misleading.

The bacteria identified included: *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Pediococcus*, and *Weissella*.

Identification of different genera varied according to the abuse temperature chosen, temperature being one of the factors influencing prevalence of one genus or another: *Lactobacillus* at 5°C; *Leuconostoc* at 5 and 16°C; *Enterococcus* at 16, 25 and 30°C; *Lactococcus* and *Weissella* at 16, 18, 25°C (Tables 1 and 2). From these isolates, the ones able to grow faster than *L. monocytogenes* at abuse temperatures in cucumber juice at 2 different salt concentrations (2 and 4%) were: *Leuconostoc mesenteroides* (LR50),

*Lactobacillus curvatus* (LR53, LR55, LR57) (Fig 2a). They also grew slowly at 5°C (data not shown). However, LR50 is a heterofermentor (*Leuconostoc mesenteroides*), and does not comply with the definition of the ideal biocontrol agent for this type of product, it will produce gas and cause bloater formation or the jars will explode due to gas pressure. LR53 is a homofermentor (*Lactobacillus curvatus*) but it does not produce bacteriocin. Two isolates (LR10 and LR11, *L. lactis lactis*) testing negative for bacteriocin-like production, could also inhibit *L. monocytogenes* in cucumber juice containing 2% salt. They grew faster than *Listeria* at 20°C (Fig 2b). This inhibition could be due to the decrease of pH due to acid production.

*L. lactis* non-nisin producer was able to inhibit the growth of *L. monocytogenes* in cucumber juice at 11°C, this inhibition was due to the low pH (Breidt and Fleming, 1998). *S. enteritidis* was not able to grow in tomatoes due to the growth of LAB, which produced lactic, acetic, propionic and formic acid and thus a decrease in pH (Drosinos *et al.*, 2000). The initial pH of tomatoes varies from 3.4-4.8, depending on the variety and state of ripeness. However, *Salmonella* could grow in tomatoes at room temperature. The two principal determinants for growth of pathogens on produce are pH and temperature (De Roever, 1999). Other factors are the nutrient composition of the produce, natural microflora, and antimicrobials present in some produce, such as carrots.

Advances in biotechnology may make possible to develop the ideal biocontrol agent with some of the candidates mentioned above. The ideal biocontrol agent could be defined as one belonging to the LAB group, being a homofermentor to avoid bloater formation,

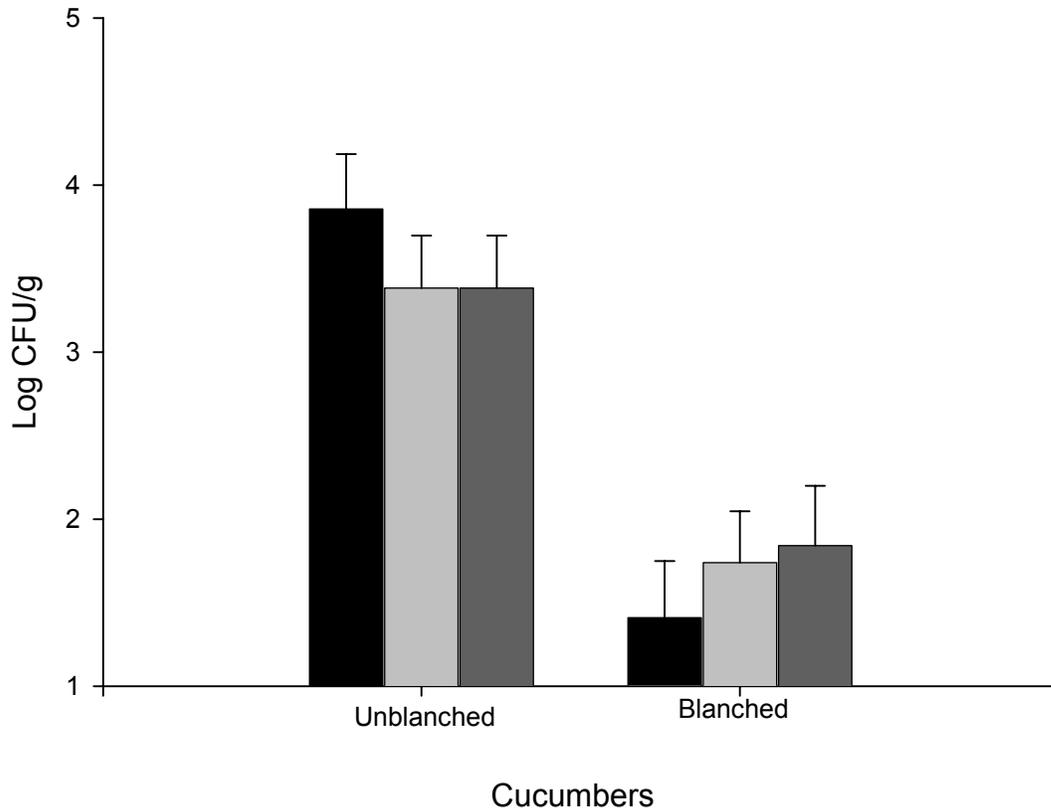
being able to survive the initial harsh environment of 4% salt and low temperatures of storage, able to grow slowly but faster than the pathogen at refrigeration storage, and able to grow faster than the pathogen and to produce inhibitory substances (bacteriocins) against the pathogen at abuse temperatures. The terminal pH should be around 4 to preserve the fresh cucumber flavor and color. Consumers of this type of product do not like the sharp acidic flavor of pickles due to high concentrations of lactic acid.

None of the isolates showed any inhibitory activity against the Gram-negative bacteria tested (*P. fluorescens* and *E. coli* K12). Five isolates showing bacteriocin-like activity against *Lactobacillus plantarum*, *L. mesenteroides*, *Pediococcus dextranicum* and *L. monocytogenes* (4b) belonged to the genus *Lactococcus* (Fig. 3 and Fig. 4). Three isolates showing bacteriocin-like activity against *Listeria* (B181, B182, B184) belonged to the genera *Lactococcus* (LR80), *Enterococcus* (LR110) and *Leuconostoc* (LR50). However, bacteriocin activity was lost after the cultures were frozen. Similar results were reported by other authors in the case of class II bacteriocin producers (Vaughan et al., 2001). This effect was due to the presence of the bacteriocin genes in plasmids, which were lost during handling of the bacteria.

## Conclusions

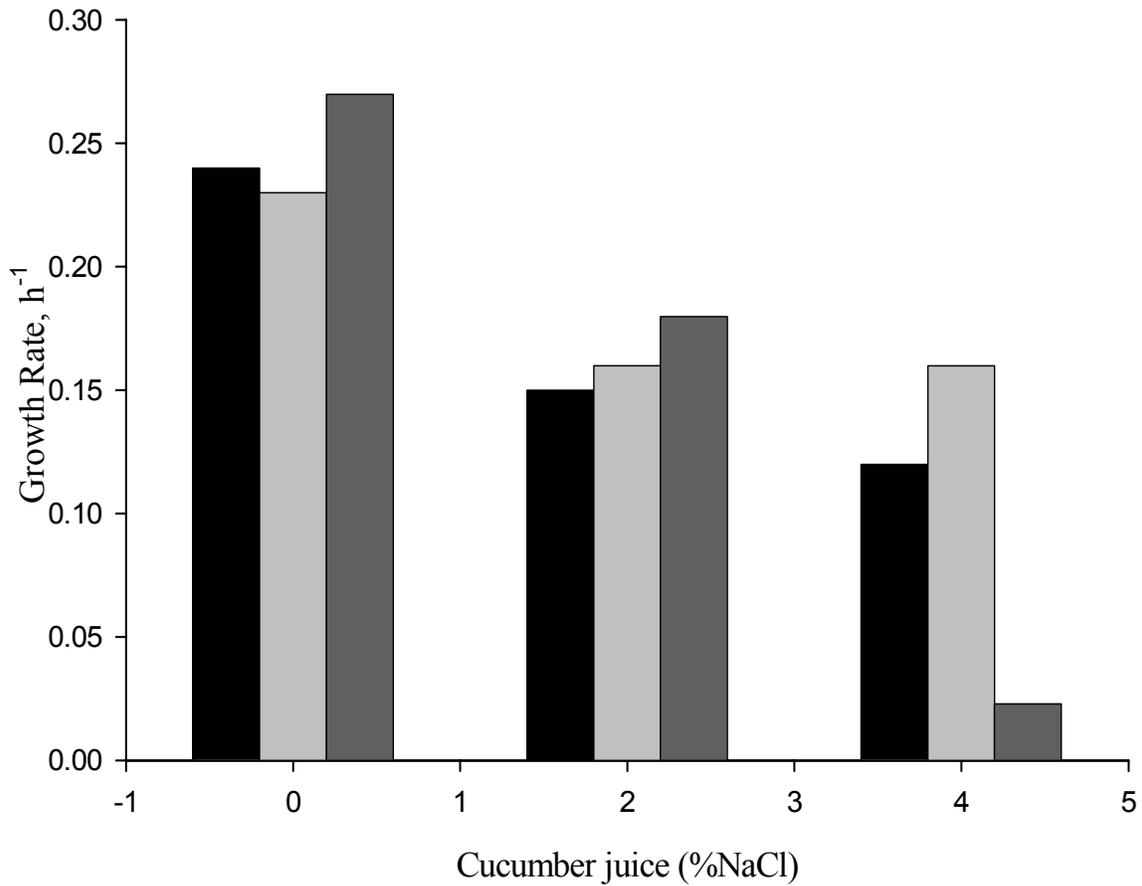
- The spontaneous fermentation of the MPBRC at abuse or refrigerated temperatures was due mainly to LAB. According to these results, the conditions under which the product was prepared and stored allowed the prevalence of LAB as the microflora responsible for the turbidity of the product. Thus, the product can be considered safe.
- One hundred and eighteen LAB from a deli-type pickle product were clearly separated in clusters according to their origin and species identification by phenotypic and genotypic characteristics. These strains are unique because they can grow in the presence of garlic, spices, and salt. They grew faster than the pathogen at temperatures considered abuse temperatures.
- Bacteriocin-like producing LAB isolated from this study could be used as biocontrol agents in this product where *L. monocytogenes* growth is of concern, and in other products where the presence of *Lactobacillus*, *Leuconostoc* or *Pediococcus* is undesirable. Further studies are needed to determine the characteristics of the putative bacteriocins produced by these LAB.

**Figure 1. Enumeration of lactic acid bacteria in pickling cucumbers by different methods**



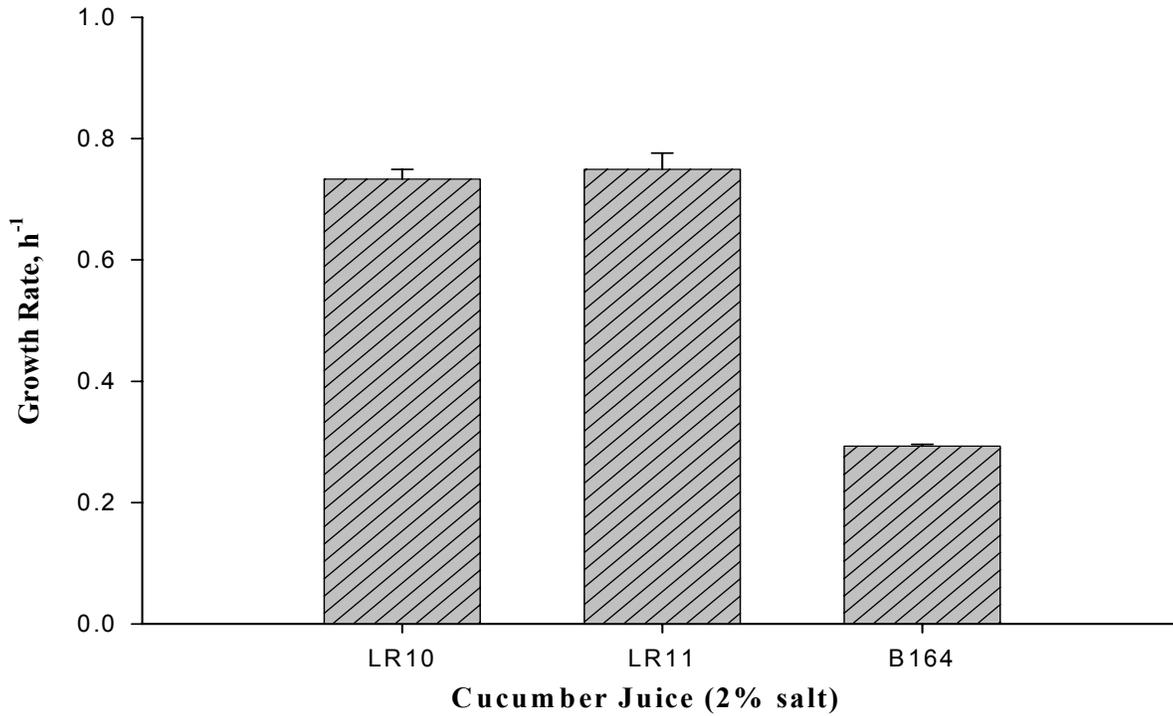
■ Spread plating, ■ Pour plating, ■ Membrane filtrate. Each bar represents the average of 2 samples. Cucumbers (200 g) were washed / washed and blanched, blended with 2x saline solution and plated by the different methods. The numbers are the average of 2 samples. Error bars are the standard deviations.

Fig 2a. Comparative growth of lactic acid bacteria in cucumber juice at 20°C



■ LR 50 (*L. mesenteroides*), ■ LR 55 (*L. curvatus*), ■ LA 218 (*L. lactis*), included as control. Each bar represents the average of 3 wells of a microtiter plate. Cells were inoculated at a concentration of 10<sup>6</sup> CFU/mL.

**Fig 2b. Growth comparison of *L.lactis* (LR10, LR11) and *L. monocytogenes* (B164) in cucumber juice at 30°C**



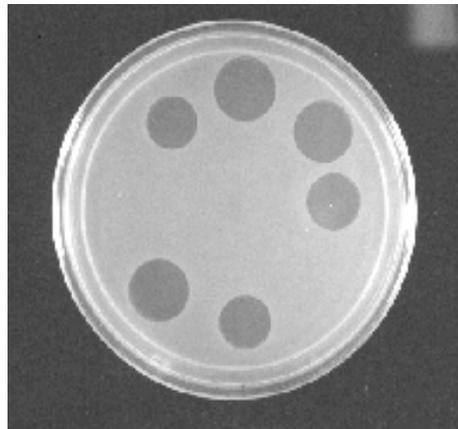
LR10, LR11 are *L. lactis lactis*, B164 is *L. monocytogenes* 4b. Each bar is the average of 3 wells of a microtiter plate. Cells were inoculated at a concentration of 10<sup>6</sup>CFU/mL. Error bars are the standard deviations.

**Figure 3. Natural LAB growing on MMRS**



LAB showing zones of inhibition on MMRS agar were isolated on MRS agar and tested for bacteriocin-like production.

**Figure 4. Supernatant of *L. lactis* isolated from minimally processed brined refrigerated cucumbers**



Lawn is *Pediococcus dextranicum*. Zones of inhibition were also obtained with other LAB (*Lactobacillus*, *Leuconostoc*) tested and *L. monocytogenes* 4b.

**Table 1. Characterization and identification of the different LAB isolated from brined refrigerated cucumbers**

| Shape              | HHD medium <sup>4</sup> | ITS pattern          | AN plate                | API 50 CHL                    | 16S rRNA sequencing                          |
|--------------------|-------------------------|----------------------|-------------------------|-------------------------------|--|
| Cocci              | Homofermentor           | <i>Enterococcus</i>  | no ID <sup>2</sup>      | no ID                         | <i>E. casseliflavus</i> (98) <sup>5</sup>    |
| Cocci              | Homofermentor           | <i>Enterococcus</i>  | no ID                   | no ID                         | <i>E. durans</i> (98) <sup>5</sup>           |
| Cocci              | Homofermentor           | <i>Enterococcus</i>  | no ID                   | no ID                         | <i>E. mundtii</i> (98) <sup>5</sup>          |
| Cocci, chains      | Homofermentor           | <i>Lactococcus</i>   | <i>L. lactis</i>        | <i>L. lactis</i> <sup>1</sup> | <i>L. lactis lactis</i> (98) <sup>5</sup>    |
| Coccobacilli       | Heterofermentor         | <i>Leuconostoc</i>   | <i>L. citreum</i>       | ND                            | <i>L. citreum</i> (97.5) <sup>5</sup>        |
| Coccobacilli       | Heterofermentor         | <i>Leuconostoc</i>   | <i>L. mesenteroides</i> | <i>L. mesenteroides</i>       | <i>L. mesenteroides</i> (98) <sup>5</sup>    |
| Short rods         | Homofermentor           | <i>Lactobacillus</i> | ND                      | <i>L. curvatus</i>            | <i>L. curvatus</i> (98) <sup>5</sup>         |
| Short rods, chains | Homofermentor           | <i>Lactobacillus</i> | ND <sup>3</sup>         | <i>L. plantarum</i>           | <i>L. plantarum</i> (98) <sup>5</sup>        |
| Cocci, pairs       | Homofermentor           | <i>Pediococcus</i>   | ND                      | ND                            | <i>P. pentosaceus</i> (96.5) <sup>5</sup>    |
| Coccobacilli       | Heterofermentor         | <i>Weissella</i>     | <i>W. confusa</i>       | <i>L. coprophillus</i>        | <i>W. confusa/kimchi</i> (96.5) <sup>5</sup> |

<sup>1</sup> Two isolates were identified as *L. lactis raffinolactis* by API50 CHL

<sup>2</sup> No identification, <sup>3</sup> Not Determined, <sup>4</sup> HHD medium (McDonald *et al.*, 1987). <sup>5</sup> Number in parenthesis indicates the % of similarity of the 300 bp of the 16S PCR product.

| <b>Table 2. Lactic acid bacteria isolated from minimally processed brined refrigerated cucumbers at different temperatures</b> |                           |                                   |
|--|---------------------------|-----------------------------------|
| <b>Strain ID</b>   | <b>Number of isolates</b> | <b>Temperature of isolation°C</b> |
| <i>Enterococcus casseliflavus</i>  | 3                         | 25                                |
| <i>Enterococcus durans</i>   | 23                        | 18                                |
| <i>Enterococcus mundtii</i>  | 7                         | 30                                |
| <i>Lactococcus lactis</i>  | 20                        | 16, 18, 25                        |
| <i>Leuconostoc citreum</i>   | 6                         | 16, 25                            |
| <i>Leuconostoc mesenteroides</i>   | 28                        | 5, 18                             |
| <i>Lactobacillus curvatus</i>  | 9                         | 5                                 |
| <i>Lactobacillus plantarum</i>   | 5                         | 18                                |
| <i>Pediococcus pentosaceus</i>   | 1                         | 5                                 |
| <i>Weisella confusa/kimchi</i>   | 16                        | 18, 25                            |
| Total isolates   | 118                       |                                   |

Colonies of different morphology growing on MMRS were re-streaked on MRS agar for further characterization.

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## CHAPTER IV

### **Biochemical and Genetic Characterization of *Lactococcus lactis lactis* strains isolated from Minimally Processed Brined Refrigerated Cucumbers**

## Abstract

*Lactococcus lactis lactis* strains isolated from Minimally Processed Brined Refrigerated Cucumbers (MPBRC) at 16, 18, and 25°C, were characterized genetically by Triplicate arbitrarily primed-PCR and plasmid profile. Biochemical characterization was done by API 50 CHL. Some of the isolates showed carbohydrate fermentation patterns different than the ATCC type strain, being able to use raffinose, xylose, melibiose, sucrose and inulin.

Bacteriocin-like production was tested against other lactic acid bacteria (LAB) and *Listeria monocytogenes* serotypes 4b, 1/2a, 1/2b. Six isolates gave positive reaction for bacteriocin-like activity against LAB and *L. monocytogenes* 4b. However, bacteriocin-like activity as demonstrated by the diameter of the zones of inhibition, was dependent on the medium used to grow the bacteria, showing more activity when grown in MRS, and less when grown in GM17, cucumber juice or brine. The substance showing inhibitory properties lost activity after treatment with proteinase K in 3 isolates, but it did not lose activity by treatment with either trypsin or heat (100°C, 2 minutes).

The isolates were shown to be resistant to nisin up to 2000 IU/mL. Primers designed to target the genes for nisin production and immunity (*nisABTCl*) and nisin immunity (*nisFEG*) gave product for 6 of the 9 isolates. However, two isolates not showing any bacteriocin-like activity showed PCR product for the immunity genes. They also could grow in concentrations of nisin of 300-2000 IU/mL.

These strains may have uses as biocontrol agents in MPBRC. Further characterization (biochemical and genetic) of the putative bacteriocin is needed.

Key words: *Lactococcus lactis* *sps lactis*, bacteriocin, Minimally-Processed Fruits and Vegetables (MPFV).

## Introduction

Minimally Processed Fruits and Vegetables (MPFV) have increased in demand lately and they rely on MAP (Modified Atmosphere Packaging) and refrigeration for quality and safety (Kelly *et al.*, 1998). However, concerns have arisen with the emergence of food-borne psychrotrophic organisms, especially *L. monocytogenes*. The development of new food technologies and the search by consumers for natural products point to the use of bacteriocins, and/or bacteriocin-producing microorganisms as a potential source of biopreservatives for food. Bacteriocin production has been observed in all genera of LAB, and has been well described for *L. lactis* *sps lactis* (Klaenhammer, 1993). Strains of *L. lactis* produce a range of bacteriocins, the most important of which is the lantibiotic, nisin, which is a broad spectrum bacteriocin, active against *Clostridium botulinum* and its spores, *L. monocytogenes*, and Gram-negative bacteria, if the cells are sensitized (Stiles and Holzapfel, 1997, Klaenhammer, 1993).

*L. lactis* *sps lactis* has been traditionally used as a starter in the manufacture of cheese and fermented milk products on account of its function in preservation and its contribution to flavor and aroma (Moreno *et al.*, 1999). Even though the most recognized habitat for the *Lactococci* is dairy products, the literature also provides evidence that green plant material is a natural source for *L. lactis* *sps lactis*. Surveys of vegetable samples have shown that *L. lactis* can be isolated from potatoes, cucumbers, sweet peas,

beans, cantaloupes, corn, and broccoli after enrichment at 21°C for 2 days under anaerobic conditions (Salama *et al.*, 1995); from sorghum and barley (Booyesen *et al.*, 2002; Muyanja *et al.*, 2002); and from unpasteurized grapefruit juice (Kelly *et al.*, 1996). Even though fresh fruit and vegetable samples yield low numbers of LAB ( $10^1$ - $10^3$  CFU/g), under certain conditions, such as under modified atmosphere packaging, LAB can reach high numbers ( $10^8$ - $10^9$  CFU/g) (Kelly *et al.*, 1996). *L. lactis* *sps lactis* was isolated in large numbers from sauerkraut fermentation at the initial stage (Harris *et al.*, 1992), and it has also been identified as the dominant microflora present in MPFV, especially sprouted seeds during the period they are ready for human consumption. Many of these isolates produced nisin. The occurrence of bacteriocin-producers may aid the dominance of these strains in the vegetable environment (Kelly *et al.*, 1998).

The ability to produce nisin is widely distributed among *L. lactis* *sps lactis* (Immonen *et al.*, 2000). Several surveys of *L. lactis* *sps lactis* for bacteriocin production reported that bacteriocin producer strains account for 8.5 % of the isolates (Moreno *et al.*, 1999). However, *L. lactis* *sps lactis* used as starters in dairy fermentation have other important properties distinctive from bacteriocin production. These include plasmid-linked traits essential to milk fermentation, such as fast acid development, phage resistance, lactose metabolism, thermotolerance, and proteinase systems (Harris *et al.*, 1992). Bacteriocin production and immunity have been found in conjugative plasmids encoding essential phenotypes for growth and survival of lactococci in milk. Such is the case of lactococcins (Klaenhammer, 1993).

When *L. lactis* nisin-producers isolated from sauerkraut were tested for those properties, they failed to produce acid or to clot milk in a short period of time (Harris *et al.*, 1992). Nisin-producing strains are reported as poor cheese starters, relatively more sensitive to phage attack, and they are used in combination with a nisin-resistant starter to ensure adequate performance (Ryan *et al.*, 1996).

Bacteriocin-producing cultures capable of growth in vegetable brines may have a potential use as biocontrol agents for MPFV.

The objectives of this research were:

- To characterize genetically and biochemically the *L. lactis* *sps lactis* strains isolated from MPBRC as potential biocontrol agents for this type of product.

## **Materials and Methods**

### **Biochemical characterization of *Lactococcus* strains**

Chemical profiles determined by the carbohydrate metabolism of bacteria were obtained with API 50 CHL (bioMerieux, Hazelwood, MI). Bacteria were grown on MRS agar under anaerobic conditions. Pure isolated colonies were used to prepare the solution to inoculate the strips of API 50, as suggested by the manufacturer. Readings of positive or negative reactions were recorded every 4, 24, and 48 hours. A control was included in

each test, *L. lactis sps lactis* ATCC 11454 (LA 119, USDA culture collection, Raleigh, NC).

Gas production was tested with Durham tubes and HHD medium (McDonald *et al.*, 1987). Salt tolerance was measured by inoculating GM17 broth, containing 0, 2, 4, 5, and 6.5 % NaCl with 1% of an overnight culture. Positive growth was determined by turbidity of the liquid. Nisin resistance was determined in an automated microtiter plate reader (Bio-Tek Instruments, Inc. Winooski, Vermont). The strains were inoculated in a 96-well microtiter plate (Costar Inc., Corning, NY) containing different concentrations of nisin. The culture was grown overnight at 30°C and diluted to  $10^7$  CFU/mL with sterile saline solution (0.85% NaCl), inoculated in each well to get  $10^6$  CFU/mL, and incubated at 30°C for up to 72 hours. Nisin was obtained from NISAPLIN (Aplin & Barrett Ltd., Beaminster Dorset, England), and prepared by placing 2 g of the powder in 100 mL of HCl 0.02N and filter sterilized. According to the manufacturer, 0.1 g of powder contains 2.5 mg of nisin or  $1 \times 10^6$  IU. That dilution (50 mg/100mL) was used to prepare the stock solution for the different nisin concentrations. Growth rates were determined by using the software, Bacterial Growth Kinetics, designed by Breidt, 2001.

### **Bacteriocin Assay**

Bacteriocin-like activity was tested by the spot test method (Fleming *et al.*, 1975). The bacteria were grown in different media: MRS (Difco, Detroit, MI), GM17 (Difco, Detroit, MI), cucumber juice (CJ), and brine (obtained from MPBRC). Cucumber juice was composed of 60% CJ, 40 distilled water, 2% NaCl, which were mixed, filter

sterilized and placed at 5°C until use. The brine was obtained by blanching 680 g of size 2B cucumbers and placing them in 46 oz jars, adding 4% sterile brine, 1 g of garlic oil (Kalsec, Kalamazoo, MI) and 5 g of irradiated pickling spices (Ba-Tampte Pickle Products, Brooklyn, NY). The jars were placed at 5°C for 5 days. One hundred mL of brine were removed from each jar, and samples were mixed and filter sterilized. Samples of brine were taken for HPLC analysis and pH measurement.

The turbid cultures of *Lactococcus* strains were centrifuged at 5000 rpm for 10 minutes, the supernatant adjusted to pH 7 with 25% NaOH, and then filter sterilized. Ten µl of the broth were spotted on MRS agar or TSG agar. Plates were allowed to dry at room temperature for 2-3 hours and overlaid with 10<sup>5</sup>-10<sup>6</sup> CFU/mL of the test microorganism in soft agar (0.75 % agar).

Test bacteria were *Pediococcus dextranicum* (LA77), *P. acidilactici* (LA61), *Leuconostoc mesenteroides* (LA81), *Lactobacillus plantarum* (LA70), *L. plantarum* MOP3 (BI7), *L. monocytogenes* serotypes 4b (B164, B181, B184), 1/2a (B182), and 1/2b (B183).

Plates were incubated at 30°C (LAB) and 37°C (*Listeria*) for 24 hours. Plates showing zones of inhibition were reported as positive for putative bacteriocin production. Positive controls were also included (broth from a nisin producer strain, LA 218, and nisin in a concentration of 50 µg/mL). A negative control was also included (broth from a non-bacteriocin producer, LA 70). Positive samples were further tested for heat stability of the

putative bacteriocin at 100°C for 2 minutes, pH stability (2-9), and digestion with proteinase K and trypsin. Briefly, 200 µl of broth were digested with either 2 µl of proteinase K or trypsin to reach a final concentration of 1mg/mL (Sigma, Chemical Company, St Louis, MO), and incubated at 37°C for 3 hours. After treatment, 10 µl of broth were spotted onto MRS or TSG agar and allowed to dry, and the plates were overlaid with the test microorganism as described above.

### **Genetic Characterization**

Bacterial DNA was extracted using the Promega Kit (Promega Corp, Madison WS). DNA fingerprinting patterns were obtained by using the method of Cusick and O'Sullivan, 2000, with only one variation: the temperature of annealing used was 40°C (one) instead of 3 different temperatures. This method uses a single arbitrarily primer at low stringency annealing conditions, which allow it to bind to genomic DNA at places to which has partial or full homology, giving a PCR product formation if two sites are within a few thousand base pairs of one another and are on opposite DNA strands. The primer used was an 18-mer that had homology to a universally conserved region of the 16S rRNA gene 5'-CAGCAGCCGCGGTAATWC-3'; where W is a degenerate base (W indicates A/T bases). PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA). PCR parameters were as follows:

92°C: 2 min, 40 cycles of: 92°C for 30 seconds, 40°C 1 minute, 68°C 2 minute, final 68°C 8 minutes. The reaction master mixture for 100 µL consisted of 3 µL of primer (52.4 µM), 10 µL of dNTPs (10 mM), 20 µL of Mg Cl<sub>2</sub> (25mM ), 3 µL of BSA (10 mg/mL), 10 µL of 10X PCR buffer, 1 µL Taq polymerase (Invitrogen life technologies, Carlsbad,

CA), 54  $\mu$ L of sterile distilled water. For each reaction 20  $\mu$ L of the master mixture was added into each tube, 4  $\mu$ L of DNA was added, and overlaid with mineral oil. The PCR products were analyzed by agarose (2%) gel electrophoresis with TBE 1% as running buffer.

### **Plasmid Profile**

Plasmid profiles were obtained in order to observe homology among the *L. lactis* strains isolated from MPBRC. Plasmids carry genes that supply the bacterial cells with new features, important for their survival in new and extraordinary conditions. Cells were grown in GM17 at 30°C overnight (14 hours). Plasmid extraction and agarose gel electrophoresis followed the method of O'Sullivan and Klaenhammer (1993). Plasmid profiles of strain NCK6540 (obtained from the culture collection of Todd Klaenhammer, NC State University, Raleigh, NC) and LA 218 (pGKC) from the USDA culture collection (Raleigh, NC), both *L. lactis sps lactis*, were included as controls.

### **Nisin genes**

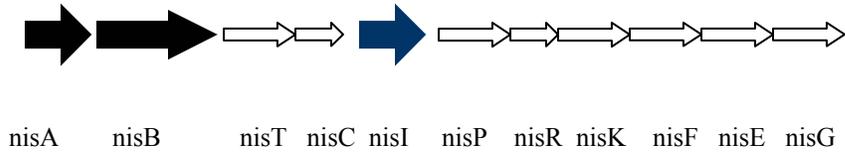
Primers targeting the *nisA* gene (Moschetti *et al.*, 1999) were *nisL* (5'→3') CGAGCATAATAACGGC and *nisR* (5'→3') GGATAGTATCCATGTCTGAAC.

Additional primers were designed to target *nisABTCl*, *nisIPRK* and *nisFEG* genes by using the program: Primer3 Output (version 0.2) ([www-genomewi.mit.edu](http://www-genomewi.mit.edu)).

Primers were obtained from Sigma-Genosys (The Woodlands, Texas, USA). PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA). PCR parameters were as follows 1 cycle 92°C: 2 min, 30 cycles at 92°C: 30 sec, 55°C: 45 sec, 72: 1

minute, 1 cycle 72: 3 min. The reaction mixture was composed of 45  $\mu$ L of master mix (Promega Corp, Madison, WS), 2  $\mu$ L of DNA, 1.5  $\mu$ L of each primer (6  $\mu$ M), overlaid with mineral oil.

**Primers used:**



ABTCI (R): (5'→3') TTTCCACCCAGGTTTGCTAC

ABTCI (L): (5'→3') GCCGATGTTGTAGTGCCTT

IPRK (R): (5'→3') CATTACCAAGAGCTGCGACA

IPRK (L): (5'→3') TTCGAAATCGGAATGGGTAG

FEG (R): (5'→3') CCTGTTTCTGCCAAACCAAT

FEG (L): (5'→3') AACGGTGCAGGAAAATCAAC

The PCR products were analyzed by polyacrylimide TBE (pre-cast) gel electrophoresis, following the instructions of Invitrogen (Invitrogen life technologies, Carlsbad, CA).

## Results and Discussion

### Biochemical Profile

The strains isolated from MPBRC had different biochemical profiles from the ATCC strain 11454 (Table 1). One isolate could use inulin, 8 could use xylose, 3 melibiose and all could ferment sucrose, a phenotypic characteristic linked to nisin production. However, only 6 of these isolates tested positive for bacteriocin-like activity (Table 2). It has been reported that conjugative transposon-like elements encoding sucrose metabolism without nisin production are present in *L. lactis* isolated from vegetables (Kelly *et al.*, 2000). In a survey of *L. lactis lactis* from dairy habitats, 35.8% were able to ferment sucrose, but were unable to produce nisin (Moschetti *et al.*, 1999). Other surveys reported that all strains able to ferment sucrose were nisin-producers and had nisin immunity. However, great variability was found in the natural nisin production levels, when different strains were compared ranging from 0, 100, 500, 1900 IU/mL of medium (De Vuyst, 1994).

Two isolates could use raffinose, a characteristic used to differentiate *L. lactis* from *L. raffinolactis*. Raffinose is found in the seeds of cucumbers (Handley *et al.*, 1983), possibly indicating the ability of these strains to survive in the cucumber. This characteristic was kept even though the strains were cured of plasmids. According to the literature, *raf* genes are generally located in plasmids. Genetic analysis of strains able to metabolize raffinose demonstrated that the genes were located in a conjugative

transposon in the chromosome, and they were probably acquired by horizontal transfer from another organism (Kelly *et al.*, 1998).

Xylose metabolism is not common in *L. lactis*, but plant isolates were able to ferment xylose (Harris *et al.*, 1992, Kelly *et al.*, 1996), supporting the link between xylose metabolism and habitat. It was reported that xylose metabolic genes were present in *Xyl<sup>+</sup>* (plant isolates) and *Xyl<sup>-</sup>* (dairy isolates) of *L. lactis*. The authors concluded that the xylose metabolic genes in the dairy strains may be becoming pseudogenes because there is no selective pressure to maintain xylose metabolism in the dairy environment, or that the xylose genes are evolving new catalytic functions in dairy strains (Erlandson *et al.*, 2000).

All the strains could grow at 4% NaCl but not at 6.5% (Table 1). All strains fermented lactose, a trait known to be encoded in a plasmid (Klaenhammer and Sanosky, 1985). Some of them fermented melibiose, a characteristic not recognized in *L. lactis* (Kelly *et al.*, 1998).

### **Genetic characterization**

Plasmid profile for the strains studied showed no evidence of plasmid for 7 strains, and a weak band was observed for 2 isolates of approximately 3990 bp (Fig. 1a). Upon repeating plasmid isolation for these 2 isolates (LR10 and LR11), it can be seen that they had plasmids (Fig 1b), in contrast to the control strain (NCK6540), which had a complex plasmid profile, typical of *Lactococci*. However, other methods for plasmid isolation were not tested.

DNA fingerprinting of the strains was done by using a modification of the TAP (TriPLICATE arbitrarily primed)-PCR (Cusick and O'Sullivan, 2000), with one annealing temperature (40°C). This technique has previously been shown to differentiate organisms to the strain level. As shown in Fig. 2, all the isolates have a similar fingerprinting profile. However, LR5 had one more band, being different from the others. Six isolates gave positive reaction for bacteriocin-like activity.

PCR targeting the *nisA* gene showed product at approximately 300 bp in all the strains with exception of LR3 (data not shown). However, strains LR10 and LR11 also showed a weak band but they did not give positive results for bacteriocin-like activity. Other studies have also reported the presence of non-functional nisin genes in strains of *Lactococcus* that were also sensitive to nisin (Moschetti *et al.*, 1996). PCR product was obtained when targeting the *nisABTCI* genes, only three strains (LR3, LR10, LR11) did not show product, and these strains did not show any bacteriocin-like activity. However, when targeting the *nisFEG* genes (immunity), all the strains gave product, including LR10 and LR11. Only one strain (LR3) did not show any product, and it was nisin sensitive, even to low nisin concentrations (data not shown). The strains showing PCR product for the immunity genes were resistant to nisin in broth (Fig. 4). Nisin resistance genes had been found in conjugative plasmids with lactose positive and phage resistant phenotypes (Klaenhammer and Sanosky, 1985). It was also shown the integration of FEG genes in a plasmid can increase immunity in a nisin-producer strain (Kim *et al.*, 1998). It has been shown that in some streptococci, nisin resistance was due to a change in the lipoteichoic acid composition of the cell membrane, such is the case of *S. bovis* and not to nisinase (Mantovani and Rusell, 2001). In other bacteria, nisin resistance was due to

activity of proteases secreted by the bacteria. However, test for proteases was not done in this study.

LR10 and LR11 were resistant to nisin, and thus, these strains can be used in combination with a nisin-producer strain to achieve the desired goal in a food system. However, further studies are needed to determine if they can grow together.

### **Bacteriocin-like production**

Six strains were positive for bacteriocin-like production, demonstrated by the zones of inhibition. The substance could inhibit other LAB and *L. monocytogenes* serotype 4b; strains of serotypes 1/2a and 1/2b were shown to be resistant. The serotypes differ in cell wall composition. In serotype 4b, teichoic acid has a unique composition, with both glucose and galactose substituents attached to N-acetylglucosamine in the teichoic acid chain, whereas serotypes 1/2a and 1/2b have rhamnose and N-acetylglucosamine. The rhamnose units in serotype 1/2 are essential for phage attachment. Galactose units replaced or not present in the cell wall of serotype 4b rendered the bacteria insensitive to phage attack and incapable of invading mammalian cells in culture (Kathariou, 2002). However, studies with other bacteria reported no relation between serotype and sensitivity to bacteriocins. Sensitivity was strain dependent, and a large variation in sensitivity to curvacin was observed (Bouttlefroy *et al.*, 2000). *Listeria* isolates of serotype 1/2a, tended to be more resistant to bacteriocins produced by *Lactobacillus sake* than serotype 4b at 4°C; no differences were observed at 37°C (Buncic *et al.*, 2001). This could indicate that serotype 1/2a may have a competitive advantage over serotype 4b in

cold-stored foods with background microflora containing antilisterial-producing LAB (Buncic *et al.*, 2001). Most listeriosis outbreaks have reported to be caused by serotype 4b, followed by serotype 1/2a, and by serotype 1/2b (Doyle, 2001).

Bacteriocins kill bacteria by disruption of the cell membrane and pore formation, dissipating the proton motive force. The type-A lantibiotics act in a voltage dependent manner without the requirement of a specific protein receptor. Recent work has shown that activity of nisin is dependent on the concentration of lipid II in the membrane of sensitive cells (McAuliffe *et al.*, 2001). The class II bacteriocins are thought to interact with membrane receptor proteins prior to insertion into the cytoplasmic membrane in a voltage independent fashion. Further studies are needed to determine the class of bacteriocin produced by the *Lactococcus* strains isolated from MPBRC. However, the genetic analysis and chemical tests point towards nisin, in the case of 3 strains. Nisin is resistant to trypsin and sensitive to proteinase K (Harris *et al.*, 1992). The other strains produced a substance that was resistant to both enzymes (Table 3).

When the *Lactococcus* strains were grown in MRS, there was a zone of inhibition (test microorganism, *Listeria monocytogenes* 4b). In GM17 or CJ (cucumber juice, 2% salt), the zones of inhibition were smaller, almost not noticeable, and no zone was observed when the cells were grown in brine. In comparison to GM17, MRS has 2 times more yeast extract, and is expected to have much more than CJ or brine. When yeast extract (3%) was added to cucumber juice, there was bacteriocin-like production, demonstrated by zones of inhibition comparable in size to the ones obtained with the bacteria were

grown in MRS (13-14 mm in diameter). In addition to that, GM17 has more buffering capacity than MRS, this could also affect bacteriocin detection. Since nisin is more active at low pHs. These results indicate that enrichment of the vegetable broth (cucumber juice) with yeast extract provided the nutrients necessary for bacteriocin production by the strains of *L. lactis*. However, in the real product (MPBRC) these nutrients will be lacking, suggesting that these bacteria may not be able to produce large amounts of bacteriocin in situ. Tests done growing the bacteria in brine, obtained from the product showed no zones of inhibition (data not shown).

It has been shown that bacteriocin production is dependent on the carbon source available and on the nitrogen source (Cheigh *et al.*, 2002). Nisin production was found to be inhibited by an increase in nisin concentration, and when the medium contained excess of nutrients, even though growth of the bacteria continued because nutrient limitation was not operating (Kim *et al.*, 1997). In low nutrient concentration, nisin production was limited by nutrient depletion (Kim *et al.*, 1997). Growth of *L. lactis* in a medium containing galactose or lactose as carbon source induced the nisin promoter (*nisA*) (Chandrapati and O'Sullivan, 1999). Higher bacteriocin activity was detected when *L. lactis* was grown in MRS or in GM17 when yeast extract was added in the medium (Cheigh *et al.*, 2002).

In *L. lactis* isolated from sauerkraut, nisin production was less when the bacteria were grown in cabbage juice, even though the strains grew well in the broth, as determined by the turbidity of the cultures. No nisin was detected in two strains when grown in BHI or

GM17 (Harris *et al.*, 1992). This means that bacteriocin production is affected by environmental conditions and sugar availability, things to be considered when trying to use a culture as biocontrol agent in MPFV. Bacteriocin stability increased or decreased at low levels of complex nutrients or sugar, respectively. In *Enterococcus faecium*, salt-induced stress decreased bacteriocin production, but moderate levels of sodium chloride improved bacteriocin activity (Leroy *et al.*, 2002).

Each food system will be different, and one strain cannot fit or cannot be used in every fruit or vegetable system. Thus, it is important to determine the best conditions for growth and bacteriocin production of the potential biocontrol agent. The brine used in this study contained fructose and glucose as carbon sources, as well as malic acid. Under these conditions, *L. lactis* LA 218 isolated from sauerkraut fermentation used preferentially malic acid to produce lactic acid (Table 4). However, the role of malic acid in bacteriocin production has not been determined.

## Conclusions

- These (LR1, LR10, LR11), isolates could be useful in vegetable fermentations where raffinose, xylose or melibiose is the main source of carbon, such as in legume-based products or vegetable juices. The two strains able to utilize raffinose (LR10 and LR11) were able to grow in wheat grass juice.
- More studies are needed to see if the strains that are bacteriocin producers are able to work as biocontrol agents in this type of product, as the circumstances of how these organisms were isolated may not be the ideal ones when used in the real product: initial salt concentration 4 %, little or no nutrients available for the first 24 hours, low temperatures of storage.
- Further studies are needed to characterize the bacteriocin the *Lactococcus* strains are producing and their antimicrobial activity against different *Listeria* strains and serotypes. Serotypes other than 4b can be frequently isolated from food or food processing plants, additional bacteriocins would be desirable as preservatives in food systems.

**Table 1. Biochemical profile of *L. lactis lactis* strains isolated from minimally processed brined refrigerated cucumbers**

| Strain           | 2% salt | 4% salt | Inulin | Lactose | Raffinose | Xylose | Melibiose | Sucrose |
|------------------|---------|---------|--------|---------|-----------|--------|-----------|---------|
| <i>L. lactis</i> |         |         |        |         |           |        |           |         |
| LR1              | +       | +       | +      | +       | -         | -      | +         | +       |
| LR2              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR3              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR4              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR5              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR6              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR7              | +       | +       | -      | +       | -         | +      | -         | +       |
| LR10             | +       | +       | -      | +       | +         | +      | +         | +       |
| LR11             | +       | +       | -      | +       | +         | +      | +         | +       |
| ATCC 11454       | +       | +       | -      | +       | -         | +      | -         | +       |

Bacteria were grown overnight in Bromcresol Purple broth containing the different sugars. Color change to yellow indicated positive for carbohydrate utilization. Salt tolerance was measured in GM17 with sodium chloride. Positive growth was determined by turbidity of the culture.

**Table 2. Bacteriocin-like activity of *L. lactis lactis* strains isolated from minimally processed brined refrigerated cucumbers**

| Test microorganism                   | LR1 | LR2 | LR4 | LR5 | LR6 | LR7 |
|--------------------------------------|-----|-----|-----|-----|-----|-----|
| <i>L. plantarum</i> (LA70)           | ++  | +   | +   | +   | +   | -   |
| <i>L. plantarum</i> (BI07)           | ++  | +   | +   | ++  | ++  | +   |
| <i>L. mesenteroides</i><br>(LA81)    | ++  | ++  | ++  | ++  | ++  | +   |
| <i>P. pentosaceus</i> (LA61)         | ++  | ++  | ++  | ++  | ++  | +   |
| <i>P. dextranicum</i><br>(LA 77)     | +++ | +++ | +++ | +++ | +++ | +++ |
| <i>L. monocytogenes</i> 4b<br>(B164) | ++  | ++  | ++  | +++ | +++ | +++ |

Diameters of the zone of inhibition: + = 1-2 mm, ++ = 5-7 mm, +++ = 13-15 mm. Bacteria grown overnight in MRS. The diameters of the zones of inhibition were smaller when the bacteria were grown in CJ, GM17. No zones were observed when grown in brine. pH adjusted to 2, 7, 8, and 9. At pH 8 activity was reduced, at 9 no activity. Activity lost after 5 days at 5°C at pH 7 and after 20 days at pH 4.

**Table 3. Factors affecting putative bacteriocin activity of the crude supernatant**

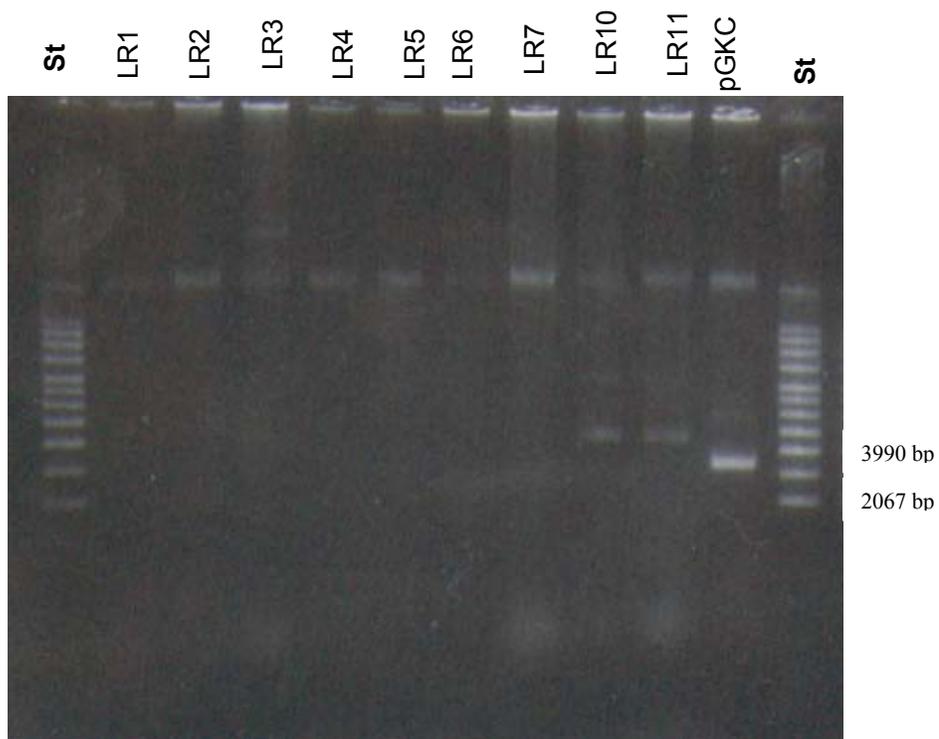
| Bacteriocin-like activity from strain | Broth Treatment (grown in MRS) |          |         |            |
|---------------------------------------|--------------------------------|----------|---------|------------|
|                                       | Heated                         | Unheated | Trypsin | Protease K |
| LR1                                   | +                              | +        | +       | -          |
| LR2                                   | +                              | +        | +       | -          |
| LR4                                   | +                              | +        | +       | -          |
| LR5                                   | +                              | +        | +       | +          |
| LR6                                   | +                              | +        | +       | +          |
| LR7                                   | +                              | +        | +       | +          |
| <i>L. lactis</i> ATCC 11454           | +                              | +        | +       | -          |

Test microorganism *L. monocytogenes* (B164). One mL heated at 100°C for 2 min. Enzyme treatment: 200 µL of broth with 2 µL of enzyme, placed at 37°C for 3 hours. Ten µL were spotted onto TSG agar, allowed to dry and overlaid with the test microorganism. + (zone of inhibition); - no zone.

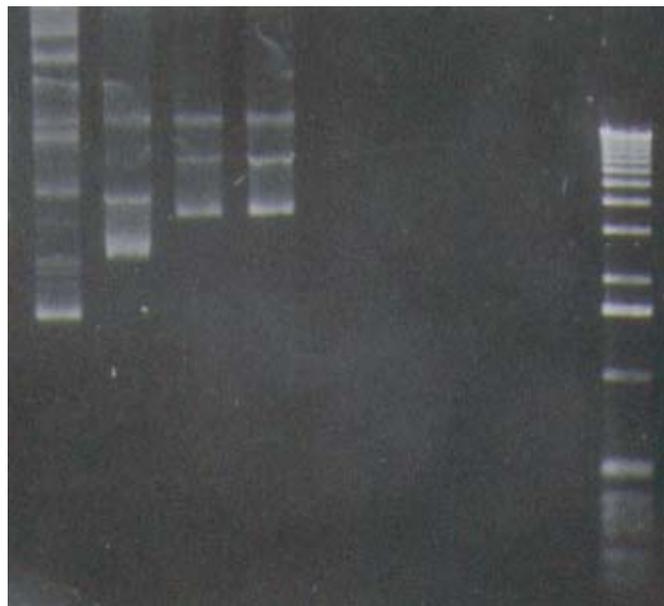
**Table 4. Fermentation pattern of *L. lactis* sps *lactis* (LA218) in different media**

| Medium             | Glucose (mM) | Fructose (mM) | Malic ac (mM) | Lactic Ac (mM) | Acetic Acid (mM) |
|--------------------|--------------|---------------|---------------|----------------|------------------|
| Uninoculated GM17  | 37           | 13            | 0             | 1              | 0                |
| Inoculated GM17    | 1.3          | 0             | 0             | 98             | 0                |
| Uninoculated Brine | 19           | 25            | 13            | 0              | 0                |
| Inoculated Brine   | 12           | 23            | 0             | 30             | 0                |
| Uninoculated MRS   | 85           | 13.4          | 0             | 2.9            | 79.4             |
| Inoculated MRS     | 44           | 5.1           | 0             | 65             | 72               |

*L. lactis* isolated from sauerkraut (LA218). Different media inoculated with 1% of overnight culture and incubated at 30°C. Biochemical profile obtained by HPLC. Numbers are the average of 2 samples.



**Fig 1a. St is supercoiled DNA ladder. Plasmid Profiles of *L. lactis* strains isolated from MPBRC**



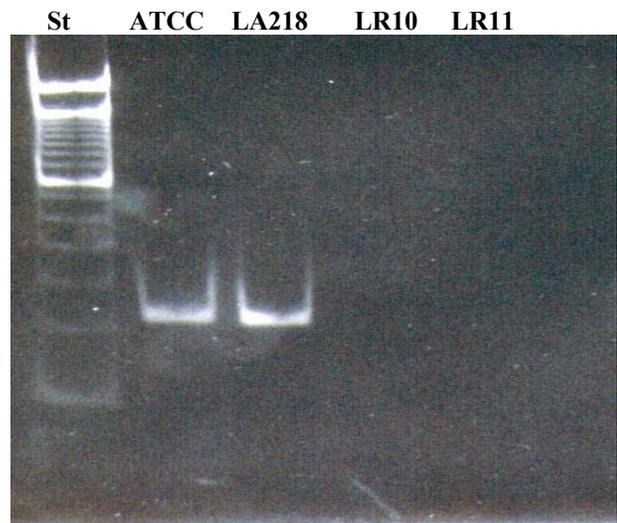
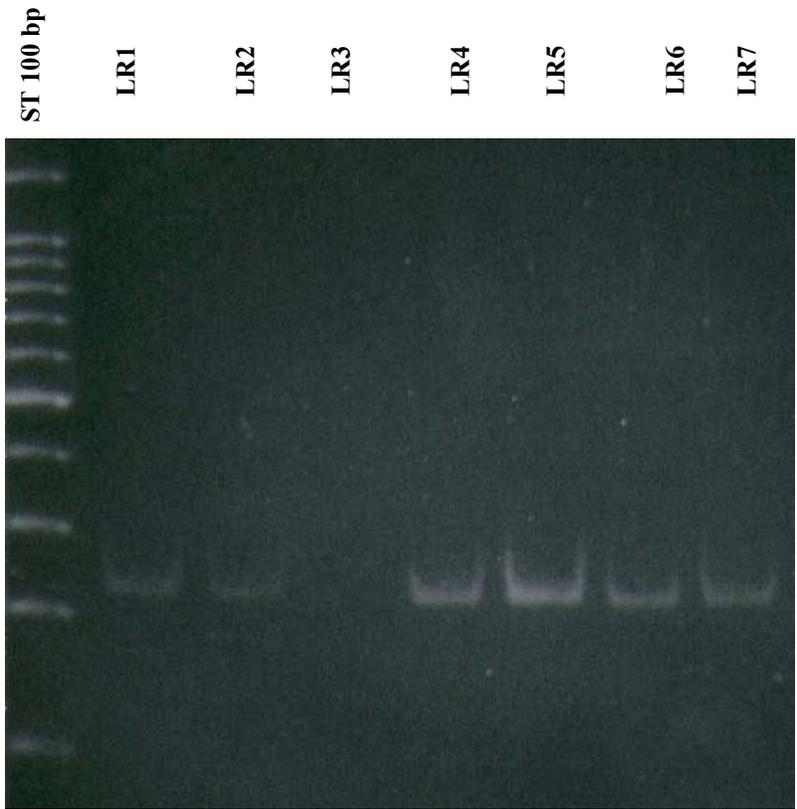
**Fig 1b. Plasmid profiles: 1-*L. lactis* ATCC11454, 2-pGKC, 3-LR10, 4LR11, St 1Kb**

**Fig 2. PCR profile of different *L. lactis* isolates from MPBRC**



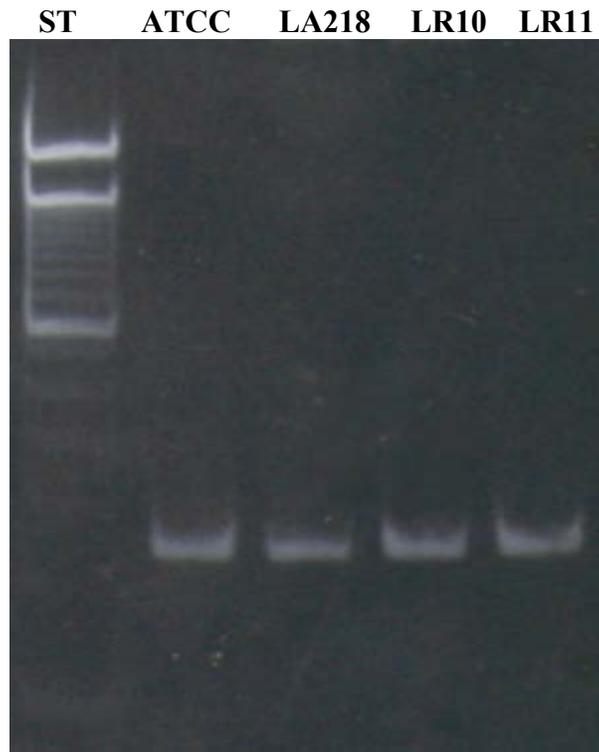
From left to right: standard 1KB, LR1,LR2, LR3, LR4, LR5, LR6, LR7, LR10, LR11, *L. lactis*

**Fig 3a. PCR detection of *nis* ABTCI genes**



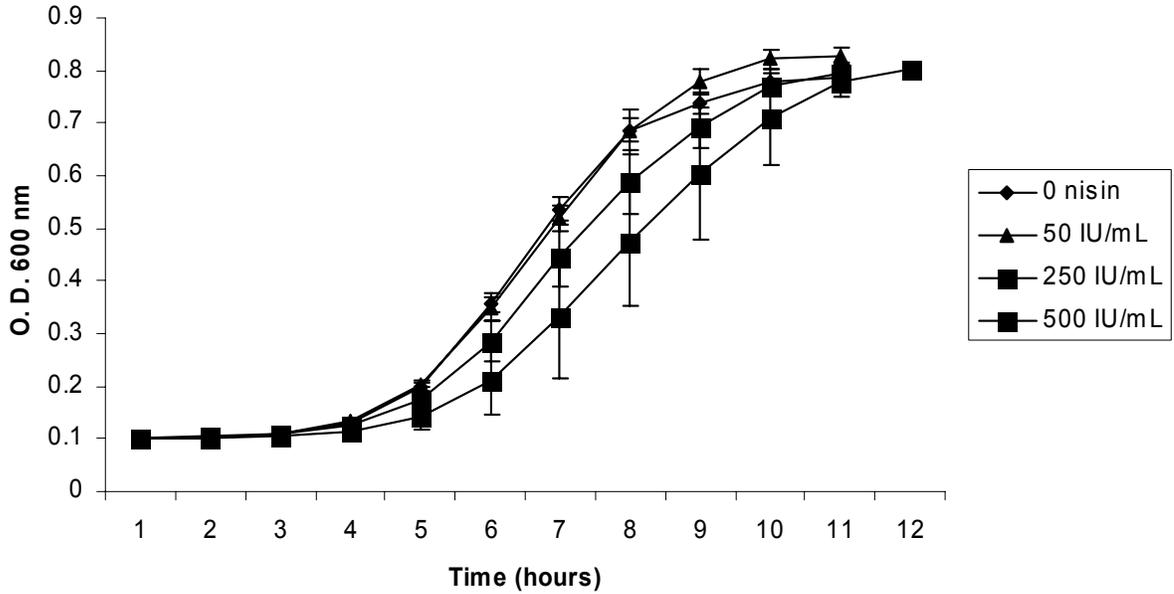
St 100 bp, Positive controls (*L. lactis* ATCC 11454), *L. lactis* 218, LR10, LR11

**Fig 3b. PCR detection of the *nis* FEG**



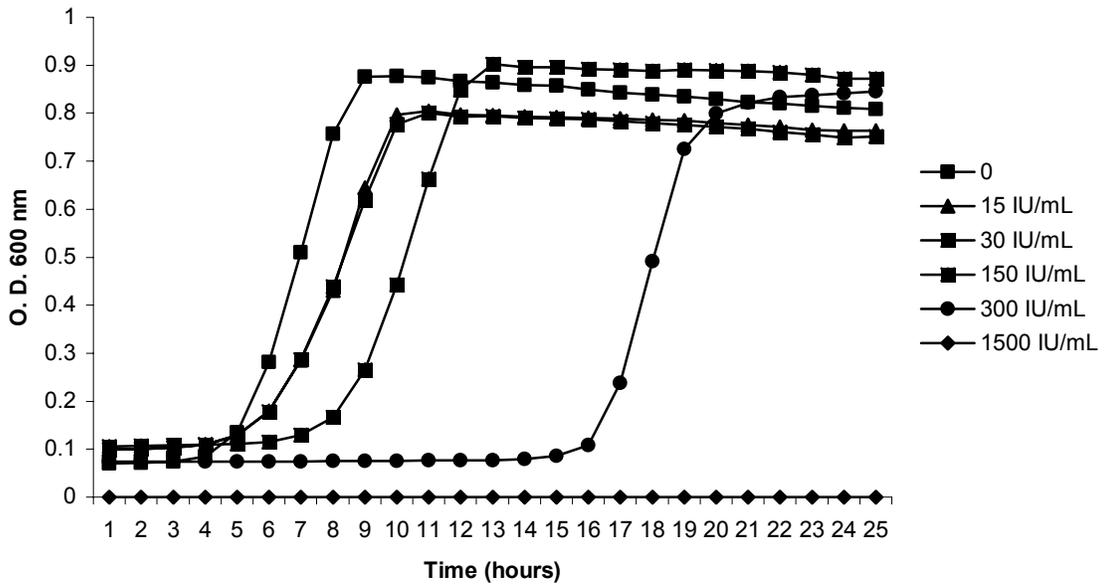
From left to right: Standard 100 bp, ATCC 11454, LA218, LR10, LR11

Fig 4a. Nisin resistance of LR1



This strain showed bacteriocin-like activity. Numbers are the average of 4 wells of a microtiter plate.

Fig 4b. Nisin resistance of LR10, *L. lactis*



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## **CHAPTER V**

### **Biocontrol of *Listeria monocytogenes* by LAB in Minimally Processed Brined Refrigerated Cucumbers**

## Abstract

Nisin-producing *Lactococcus lactis* or *Lactobacillus curvatus* was inoculated into Minimally Processed Brined Refrigerated Cucumbers (MPBRC) at a concentration of  $10^6$  CFU/mL as a biocontrol agent of *Listeria monocytogenes*. The product was prepared with washed unblanched or blanched cucumbers containing 5 g of irradiated spices and 1 g of garlic aquaresin. It was stored at 5°C for 3 weeks, and then transferred to 18°C (abuse temperature) for 10 days. *L. lactis* counts decreased by 2-3 log cycles at 5 °C, whereas *L. curvatus* counts decreased by less than 1 log under these conditions. *Listeria* survived at 5°C during the period of storage, even in the presence of the biocontrol agent; and when the jars were transferred to 18°C, *Listeria* grew in the absence of the biocontrol agent or the natural LAB. The biocontrol agent reached  $1 \times 10^8$  CFU/mL. *L. monocytogenes* counts decreased by 4-5 log cycles after 48 hours at 18°C in the jars containing *Lactococcus*. In the case of *L. curvatus*, *Listeria* counts decreased slowly to undetectable levels after 7 days. These results indicated that *Listeria* died faster in the jars containing *L. lactis* due to one or more factors, such as low pH, bacterial competition, and nisin produced by *Lactococcus*.

Blanching the cucumbers to reduce the initial microflora helped in *Lactococcus* survival, *L. curvatus* was not affected by the natural LAB. When nisin was added into the product to inhibit the natural LAB, *Lactococcus* survived better at 5°C and at 18°C, than when not added. However, other nisin resistant LAB also grew in the product and lowered the pH.

Key words: biocontrol agent, minimally-processed vegetables, lactic acid bacteria.

## Introduction

The increased demand of fresh fruits and vegetables in the U.S. has been accompanied by an increase in the number of food-borne outbreaks due to their consumption (Beuchat, 1997). Fruits and vegetables carry a natural non-pathogenic microflora, and perhaps human pathogens, depending upon growing conditions. However, during growth, harvest, transportation and further processing and handling, the produce can be more highly contaminated with pathogens from human or animal sources. Microbial background is not eliminated from the fruits or vegetables during washing, and sometimes can increase during processing. Fresh produce has been implicated in a number of outbreaks caused by bacteria, viruses, and parasites. Bacterial pathogens include *Salmonella*, *Shigella*, *E. coli* 0157H7, *L. monocytogenes*, and *Campylobacter* (De Roever, 1998). The major safety concern of refrigerated ready-to-eat products is the possible outgrowth of psychrotrophic pathogens, such as *Listeria monocytogenes* (Kelly *et al.*, 1998) and mesophilic pathogens at abuse temperatures.

The development of novel food technologies and consumers' search for natural products have directed attention to the use of LAB as biocontrol agents for minimally processed fruits and vegetables (MPFV) (Moreno *et al.*, 1999; Breidt and Fleming, 1997). LAB are able to produce a number of inhibitors in addition to bacteriocins, such as organic acids, diacetyl, hydrogen peroxide, enzymes, and lytic agents (Klaenhammer, 1993). LAB are considered GRAS, and have been used for a long time in fermented food. The use of LAB in vegetable broths has been shown to inhibit the growth of different pathogens at

different temperatures. Results were different when the LAB were applied to the real vegetable product (Bennik *et al.*, 1999, Palmai and Buchanan, 2002). *L. monocytogenes* was inhibited by *L. lactis* during sprouting of alfalfa seeds, but this inhibition was lower than the one obtained in a sterile broth model system of sprouts. This may be the result of inhibitory activity associated with the microflora of the sprouting seeds against the inoculated culture (Palmai and Buchanan, 2002). Breidt and Fleming (1998) studied the effect of *L. lactis* on *L. monocytogenes* in cucumber juice, and found that *L. lactis* inhibited the growth of *L. monocytogenes* at 10°C. However, Wessels and Huss (1996) inoculated salmon slices with *L. lactis* and *L. monocytogenes*, and found that the culture was not suitable as a biocontrol agent at 5°C due to the effect of salt and low temperatures on the culture. They also found, however, that this culture could work at abuse temperatures. Babji and Murphy (2000) reported that meat inoculated with *L. lactis* or *L. plantarum* at concentrations of  $1 \times 10^7$  CFU/g went down 4 log cycles at 7 and 18 days at 4°C, and other LAB predominated spoilage of the meat. It seems that a bacteriocin-producer is less efficient in a food system than under optimal laboratory conditions.

*L. curvatus* was isolated from spoiled fish products at 3 and 8°C, and represented the second largest group responsible of spoilage (Lyhs *et al.*, 2002). It was also isolated from vacuum-packaged beef (Yost and Nattress, 2002), and from raw goat's milk and artisanal (home made) cheeses (Casla *et al.*, 1996). In addition, together with *L. sake*, *L. curvatus* dominates the fermentation of sausage; it develops during the initiation stage of fermentation and does not change during the process. These two strains were originally

isolated from sauerkraut fermentation and used in sausage fermentation (Vogel *et al.*, 1993). The strains were able to outnumber the meat-borne flora, and dominated the fermentation. *L. curvatus*, curvacin-producer has been used as a biocontrol agent in sausage and other fermented meats. It was successful in inhibiting *L. monocytogenes* and *E. coli* O157H7. The inhibitory effect was due to the bacteriocin produced and the rapid growth of the strain in the meat system, lowering the pH, thus inhibiting the Gram-negative pathogen (Leroy *et al.*, 2001).

A deli-style pickle product (MPBRC) was chosen as a model of MPFV. It is a ready-to-eat, highly perishable pickle product containing fresh cucumbers, garlic, spices and about 2% brine after equilibration. It is non-acidified, and relies on refrigeration and sanitation to ensure quality and safety of the product (Etchells and Moore, 1976). *L. monocytogenes* was able to grow in this product stored at 5°C, when LAB were in low numbers. *Listeria* growth was inhibited by the natural LAB or by the biocontrol agent added into the product (Romick, 1994).

The objectives of this research were:

- To study the effect of a nisin-producing *L. lactis* as a biocontrol agent or *L. curvatus* non-bacteriocin-producer in MPBRC as a model of minimally-processed vegetables in order to determine how the natural microflora and *L. monocytogenes* are affected by the inoculated bacteria.

- To study the effect of blanching of cucumbers to reduce the natural microflora on the survival of the inoculated biocontrol agent.
- To study the effect of nisin in combination with *L. lactis* on the growth of competitive microflora (natural LAB) and on the survival of *L. monocytogenes* in MPBRC prepared with unblanched cucumbers.

## **Materials and Methods**

### **Deli-type Pickle product (MPBRC)**

Cucumbers (size 2 B, 3.5-3.8 cm diameter, 680±5 g) were washed and blanched for 15 seconds at 80°C (Breidt *et al.*, 2000), then immediately placed in 46 ounces glass jars and cooled with 680± 5 g sterile 4% cold brine containing 1 g of sterile aquaresin garlic (Kalsec, Kalamazoo, MI) and 5 g of irradiated pickling spices (13 KGy) (Ba-Tampte Pickle Products, Brooklyn, NY) of undefined composition due to proprietary rights of the company. Jars were sealed with caps containing septa and placed at 5°C after inoculation with *L. lactis lactis* (LA218) or *L. curvatus* (LR53).

### **Inoculum preparation**

*L. lactis* (LA218, USDA-ARS Culture collection, Raleigh, NC), isolated from sauerkraut fermentation by Harris *et al.*, 1992, was transformed with plasmid pGKC (Breidt and

Fleming, 1992). The culture was grown in M17 (Difco, Detroit, MI) broth containing 1% glucose, and 5 mM GB (glycine betaine) (Aldrich Chemical Company, Inc., Milwaukee, WI), 4% salt (NaCl) and 5 µg/mL of chloramphenicol at 20±1°C. The cells were centrifuged and resuspended in cold GM17 containing salt and GB, and placed at 5°C for 2 hours before their inoculation into the product. *L. curvatus* (LR53, USDA-ARS Culture collection, Raleigh, NC) was isolated from MPBRC; it does not produce bacteriocins. The culture was grown in MRS (Difco, Detroit, MI) broth and transferred to cucumber juice containing 4% salt and grown at 20±1°C.

A control was prepared without GB, salt or cold shock. Jars were sampled periodically by syringe (Becton Dickinson, Portland, CT) to obtain 1 mL samples for chemical and microbiological analyses. Samples were appropriately diluted in sterile saline solution (0.85%) and plated by spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) on MRS with chloramphenicol (5 µg/ml) (Difco, Detroit, MI) agar and incubated at 30°C for 48 hours under anaerobic conditions to enumerate *L. lactis*. Samples were plated on MRS agar with 2% salt to enumerate *L. curvatus*; plates were incubated in anaerobic jars. Experiments were done in triplicate. Total aerobic count was done on PCA agar (Difco, Detroit, MI), Total *Enterobacteriaceae* on VRB agar with 1% glucose (Difco, Detroit, MI), and LAB were enumerated on MMRS (MRS with 0.02% sodium azide) (Difco, Detroit, MI). Plates were incubated aerobically at 30°C for 24 and 48 hours and counted with a colony counter (Protos Plus, Bioscience International, Rockville, MD).

### **Biocontrol of *L. monocytogenes***

*L. monocytogenes* (B164, USDA-ARS Culture collection, Raleigh, NC), which carries an erythromycin resistant marker, was used for these studies (Romick, 1994). *Listeria* was grown at 37°C in BHI containing 5 µg/mL erythromycin, 1% of the inoculum was transferred into cucumber juice containing 2% salt and grown for 12 hours. Cells were centrifuged and resuspended in cucumber juice and diluted with 0.85% sterile saline solution to get a final concentration of  $1 \times 10^5$  CFU/ml in the jars. A total of 10 jars were inoculated with *Listeria*; 5 jars contained *Listeria* and *Lactococcus* ( $1 \times 10^6$  CFU/mL), or *L. curvatus* ( $1 \times 10^6$  CFU/mL), and 5 contained only *Listeria*. To obtain *Listeria* counts, samples were plated by spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) onto Modified Oxford agar (MOX) (Difco, Detroit, MI) containing 5 µg/mL of erythromycin and the antibiotic supplement containing 10 mg of colistin sulfate and 20 mg of moxalactam (Difco, Sparks, MD). Plates were incubated at 37°C for 24 hours.

To make sure that only *Listeria* or *Lactococcus* were growing on the selective media, jars without inoculation were included in each experiment and plated onto MMOX em and MRS cm. Colonies were picked periodically from the different media and re-streaked for isolation on non-selective medium; after that, they were streaked onto selective media. Microscopic observation, and catalase and oxidase tests were performed.

*Lactococcus* presence was verified by PCR fingerprinting following the procedure described by Cusick and O'Sullivan 2000, with one annealing temperature (40°C). This method uses a single arbitrarily primer at low stringency annealing conditions, which allow it to bind to genomic DNA at places to which has partial or full homology, giving a

PCR product formation if two sites are within a few thousand base pairs of one another and are on opposite DNA strands. The primer used was an 18-mer that had homology to a universally conserved region of the 16S rRNA gene:

5'-CAGCAGCCGCGGTAATWC-3' that contains a degenerate base W. PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA). PCR parameters were as follows:

92°C: 2 min, 40 cycles of: 92°C for 30 seconds, 40°C 1 minute, 68°C 2 minute, final 68°C 8 minutes.

### **Nisin determination**

Nisin was qualitatively measured with the lawn test (Tramer and Fowler, 1965) with *L. cremoris* as the sensitive strain. Samples of brine were centrifuged, and the supernatant transferred to a new tube, filter sterilized and boiled for 1 minute. Five µl of this brine was spotted onto MRS agar (1.5%) and allowed to dry. Ten µl of the sensitive strain (*L. cremoris*) was inoculated into 3 mL of MRS (0.75%) agar, mixed and overlaid on MRS agar. Plates were incubated at 30°C for 24 hours. A positive test was a clear zone of inhibition. Results were reported as + / -.

### **Effect of nisin on the survival of *L. monocytogenes***

Nisin in 2 different concentrations (50 IU/mL and 1000 IU/mL) was added to jars (4) containing washed, unblanched cucumbers (size 2B) and both cultures (*Listeria* and *Lactococcus*) at concentrations of 10<sup>5</sup> CFU/mL. Control jars were also prepared containing only *Lactococcus* or *Listeria*. Control jars without bacteria were also included

to determine the natural LAB growing in the product. Jars containing only nisin, 50 IU/mL, were also included to determine the effect of nisin on the natural LAB.

The strains to be inoculated were grown as indicated previously in the material and methods section. The jars were incubated at 5°C for 3 weeks and transferred to 18°C for 5 days. Bacteria were enumerated on MMOX agar plus erythromycin (5 µg/mL), on MRS agar with chloramphenicol (5µg/mL), and on MMRS agar (0.02% sodium azide).

Nisin was obtained from NISAPLIN (Applen & Barret Ltd. Trowbridge, Wilts., England) and prepared by diluting the powder into HCl 0.02 N, filter sterilized and frozen at -20°C until use.

### **Statistical analysis**

The analysis of variance was computed by the General Linear Models Procedure and Tukey's Multiple Comparison Test of SAS version 8.0 (SAS Inc., Cary, NC).

## Results and Discussion

### Survival of *L. lactis lactis* and *L. curvatus* in MPBRC at 5°C

The survival of *L. lactis* (LA218) in MPBRC was improved by growing the strain in GM17 containing 5mM GB (glycine betaine) and salt (O'Collagen and Condon, 2000, Ugen *et al.*, 1999). Untreated *L. lactis* (grown without salt or GB) inoculated into MPBRC decreased in numbers by 99.9% after 2 days of inoculation at 5°C and no cells were detected after a week of storage (data not shown). The initial decrease in cell numbers (Fig. 1a) could be due to other factors in addition to salt concentration: the lack of nutrients in the brine, and the period of time it takes for the diffusion of nutrients from the cucumber to the brine, the rate varying with temperature, being slower at low temperatures. It takes 150 hours for the solutes to approach equilibrium with the cover brine (Fasina *et al.*, 2002).

Cucumbers were blanched to reduce the natural microflora to allow the *Lactococcus* to succeed. However, *Lactococcus* were affected by the natural LAB regardless on whether the cucumbers were blanched or unblanched (data not shown). LAB able to grow at 5°C grew slowly and decreased the pH. This fact could be the major factor for the inhibition of *Lactococcus*. Ideally the reduction of the initial microflora will be an asset to allow the biocontrol to succeed (Breidt and Fleming, 1997). However, some bacteria are protected within the structures of the fruit, and are able to grow under the appropriate conditions. In reality, there is no method available to reduce the natural microflora below to 10. Gamma radiation has been used to decontaminate cucumber fruit. Studies done on the effect of

radiation on the natural microflora suggested that bacterial spores are more resistant than non-sporeforming bacteria. No organisms were recovered after treatment with high doses of radiation (3.0 megareps), but the quality of the cucumbers was affected by the loss of 60% of their initial firmness (Etchells *et al.*, 1961). Blanching cucumbers at 77°C for 3 minutes allowed the fermentation of cucumbers by the starter added without the addition of salt (Fleming *et al.*, 1994).

Micro-encapsulation of the *Lactococcus* with a material sensitive to high temperatures could be the ideal method to ensure its survival at low temperatures. With the increase in temperature, the cells would be released and grow at abuse temperatures.

In the case of *L. curvatus* (LR53), the counts decreased slightly at the beginning of the study but they remained the same during the storage period at 5 °C and started growing after 15 days (Fig. 1b). *L. curvatus* was able to survive and grow slowly to a level of 10<sup>6</sup> CFU/mL at 15 days when the product was prepared with unblanched cucumbers, demonstrated by the bacterial counts compared to the control jars that did not have any culture added. Perhaps this strain is more salt and pH tolerant than *L. lactis*. The fact that the bacteria were isolated from the product can be an advantage, as they were already adapted to live or grow in that environment. *L. curvatus* has been isolated from meat fermentation, where the salt concentration varies between 3-6% (Vogel *et al.*, 1993).

### **Behavior of *L. lactis* and *L. curvatus* MPBRC at 18°C**

Bacterial growth in the product noticed by turbidity at abuse temperatures was due to the growth of *L. lactis* or *L. curvatus* or the natural LAB from the cucumbers that were protected from blanching and could tolerate garlic, salt and cold temperatures of storage. The presence of LAB in the cucumbers varied with the cucumber source and the season of harvesting. When the bacterial load was low, indicated by the Total aerobic count ( $10^{3-4}$  CFU/g) before blanching, the non-inoculated jars showed no growth even after 6 months of storage at 5°C. When the Total aerobic count in the cucumbers was  $10^{6-7}$  CFU/g before blanching, natural LAB grew after 3 weeks of storage at 5°C (data not shown) and dominated the fermentation at abuse temperatures. However, LAB presence in cucumbers cannot be ensured. Therefore, it is important to add a biocontrol agent in MPBRC to ensure the safety of this product. *L. monocytogenes* was able to grow at different temperatures in this product when LAB were not present (Romick 1994).

Even though the *Lactococcus* counts were low at 5°C, when the inoculated jars were transferred to 18°C, the numbers of *L. lactis* reached  $1 \times 10^8$  CFU/mL in some jars (Fig. 2a). However, the numbers were not the same in all jars. The variability in the *Lactococcus* counts could be due to the presence of other LAB in the product that had higher growth rates at low temperatures, taking over the fermentation at abuse temperatures, demonstrated by the presence of colonies of different morphology on MMRS agar. LAB are also known to produce inhibitory substances, such as ethanol, CO<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub>, organic acids and bacteriocins, which could be inhibiting the

*Lactococcus*. Therefore, a blanching step is essential to achieve the growth of the biocontrol agent in the case of *L. lactis*.

In the case of *L. curvatus*, the numbers reached  $10^8$  CFU/mL after 24 hours at 18°C. However, other LAB could also be counted on MMRS plates, as shown by the different morphology of the colonies growing on the plates. However, they did not affect the number of *L. curvatus* (Fig. 2b). This strain was more stable in the MPBRC, and it was not affected by the background microflora. Perhaps they are more acid tolerant than *Lactococcus*. *L. curvatus* has been isolated from sausage, where the initial pH of the meat is around 5.5-5.9, similar to the one found in MPBRC; and it grew well at pHs 4.8-5 (Leroy *et al.*, 2001).

### **Biocontrol of *L. monocytogenes* in MPBRC**

Cucumbers used in this experiment came with low numbers of bacteria, as seen in the control jars with no inoculation (data not shown). *L. lactis* counts went down 3 log cycles (from  $10^7$  to  $10^4$  CFU/mL) during the period of storage at 5°C. *L. monocytogenes* remained the same for 5 weeks at 5°C (Fig. 3a). Even though *Listeria* survived, population levels did not increase. This could be due to the addition of garlic in the product. Garlic is an inhibitor of important enzymes, and growth of *Listeria* at low temperatures depends on the change of fatty acid (FA) composition of the cell membrane, done by de novo synthesis of FA via FA-synthetase (Mastroniculis *et al.*, 1998).

In the control jars with no *Lactococcus* or *Listeria*, the counts for total aerobes and total *Enterobacteriaceae* were below the limit of detection by spiral plater (<400 CFU/ mL); the

same was observed for the LAB counts. Two jars developed turbidity after 2 weeks of storage at 18°C due to the presence of natural LAB (data not shown).

In the control jars containing only *Listeria* (Fig. 3b), *Listeria* grew almost one log cycle, but started to decrease after 8 days, when the natural LAB grew. In one jar that contained only *Listeria*, turbidity was due to LAB growth at 5°C. *Listeria* counts went down 5 log cycles at this temperature. At 18°C *Lactococcus* reached  $1 \times 10^8$  CFU/mL after 48 hours, and *Listeria* counts were undetectable after that period. Nisin was detected in all the jars (data not shown). The rapid decrease in *Listeria* counts could be due to a synergistic interaction between nisin and garlic in the product. It was demonstrated that fresh garlic and nisin can be more bactericidal against *L. monocytogenes* when added in combination than separately (Singh, *et. al.*, 2001). The mechanism of interaction of garlic and nisin is not known, but nisin acts on the cell membrane making pores on it, and perhaps allowing the compounds from garlic to diffuse freely and faster into the cell. Once inside the cell, these compounds bind important enzymes, causing bacterial death.

Two jars that contained only *Listeria* showed turbidity, and LAB were responsible for the turbidity, as demonstrated by the LAB ( $10^8$  CFU/mL) counts. *Listeria* counts were below the limit of detection with spiral plater. The pH of those jars was 3.5. In the other jars, *Listeria* counts decreased slowly at 18°C, demonstrating that the conditions under which MPBRC were prepared and the presence of garlic did not support the growth of *Listeria* either at refrigeration storage or at abuse temperatures. Kumar and Berwal (1998) showed that garlic was inhibitory for *L. monocytogenes*. In case of the natural LAB, the presence of garlic

increased their growth rate (this thesis, chapter II, Fig. 2c). However, it is risky to rely on the natural LAB for the safety of this product.

When *L. curvatus* was used as biocontrol agent of *L. monocytogenes*, the biocontrol agent was responsible for turbidity at abuse temperatures, but *Listeria* counts decreased more slowly than with *L. lactis* (data not shown). This could indicate that the nisin produced by the *Lactococcus* could have potentiated the effect of the competition and the lowering of pH.

In vitro studies done in sterile cucumber juice containing 4% salt, *Listeria* survived at 5°C and grew at 20°C when the tubes were transferred to abuse temperatures (control tubes). In the tubes containing *L. curvatus* and *L. monocytogenes*, the biocontrol agent grew faster, and *Listeria* counts went down slowly to below the limit of detection with spiral plater after 10 days. When the cultures were inoculated in cucumber juice at 20°C in 2% salt cucumber juice, *Listeria* died faster (72 hours) (Table 1). This indicates that pre-adaptation at 5°C and high salt environment could have made *Listeria* stronger to resist low pHs. This was also demonstrated by other investigators. *Listeria* pre-adapted to low pHs can survive better in other stress environments (Hill, 2002). In this case, pre-adaptation of *Listeria* to low temperatures and 4% salt improved survival of *Listeria* in low pH environments. The pH of the cucumber juice was 3.81. It was demonstrated that exposure of *L. monocytogenes* LO28 to pH 5 enhanced its survival at pH 3.5 (Hill *et al.*, 1995). The initial pH of the product was 5.5, which could be low enough to induce ATR (Acid Tolerance Response) in *Listeria*. It is also possible that salt stress induced other stress responses. Proteins induced by one stress would be capable of protecting cells

against other environmental challenges, including thermal stress, osmotic stress, and to the action of surface active agents, such as crystal violet (Hill *et al.*, 1995).

In *L. monocytogenes*, sigma B ( $\sigma^B$ ) factor stimulates expression of numerous genes under environmental stressed conditions, and upon entry into stationary phase (Ferreira *et al.*, 2001).  $\sigma^B$  is stimulated by high osmolarity, and is necessary for the efficient uptake of osmoprotectants (carnitine and betaine), which are also cryoprotectants (Becker *et al.*, 2000) and play a role in the ability of the bacteria to grow at low temperatures. However, betaine is more effective than carnitine in providing tolerance to low temperatures. Among the salt stress-induced proteins identified in *L. monocytogenes*, 4 proteins are over expressed after cold shock in other bacteria (DnaK, CysK, CcpA, and Gap) (Duche *et al.*, 2002).

In MPBRC *Listeria* encounters many preservation obstacles: high salt concentration (4%), lack of nutrients in the brine, garlic which has antibacterial properties (Ross *et al.*, 2001), and low temperature. *Listeria* needs to adapt to this environment either by taking up the substances from the medium required for osmo- and cryo-protection or by de novo biosynthesis of these compounds. In both cases, *Listeria* will not be able to accumulate those substances required to maintain physiological processes. However, in this study *Listeria* was added into the brine. In real life, *Listeria* will become attached to the fruit surface. Therefore, it is advisable to see if other methods of contamination of the fruit with *Listeria* will have the same effect when preparing brined refrigerated cucumbers.

One approach could be immersing the cucumbers in a solution containing *Listeria*, allowed them to dry, and proceed with washing, blanching and brining.

### **Effect of nisin added on the growth of *L. monocytogenes* in MPRC**

Two concentrations of nisin (50 IU/mL and 1000 IU/mL) in addition to *L. lactis* were added into the jars. The addition of nisin may inhibit the growth of natural LAB present in the cucumbers, and at the same time allow the *Lactococcus* to survive. Being a nisin- producer, *Lactococcus* will also be immune to nisin. According to the results (Fig. 4), the bacteria survived in the jars during storage at 5°C. However, in the jars that contained more nisin (1000 IU/mL) the *Lactococcus* were enumerated 2 logs lower than the jars containing 50 IU/mL (significantly different,  $P \leq 0.05$ ). It is important to determine the ideal concentration of nisin to be used without affecting the biocontrol agent.

Survival of *Listeria* in MPBRC was also affected by the presence of high concentrations of nisin in the jars ( $P \leq 0.05$ ). There was no difference in *Listeria* death rate when the other treatments were compared by Tukey's Multiple Comparison Test (Fig. 5).

In home-made tofu, *L. monocytogenes* numbers decreased by 2 log cycles at 10°C in the presence of 100 IU/mL nisin, and after 48 hours started to grow, but counts were below the limit of detection after 24 hours, when the nisin concentration was 2000 IU/mL. Less nisin (700 IU/mL) was required to achieve the same effect when starter cultures resistant to nisin were added into the tofu (Schillinger *et al.*, 2001).

In this study, *Listeria* did not grow at 5°C nor at 18°C in MPBRC, when cucumbers were unblanched. At abuse temperatures the population decreased drastically regardless of the

treatments. This could be due to a combination of factors, such as, salt, garlic, and the natural LAB growing in the jars, which acted synergistically to inhibit *Listeria* (Fig. 6). In the previous study, cucumbers were blanched and *Listeria* grew 1 log cycle at 18°C; they declined when LAB started growing. LAB are known to produce metabolites that inhibit other bacteria (Klaenhammer, 1993).

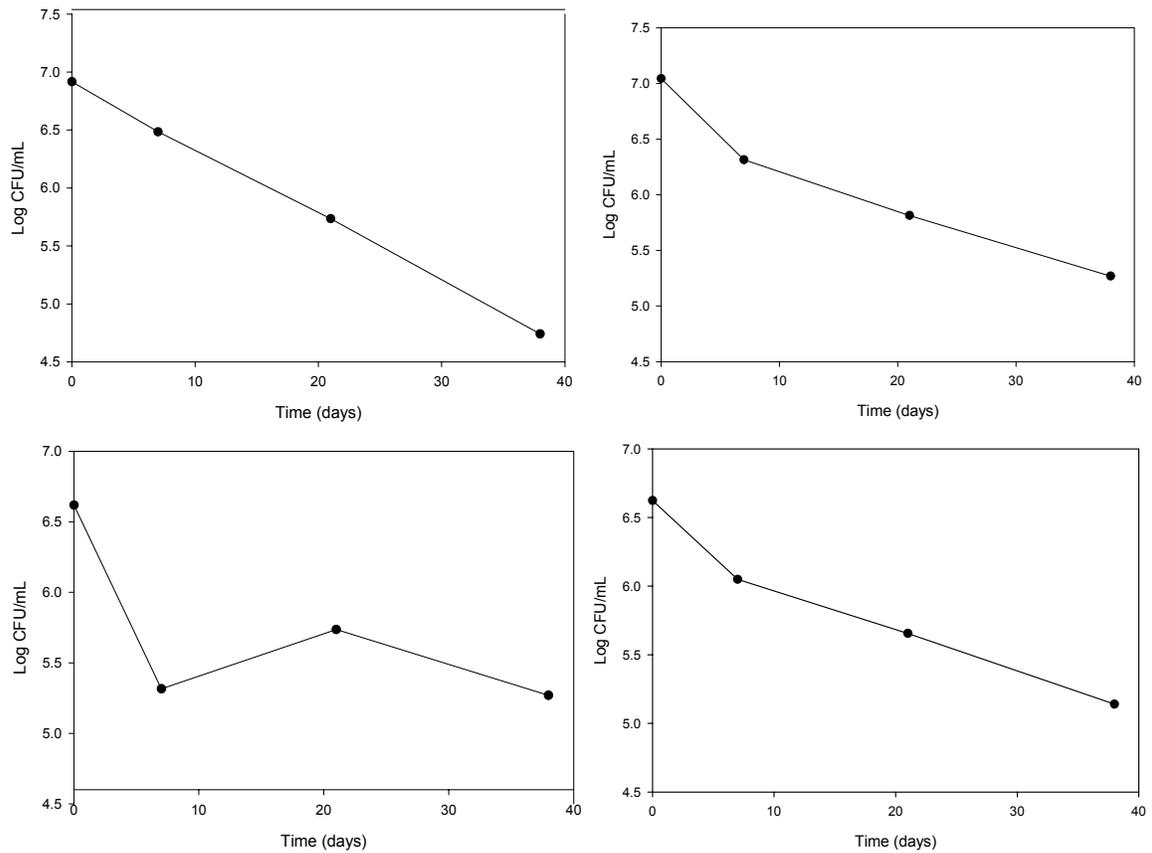
LAB naturally resistant to nisin grew on MMRS agar. Colonies were uniform, compared to the LAB growing in the jars used as controls, where 3 types of colonies were observed. There was a significant effect on the growth of LAB due to the presence of nisin ( $P \leq 0.05$ ) compared to the control jars with no nisin (Fig. 7). Finding natural LAB resistant to nisin is not unusual; nisin-resistant *Leuconostoc mesenteroides* was isolated from sauerkraut fermentation and was used to inhibit the homofermentative bacteria in sauerkraut fermentation (Harris *et al.*, 1992). Nisin proved to be effective in reducing *L. monocytogenes* in yogurt without affecting the yogurt starter cultures used (Benkerroum *et al.*, 2003).

## Conclusions

- Even though *Lactococcus* was not found in high numbers in all the samples of MPBRC, they grew faster than the pathogen at abuse temperatures. However, natural LAB present in the product and growing at 5°C inhibited *Listeria*.
- Other methods of inoculation of the bacteria should be tested to be able to use *Lactococcus* as a biocontrol agent.
- Blanching to reduce bacterial load in cucumbers and the use of sterile irradiated spices and garlic oil are a good approach to ensure the growth of the biocontrol agent (*L. lactis*) in the product in case of temperature abuse. However, *L. curvatus* behaved the same when the product was prepared with blanched or unblanched cucumbers.
- The addition of nisin (50 IU/mL) to MPBRC prepared with unblanched cucumbers, allowed the *Lactococcus* to survive at 5°C and succeed at 18°C. Then, *L. lactis* can be used as a biocontrol agent of *L. monocytogenes* in MPBRC when the product is prepared with nisin and unblanched cucumbers or with blanched cucumbers.
- The approach of brining fruits and vegetables and keeping them under refrigeration to preserve fresh-like quality is useful because LAB were the population selected and succeeding by this method. The presence of natural LAB in the product with no biocontrol agent can be considered safe because the pH was 3.0-3.2 due to LAB growth.

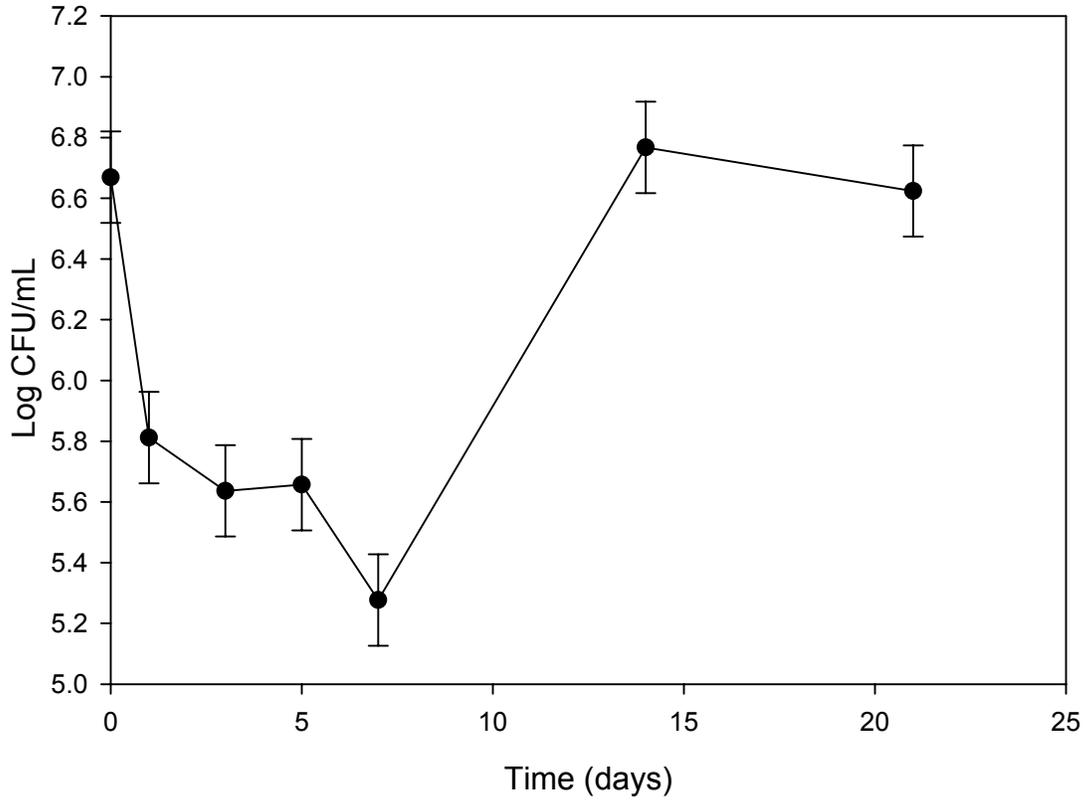
- The use of a biocontrol agent able to produce a bacteriocin will be the best approach. As seen in these results, adding more hurdles is better than relying only on bacterial competition and the decrease in pH.
- *L. curvatus* could be a suitable biocontrol agent for this type of product as demonstrated by its salt and acid tolerance. It was isolated from the product. However, making it able to produce bacteriocin would be a plus.

Figure 1a. Survival of *L. lactis* (LA218) at 5°C in minimally processed brined refrigerated cucumbers



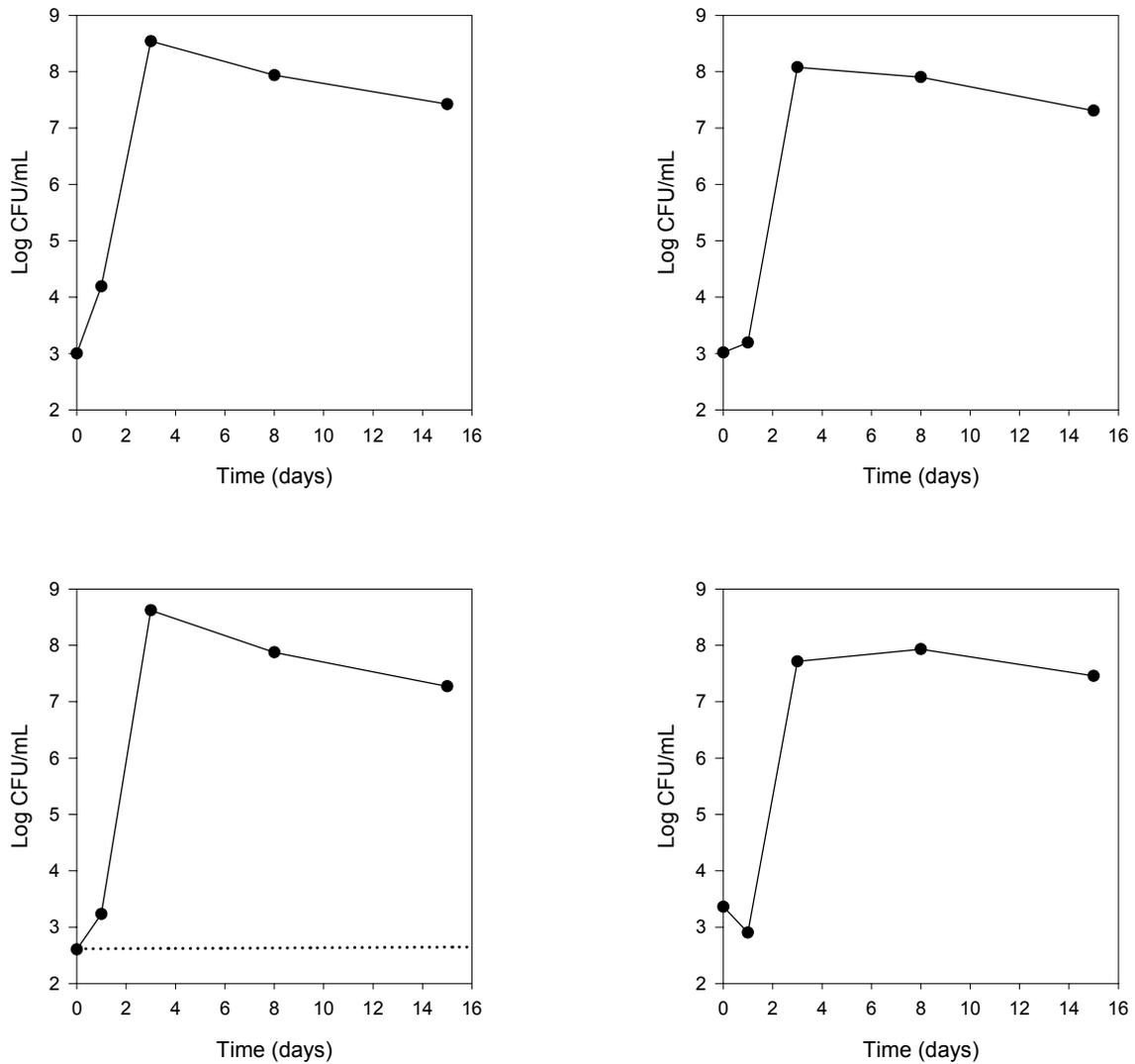
Each graph represents one jar of MPBRC. Each jar was inoculated with  $\sim 10^7$  CFU/mL. Samples for plating were taking after 2 hours of inoculation.

Figure 1b. Survival of *L. curvatus* (LR53) at 5°C in minimally processed brined refrigerated cucumbers



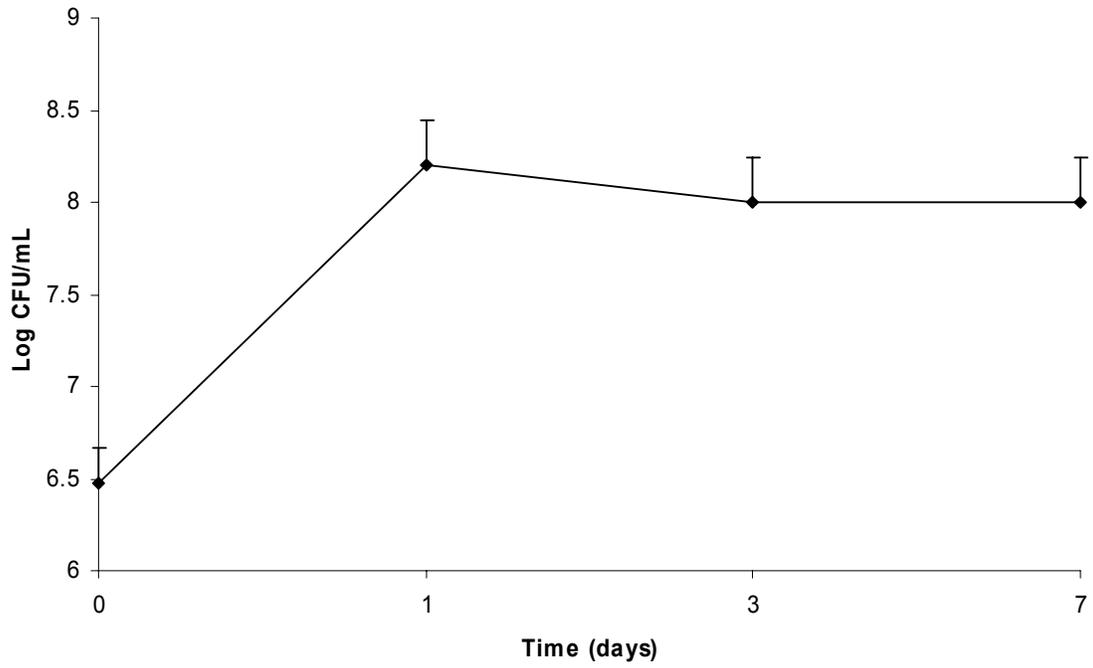
MPBRC were prepared with unblanched cucumbers, spices and garlic. Each point represents the average of 2 samples. After 21 days jars were transferred to abuse temperatures. Error bars are the standard deviations.

Fig. 2a. Minimally processed brined refrigerated cucumbers inoculated with *L. lactis* (LA218) at 18°C (Control)



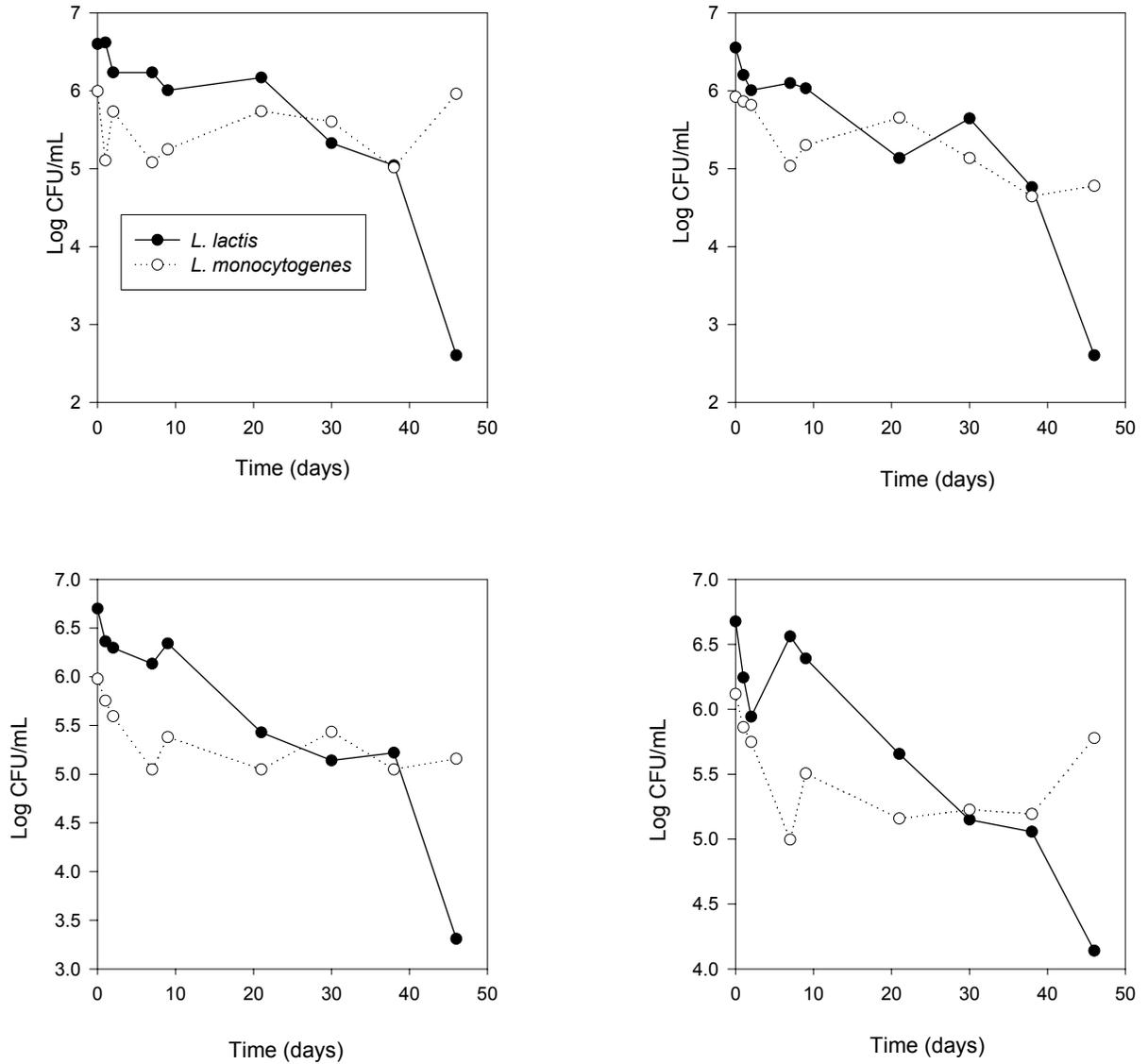
Each graph represents one jar. Dotted line means below the limit of detection with spiral plater. Jars were transferred after 5 weeks from 5°C to 18°C. Nisin was detected in all jars at 48 hours but not after that.

Figure 2b Growth of *L. curvatus* (LR53) at 18°C in minimally processed brined refrigerated cucumbers



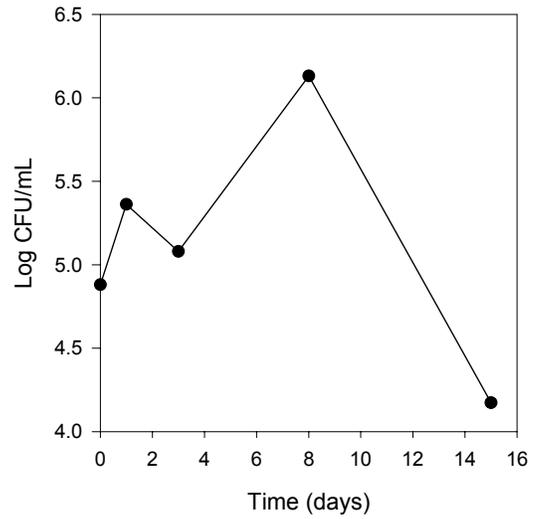
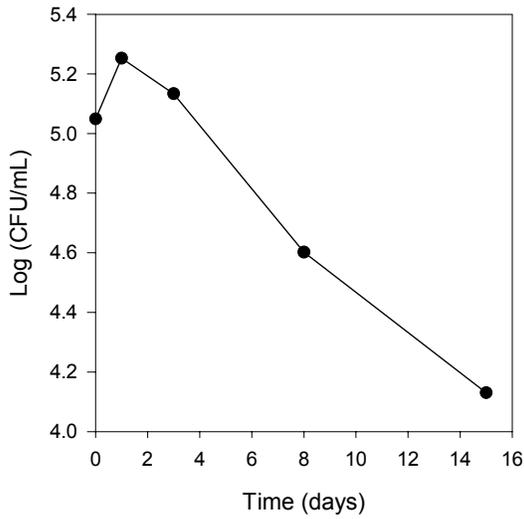
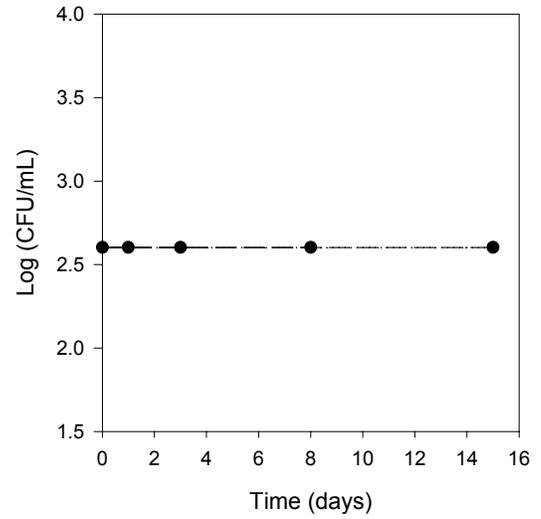
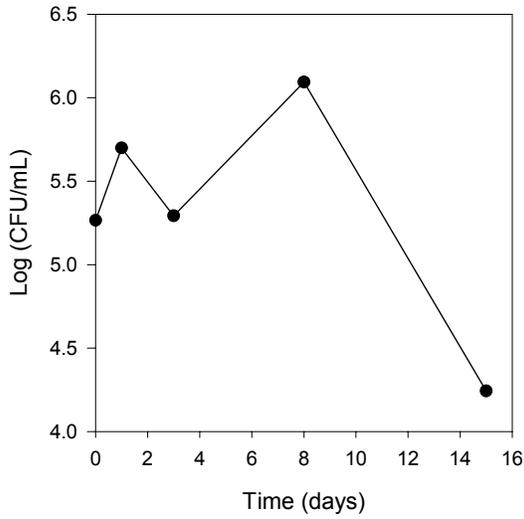
Points are the average of 2 samples. Jars were transferred after 21 days from 5°C to 18°C to simulate abuse temperatures. Bacteria enumerated on MRS + 2% salt. Plates incubated under anaerobic conditions.

Figure 3a. Survival of *L. monocytogenes* (B164) and *L. lactis lactis* (LA218) in minimally processed brined refrigerated cucumbers at 5°C



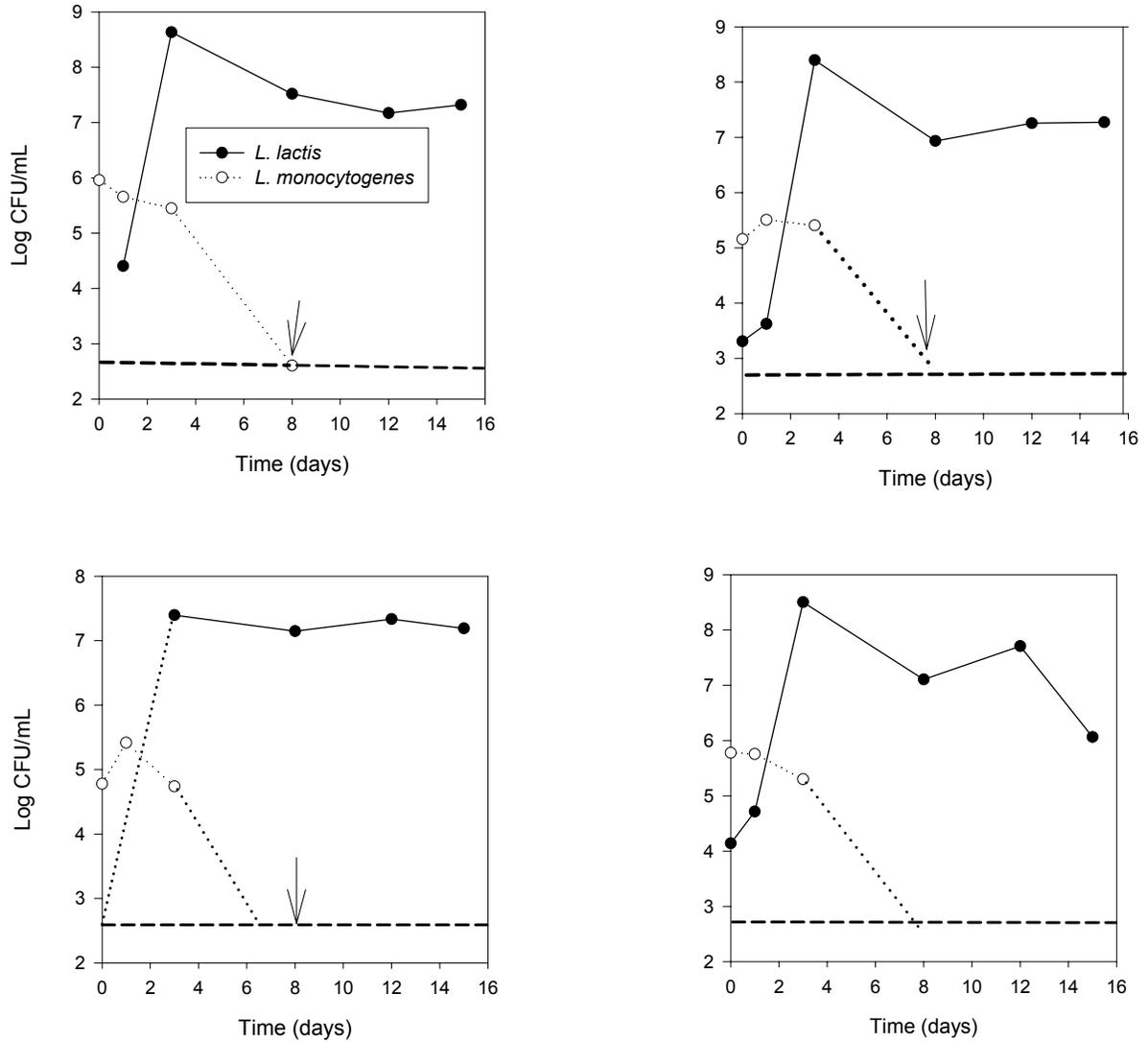
Cucumbers were blanched at 80°C for 15 seconds. Each graph represents one jar of MPBRC.

Figure 3b. Minimally processed brined refrigerated cucumbers inoculated with *L. monocytogenes* (Control), 18°C.



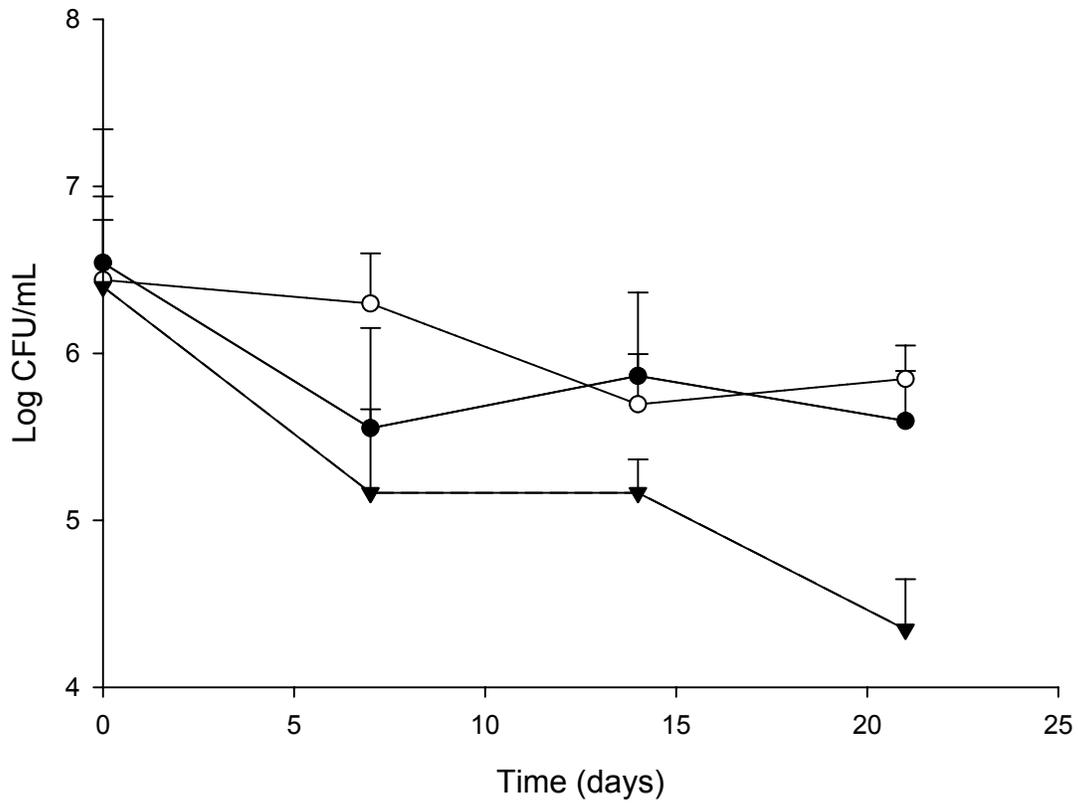
Each graph represents one jar to show variability. *L. monocytogenes* was not detected in J4. LAB grew at 5°C, it was turbid after 3 weeks at 5°C. Dotted line means below the limit of detection with spiral plater. Counts of *Listeria* decreased when LAB started growing after 8 days.

Figure 3c. Biocontrol of *L. monocytogenes* by *L. lactis* in minimally processed brined refrigerated cucumbers at 18°C.



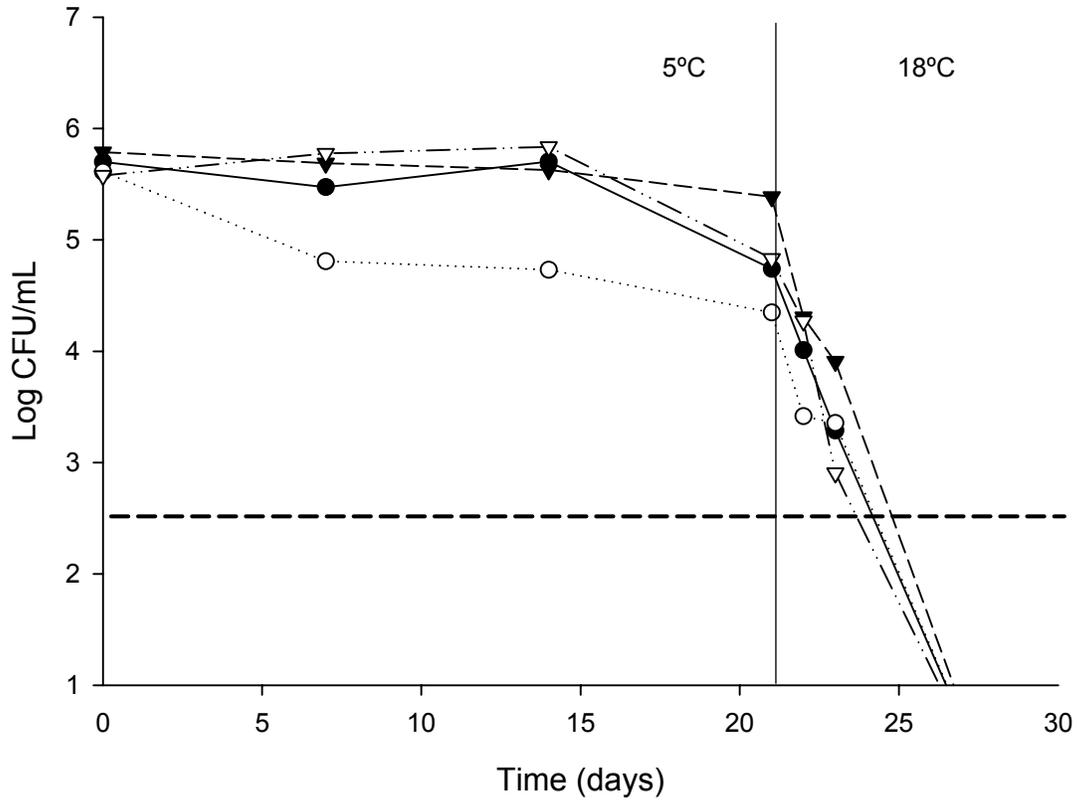
*L. lactis* dominated at 18°C and produced nisin. Dotted line means below the level of detection with spiral plater. Cucumbers were blanched. After 2 days *Listeria* decreased to undetectable levels.

Figure 4. Effect of nisin concentration on *L. lactis* survival at 5°C



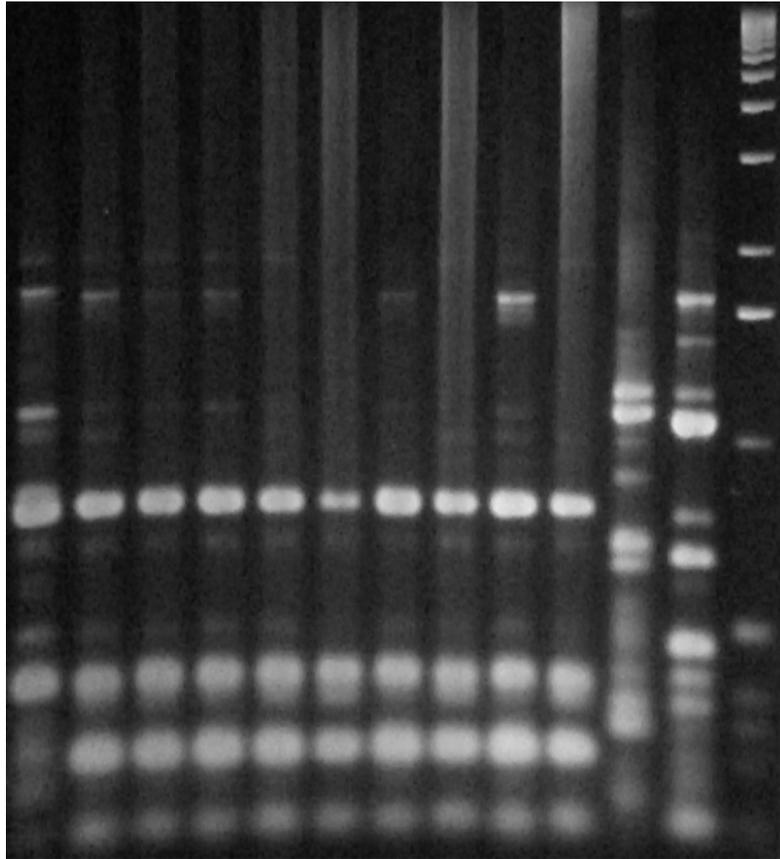
○ *L. lactis* (control, 0 nisin), ● *L. lactis* 50IU/mL nisin, ▼ *L. lactis* 1000IU/mL nisin. Each point is the average of 4 jars. Cucumbers were unwashed. *Lactococcus* was grown in GM17 with 4% salt and 5mM glycine betaine.

Fig 5. Fate of *L. monocytogenes* (B164) in minimally processed brined refrigerated cucumbers in the presence of nisin and *L. lactis* (LA218)



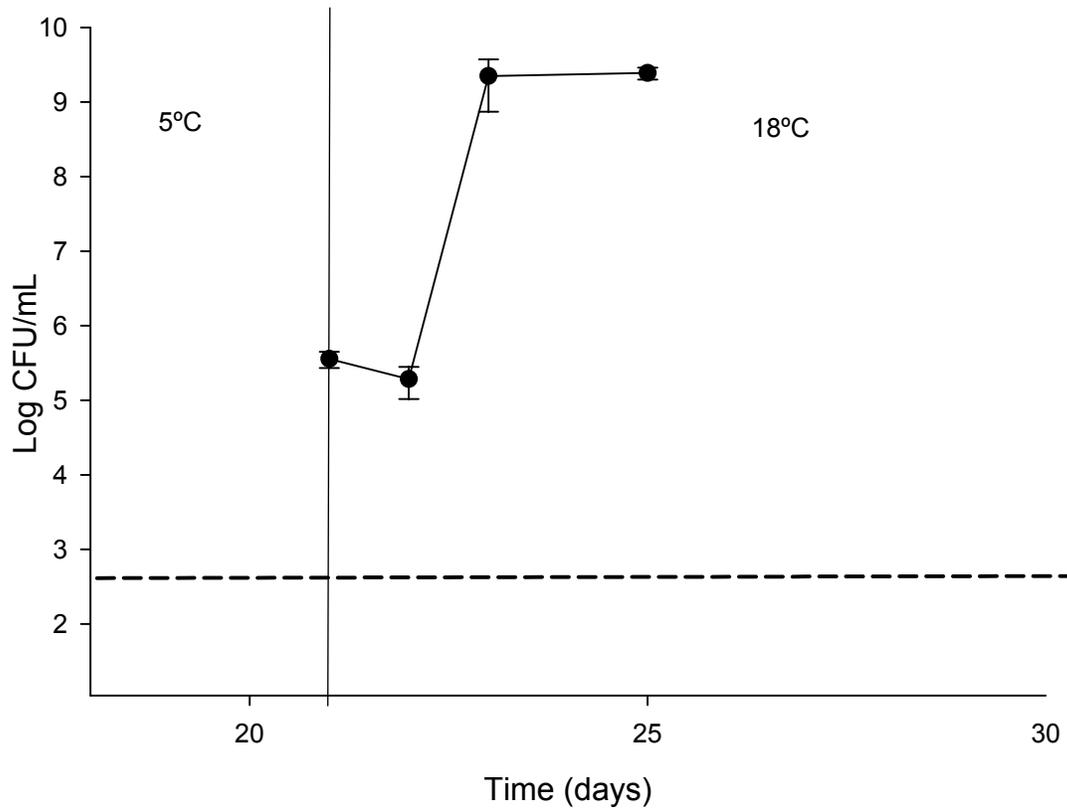
Cucumbers were unblanched. Numbers are the means of 4 samples. Jars containing *L. lactis* + 1000 IU/mL of nisin were the only significantly different ( $P \leq 0.05$ ) by Tukey's Multiple Comparison Test. (●) *L. monocytogenes* + nisin 50 IU/mL + *L. lactis*, (○) *L. monocytogenes* + nisin 1000 IU/mL, (▼) *L. monocytogenes*, (▽) *L. monocytogenes* + nisin 50 IU/mL. Jars transferred after 21 days from 5°C to 18°C. Dotted line means below the level of detection with spiral plater.

Figure 6. PCR Fingerprinting of *L. lactis* added as a biocontrol agent in minimally processed brined refrigerated cucumbers.



From left to right: 1- *L. lactis* ATCC 1454, 2- *L. lactis* (LA218), 3-10- isolates obtained from the jars at 18°C. 11- *Lactobacillus cellobiosus*, 12- *L. ruteri*, 13- 1 Kb standard.

Figure 7. Natural LAB growing in minimally processed brined refrigerated cucumbers at 5 and 18°C.



Points are the average of 4 samples. MPBRC were prepared with unblanched cucumbers, garlic and spices. Dotted line means below the level of detection with spiral plater. Jars were transferred from 5°C after 21 days to 18°C. Error bars are the standard deviations.

Table 1. Biocontrol of *L. monocytogenes* by *L. curvatus* in cucumber juice at 2% and 4% salt at 21°C

| Time                           | CJ 4% salt <sup>1</sup> | pH               | CJ 2% salt <sup>3</sup> | pH   |
|--------------------------------|-------------------------|------------------|-------------------------|------|
| 0                              | 7.2                     | 3.9 <sup>2</sup> | 6.8                     | 6.24 |
| 1                              | 7.3                     | 3.8              | 7.8                     | 4.2  |
| 2                              | ND                      | ND               | 6.9                     | 4.1  |
| 5                              | 6.3                     | ND               | ND                      | ND   |
| 7                              | 5.1                     | ND               | <1.0                    | 3.9  |
| 10                             | <1.0                    | 3.6              | <1.0                    | 3.7  |
| Control<br>( <i>Listeria</i> ) | 8.5                     | 4.6              | 8.6                     | 4.7  |

Numbers are the average of 2 samples. ND=Not determined, <sup>1</sup>Tubes were transferred after 15 days from 5°C to room temperature, *L. curvatus* was inoculated in each tube, except in the controls. <sup>2</sup>The low pH due to the growth of *L. curvatus*. <sup>3</sup>Cultures were always at room temperature. In both cases cultures were inoculated at 10<sup>6</sup> CFU/mL.

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## **Appendix 1**

### **Use of Osmoprotectants to improve Survival of a *Lactococcus lactis* strain in Minimally Processed Brined Refrigerated Cucumbers**

## Abstract

The osmoprotectants carnitine, choline, and glycine betaine (GB) were tested to improve salt tolerance of *L. lactis*, a nisin-producing strain, isolated from sauerkraut fermentation.

In vitro experiments showed that the bacteria were inhibited by salt at different concentrations (2, 4, 5 %) at 5, 10, 15, 20 and 30°C. Carnitine did not have any effect, choline had an effect similar to the one obtained with GB at 10mM.

GB was the only osmoprotectant improving salt tolerance of the strain in cucumber juice. The addition of GB at different concentrations (1-5 mM) into M17 medium with 1% glucose containing 2% and 4% NaCl enabled cells to achieve a faster maximum specific growth rate after a prolonged lag phase (7 hours), compared to the control without GB (at 20 and 30°C). Concentrations higher than 10 mM did not have a significant effect, no effect was seen at temperatures lower than 20°C.

The purpose of this study was to learn if growth/survival of *L. lactis* can be improved for use as a biocontrol agent in minimally processed brined refrigerated cucumbers (MPBRC). The product contains 4% salt initially and it is stored at 5°C. Growing the strain with 5 mM GB and 4% salt before inoculation into MPRC improved its survival at 5°C. Bacterial counts went down 2 log cycles, and at 18°C the bacteria reached  $1 \times 10^8$  CFU/mL, but after 3 days of storage other LAB predominated. Statistical analysis of the data showed that GB had a significant effect on the survival of *L. lactis* at 5°C ( $P < 0.0041$ ).

Previous treatment of the strain with GB may enhance the survival of *L. lactis* in MPBRC, and this could allow the use of the strain as a biocontrol agent in brined, non-acidified refrigerated vegetables.

## Introduction

The development of novel food technologies and consumers' search for natural products have directed attention to the use of Lactic Acid Bacteria as biopreservatives for minimally processed fruits and vegetables (MPFV) (Moreno *et al.*, 1999; Breidt and Fleming, 1998). The major safety concern of ready-to-eat products is the possible outgrowth of psychrotrophic pathogens, such as *Listeria monocytogenes*, at refrigeration temperatures (Kelly *et al.*, 1998), and mesophilic pathogens at abuse temperatures.

Breidt and Fleming (1996) studied the effect of *L. lactis lactis* on *L. monocytogenes* in cucumber juice and found that *L. lactis lactis* inhibited the growth of *L. monocytogenes* at 11°C. However, Wessels and Huss (1996) inoculated salmon slices with *L. lactis* and *L. monocytogenes* and found that the culture was not suitable as a biopreservative at 5°C, but it could work at abuse temperatures. They concluded that *L. lactis lactis* may be difficult to use as a biocontrol agent in brined products, especially at high salt concentrations (5%) and cold temperatures.

In their natural habitats bacteria are exposed to osmolality changes in the environment. The osmotic stress must be sensed and converted into an activity change of specific

enzymes and transport proteins, and/or it must trigger their synthesis, such that osmotic imbalance can be rapidly restored (Poolman and Glasker, 1998). Adaptive responses of bacterial cells to counteract low water activities ( $a_w$ ) are the accumulation of organic solvents (osmoprotectants) synthesized de novo or actively taken up from the growth medium (Picherau *et al.*, 1999). Osmoprotectants are solutes that alleviate the inhibitory effect of osmotic stress on the bacteria when they are added into the medium. They can be accumulated to high levels without interfering with vital cellular processes. They are good stabilizers of enzymes, providing protection not only against high salt, but also against high temperatures, freeze-thawing and drying. The solutes that are accumulated are: potassium ions, amino acids, amino acid analogues, methyl amines and related compounds. They do not have net charge and they should not interact specifically with the mostly negatively charged cellular macromolecules. Upon hypoosmotic stress, these compatible solutes are released from the cell, which prevents too high turgor pressure that may lead to bursting of the cell (Van Der Heide and Poolman, 2000).

Lactic acid bacteria (LAB) have a limited capacity to synthesize osmoprotectants, and a range of studies indicate that GB, carnitine, and proline are the most important compatible solutes in this group of organisms (Van Der Heide and Poolman, 2000).

O'Collagan and Condon, (2000) found that some strains of *L. lactis* can accumulate GB at low ( $a_w$ ), and that the sensitive strains that could not accumulate GB were salt sensitive. While the role of accumulation of GB in *L. lactis* under high salt concentrations has been studied (Guillot *et al.*, 2000), the effect of previous growth in GB on the subsequent survival of *L. lactis* in a food system has not been reported.

A deli-style pickle product, minimally processed brined refrigerated cucumbers (MPBRC) was chosen as a model of MPFV. It is a ready-to-eat, highly perishable pickle product containing fresh cucumbers, garlic, spices and 2% brine after equilibration, with initial pH of 5.5; that needs to be refrigerated (Etchells and Moore, 1976).

The objectives of this research were:

- To study the effect of choline, carnitine and GB (glycine betaine) on a nisin-producing *L. lactis* in order to improve salt tolerance in different media and at different temperatures.
- To be able to use the strain previously grown with the osmoprotectant as a biocontrol agent in MPBRC.

## **Materials and Methods**

### **Effect of Osmoprotectants on the growth of *L. Lactis* in the presence of salt**

Carnitine, Choline (5, 10 mM), and GB (1, 2, 5, 10, 20 mM) (Aldrich Chemical Company, Inc., Milwaukee, WI) were tested as osmoprotectants at different concentrations in brine (equilibrated brine obtained from MPBRC), cucumber juice with 2 and 4% salt, and M17 (Difco, MI) with 1% glucose (GM17) with 2 and 4% salt at 5, 10, 15, 20 and 30°C.

Experiments were done with duplicate samples and were repeated 3 times. Growth rate of *L. lactis* was determined in a microplate reader (Bio-Tek instruments, Inc.) at 600 nm optical density and rates were determined by Gompertz model, (Custom software Breidt, unpublished).

### **Strain preparation**

*L. lactis lactis* nisin-producer (LA218, USDA-ARS Culture collection, Raleigh, NC) was isolated from sauerkraut fermentation by Harris *et al.*, 1992, and was transformed with plasmid pGKC following the procedure described by Breidt and Fleming, 1992. It was grown in GM17 broth containing different concentrations of GB, carnitine or choline, 4% salt and 5 µg/mL of chloramphenicol.

### **Minimally Processed Brined Refrigerated Cucumbers (MPBRC)**

Fresh pickling cucumbers (3.5-3.8 cm diameter) were washed, weighed (680±5 g), and blanched for 15" at 80°C (Breidt *et al.*, 2000), then immediately placed in 46 ounces glass jars and cooled with 680± 5 g sterile 4% cold brine containing 1 g of sterile aquaresin garlic (Kalsec, Kalamazoo, MI) and 5 g of irradiated pickling spices (13 KGy) (Ba-Tampte Pickle Products, Brooklyn, NY) of undefined composition due to proprietary rights of the company. Jars were sealed with caps containing septa and placed at 5°C after inoculation with *L. lactis lactis* (~1x10<sup>6</sup> CFU/mL).

### **Inoculum preparation for inoculation into MPBRC**

The culture was grown in M17 (Difco, Detroit, MI) broth containing 1% glucose, and 5 mM GB (Aldrich Chemical Company, Inc., Milwaukee, WI), 4% salt (NaCl) and 5 µg/mL of chloramphenicol. The cells were centrifuged and resuspended in cold GM17 containing salt and GB, and placed at 5°C for 2 hours before their inoculation into MPBRC. To assess the effect of inoculation time, freshly prepared MPBRC jars were placed at 5°C for 5 days and inoculated once the brine had been equilibrated. A control was prepared without GB, salt or cold shock. Jars were sampled periodically by syringe (Becton Dickinson, Portland, CT) to obtain 1 mL samples for chemical and microbiological analysis. Samples were appropriately diluted in sterile saline solution (0.85%) and plated by spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) on MRS with chloramphenicol (5 µg/ml) (Difco, Detroit, MI) agar and incubated at 30°C for 48 hours under anaerobic conditions. Experiments were done in triplicate.

Total aerobic count was done on PCA agar (Difco, Detroit, MI), Total *Enterobacteriaceae* on VRB agar with 1% glucose (Difco, Detroit, MI), and LAB were enumerated on MMRS (MRS with 0.02% sodium azide) (Difco, Detroit, MI) for LAB. Plates were incubated aerobically at 30°C for 24 and 48 hours and counted with an automated colony counter (Protos Plus, Bioscience International, Rockville, MD).

### **Nisin determination**

Nisin was qualitatively measured with the lawn test (Tramer and Fowler, 1965) with *L. cremoris* as the sensitive strain. Samples of brine were centrifuged and the supernatant

transferred to a new tube, filter sterilized and boiled for 1 minute. Five  $\mu\text{l}$  of this brine was spotted onto MRS agar (1.5%) and allowed to dry. Ten  $\mu\text{l}$  of the sensitive strain (*L. cremoris*) was inoculated into 3 mL of MRS (0.75%) agar, mixed and overlaid MRS agar. Plates were incubated at 30°C for 24 hours. A positive test was a clear zone of inhibition. Results were reported as + / -.

### **Statistical analysis**

Differences between the treatments (GB or no) in the survival of the strain were analyzed by analysis of variance, determined by the General Linear Model, SAS, version 8 (Institute, Cary, NC).

## **Results and Discussion**

### **Growth of *L. lactis* in the presence of salt**

The growth rate of was significantly affected by salt in the medium, *L. lactis* grew slowly when 2% salt was added into GM17 at 30°C ( $0.52 \text{ h}^{-1}$ ). The growth was slower at 4% salt ( $0.15\text{-}0.2 \text{ h}^{-1}$ ) compared to the control ( $0.71\text{h}^{-1}$ ) (Fig 1), and no growth occurred at 5% salt (data not shown). *L. lactis* strains vary in their salt tolerance and they have been divided into two groups based on their ability to grow in high salt concentration, tolerant (0.6 M), or sensitive (0.3 M) (O'Callaghan and Condon, 2000).

Van Der Heide and Poolman, 2000, described a GB transporter system in *L. lactis* that responds to water stress, and which is activated at high salt concentrations. The activation is mediated by changes in membrane properties and protein-lipid interactions.

In this study, the bacteria adapted faster to high concentrations of salt (4%) when GB was added into CJ (Fig 2a and 2b), indicating that GB was taken up from the medium. However, concentrations higher than 5 mM did not improve the growth rate (Fig 3). Guillot *et al.*, 2000, studied the effect of temperature on GB accumulation, and reported that *L. lactis* grown at 40°C was able to accumulate higher concentrations of GB than cells grown at lower temperatures, but this accumulation did not increase the growth rate. Other studies reported that accumulation of the osmoprotectant inside the cells, inhibited further uptake of the osmoprotectant from the medium.

Even though 5 mM GB did not have an effect on the lag phase when the cells were grown in cucumber juice containing 4% salt at 20°C, it relieved the inhibitory effect of salt on the specific growth rate (Fig 2a). Our experiments included temperatures of 5, 10, 15, 20 and 30°C. As temperature decreased, the effect of GB was less noticeable, especially at 2% salt (data not shown). Depending on the medium used, the effect of GB was less or more noticeable. This effect could possibly be attributed to the presence of GB in yeast extract contained in GM17 and not in CJ. Yeast extract provides significant amounts of GB, choline and amino acids that can be accumulated inside the cell under stress conditions.

Carnitine or choline had no effect on the growth of *L. lactis* when added into the medium in the presence of salt, but choline had an effect at 10 mM (Table 1). It was demonstrated that choline can be converted to GB due to the presence of betaine aldehyde dehydrogenase and choline dehydrogenase in some bacteria (Caldas *et al.*, 1999). However, in *L. plantarum* choline was accumulated without converting it to betaine under salt stressed conditions (Kets *et al.*, 1997).

In the present study, the amount required to improve salt tolerance in *L. lactis* was 10 mM of choline but the effect was not the same as the one obtained with GB. In cucumber juice there was no effect of choline even at high concentrations (data not shown). This could indicate that CJ contains choline, or that other substances present in CJ could have inhibited choline uptake from the medium, or that *L. lactis* preferentially uptake other substances rather than choline from CJ. Organic compatible solutes in addition to betaines that the cell can accumulate include amino acids and sugars. There are not reports in the literature on the amount of choline present in cucumber juice or cucumbers.

#### **Survival of *L. lactis lactis* in MPBRC at 5°C**

The survival of *L. lactis* in MPBRC was improved by growing the strain in GM17 containing 5mM GB. Untreated *L. lactis* (grown without salt or GB) inoculated into MPBRC decreased in numbers by 99.9% after 2 days of inoculation at 5°C. Treated *L. lactis* (grown in GM17 containing GB and salt) decreased by 90% (Fig 4). Statistical analysis of these data showed that there was a small but significant difference ( $P < 0.0041$ ) in the survival of the treated versus untreated strains at 5°C. The accumulation of betaine

in *L. monocytogenes* confers the bacteria osmotic and chill stress tolerance (Ko and Smith, 1999). This is in contrast to the effect on *Enterococcus faecalis*, where accumulation of betaine inhibited the salt induced cross-tolerance to bile salts (Pichereau *et al.*, 1999).

These results suggest that other factors in addition to salt may play a role in the survival of the bacteria in MPRC. The presence of garlic and the lack of nutrients in the brine initially could be interacting with the salt affecting *L. lactis*' survival. It takes more than 5 days for the sugars to reach equilibrium with the cover brine. However, the same results were obtained when the brine was allowed to equilibrate (data not shown).

#### **Behavior of *L. lactis* in MPBRC at 18°C**

Bacterial growth in the product noticed by turbidity at abuse temperatures was due to the growth of *L. lactis* or LAB from the cucumbers that were protected from the heat during blanching and could grow in the presence of garlic, salt and cold temperatures of storage. The presence of LAB in the cucumbers varied with the cucumber source and the season of harvesting. When the bacterial load was low, shown by the total aerobic count ( $10^{4-5}$  CFU/g) before blanching, the non-inoculated jars showed no growth even after 6 months of storage at 5°C. When the total aerobic count in the cucumbers was  $10^{6-7}$  CFU/g before blanching, natural LAB grew after 4 weeks of storage at 5°C (data not shown) and dominated the fermentation at abuse temperatures.

When the inoculated jars were transferred to 18°C, the numbers of *L. lactis* reached  $1 \times 10^8$  CFU/mL in some jars. This depended on the natural LAB growing in the jars and lowering the pH. However, the numbers of *L. lactis* were not the same in all jars. The effect of the growth of the treated strain could be seen on the numbers of LAB, which were lower than in the jars with untreated or no *L. lactis* (data not shown). Nisin was detected in the jars where the *L. lactis* numbers reached  $1 \times 10^8$  CFU/mL. Control jars with no *L. lactis* had higher numbers of LAB. The treated *Lactococcus* declined faster at 18°C than the untreated one. Perhaps the presence of nisin in the jars had an effect on the cells. However, no further studies were done to find out the real effect of nisin on the culture (Fig 4b).

The variability in the *Lactococcus* counts could be due to the presence of other LAB present in the product that had higher growth rates at low temperatures taking over the fermentation at abuse temperatures. Therefore, a blanching step is essential to achieve the growth of the biocontrol agent (Breidt *et al.*, 2000).

Even though *Lactococcus* was not found in all the jars at 5 °C, the presence of the natural LAB in the jars with no *Lactococcus* can be considered safe because the pH was 3.0-3.2 due to LAB growth.

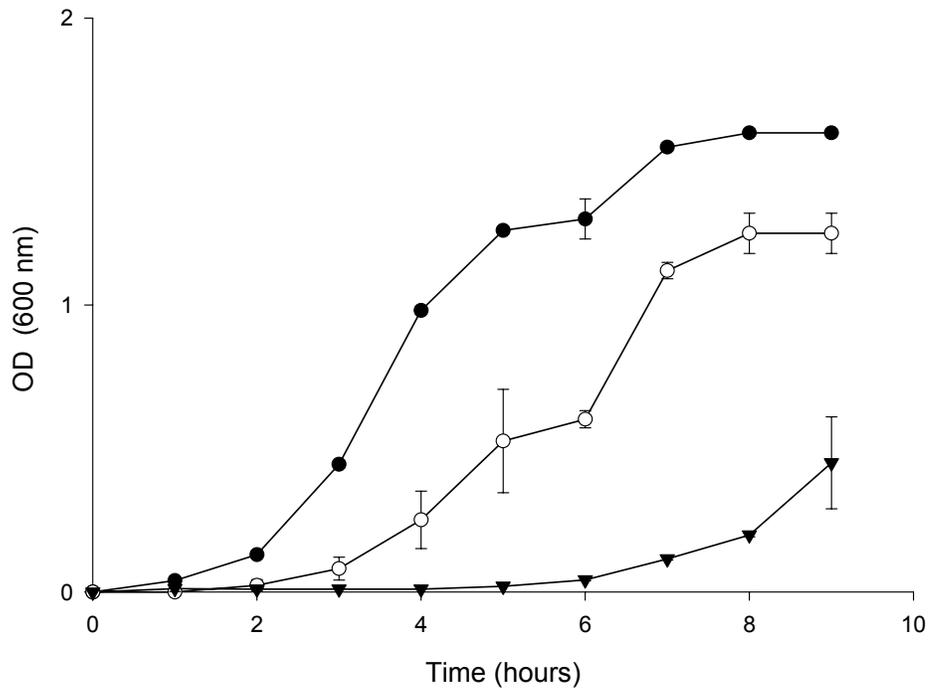
## CONCLUSIONS

- In vitro studies showed that GB was the only osmoprotectant of those tested that improved salt tolerance of *L. lactis*. However, its survival in MPBRC depended

on other factors, such as low temperature, lack of nutrients at the beginning of storage, and bacterial competition.

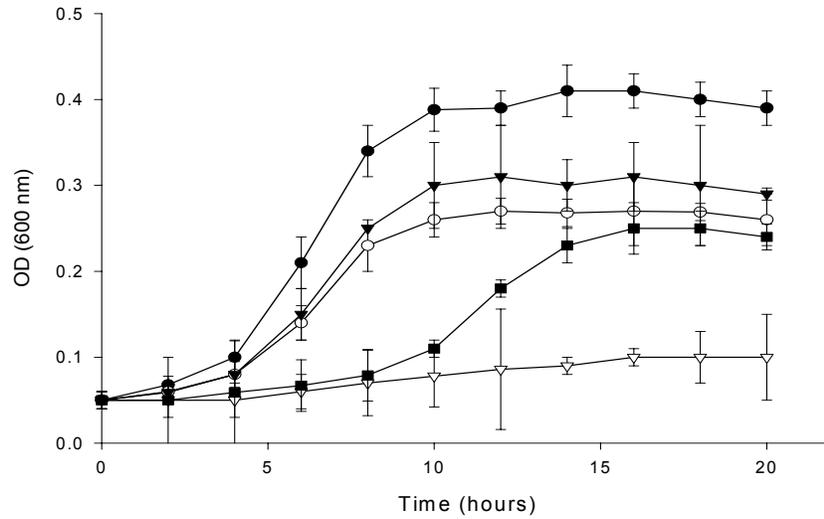
- Blanching the cucumbers reduced but it did not eliminate the natural microflora. The presence of other LAB growing in MPBRC at 5°C inhibited the *L. lactis* by lowering the pH.
- *L. lactis* had a short stationary phase in MPBRC; according to these results it reached  $10^8$  CFU/mL after 48 hours at abuse temperatures (18°C) but started to decline immediately after that. However, its mission as a biocontrol agent was accomplished as being the one responsible for turbidity at abuse temperatures.
- Other methods of inoculation need to be explored to be able to use this culture in this type of products. Micro-encapsulation of the culture with a temperature sensitive material that will melt with temperature increase and release the culture could be used to ensure that *L. lactis* will succeed.

Figure 1. Growth of *L. lactis lactis* in the presence of salt in GM17 at 30°C



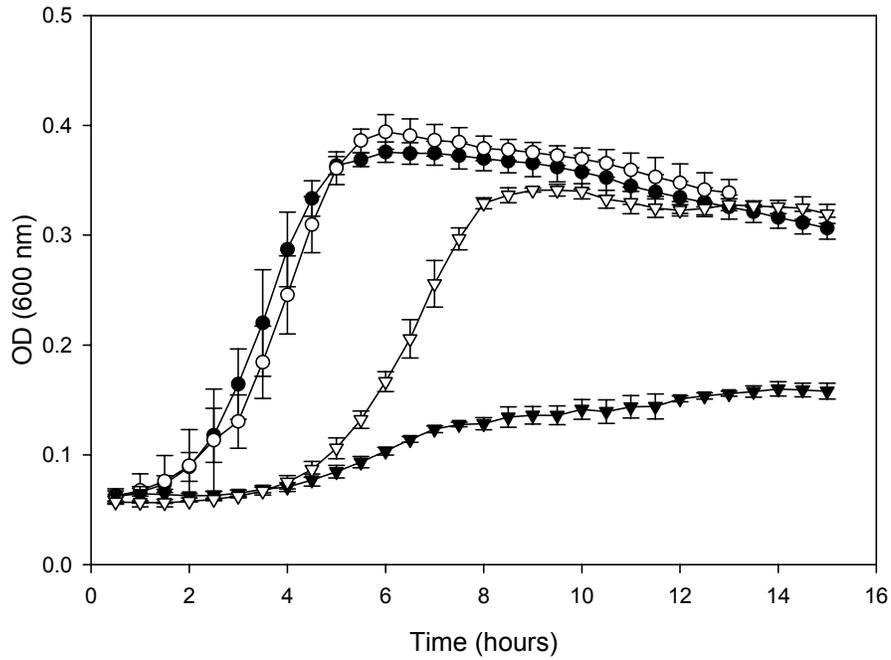
● GM17, control, ○ GM17 2% NaCl, ▼ GM17 4% NaCl. Numbers are the average of 2 samples. Experiment done 3 times. Error bars are the standard deviations.

Figure 2a. Growth of *L. lactis lactis* in the presence of salt and glycine betaine in Cucumber Juice at 20°C.



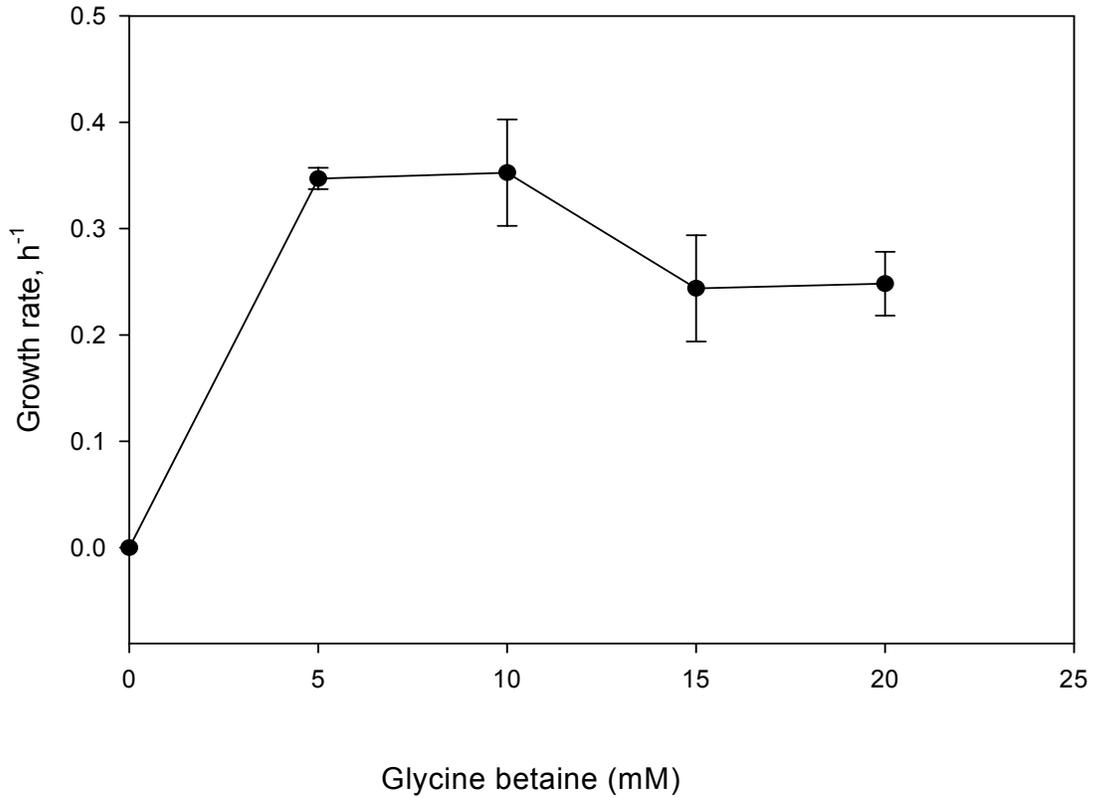
● CJ, control, ▼ CJ 2% salt + GB, ○ CJ+2%NaCl, ■ CJ+4%NaCl+GB, ▽ CJ+4% NaCl  
 Experiments done 3 times, Numbers are the average of 3 wells of a microtiter plate. Error bars are the standard deviations.

Fig 2b. Effect of glycine betaine on the growth of *L. lactis* in cucumber juice at 30°C



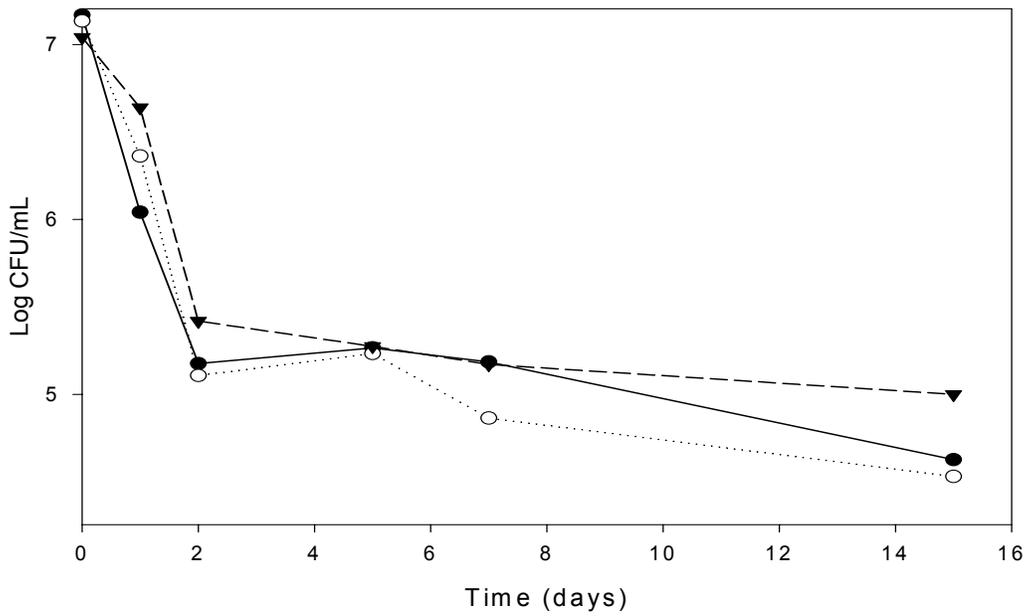
● CJ, control 2% salt, ○ CJ+2%NaCl+GB, ▽ CJ+4%NaCl+GB, ▼ CJ+4% NaCl  
Experiments done 3 times, Numbers are the average of 3 wells of a microtiter plate. Error bars are the standard deviations.

Figure 3. Effect of different concentrations of glycine betaine on the growth of *Lactococcus lactis* (LA 218) in GM17 4% salt at 30°C.



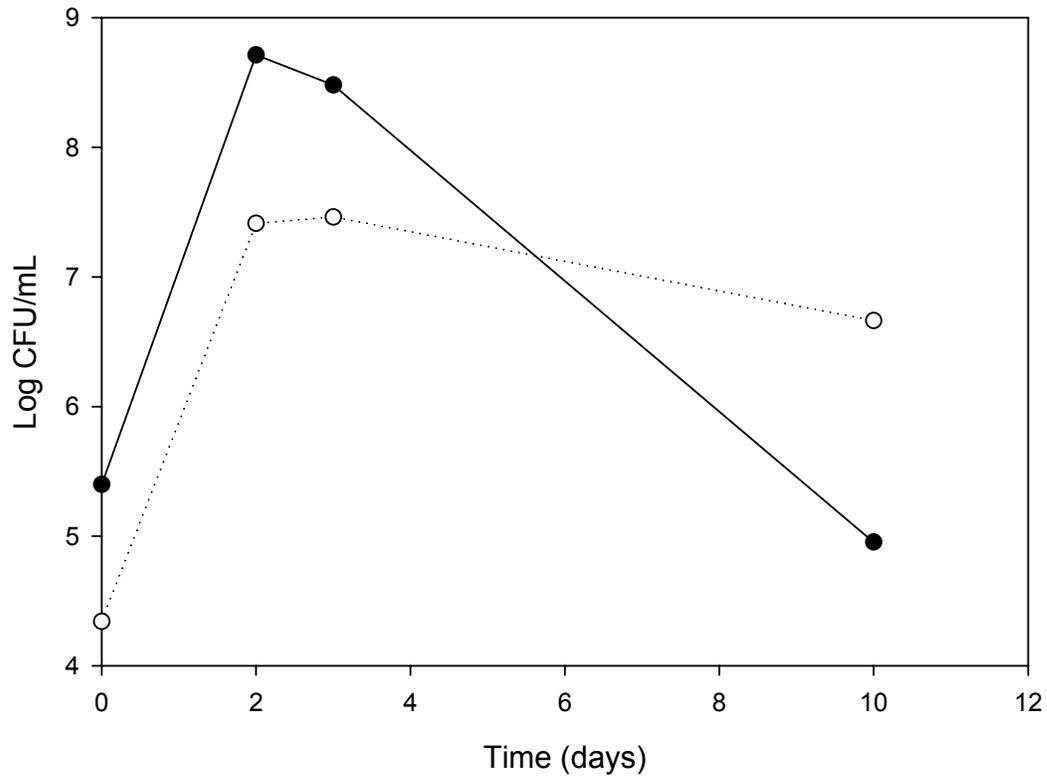
Data were analyzed by Duncan's New Multiple Range test. Five mM was significantly different from the control (0) ( $P \leq 0.05$ ), the 10- 20 mM were not significantly different from the control ( $P \geq 0.05$ ). Numbers are the average of 2 wells. Experiment repeated 3 times. Error bars show standard deviations.

Figure 4a. Survival of *L. lactis lactis* at 5°C in minimally processed brined refrigerated cucumbers



○ *L. lactis* (control, without GB), ● *L. lactis* treated with 1mM GB, ▼ *L. lactis* treated with 5 mM GB. Cells were grown in GM17 with or without GB. See materials and methods. Each line represents one jar of MPBRC. Each jar contains 680 g of cucumbers.

Fig 4b. Survival of *L. lactis* at 18°C in minimally processed brined refrigerated cucumbers



○ *L. lactis* not treated, ● *L. lactis* treated with GB. Jars were transferred from 5°C to 18°C. 0 time is the last count at 5°C.

Table1. Effect of different osmoprotectans on the growth of *L. lactis* at 30°C.

| Samples                      | <sup>1</sup> Growth Rate h <sup>-1</sup> |
|------------------------------|--|
| GM17                         | 0.71 (0.04)                              |
| GM17 2% salt                 | 0.52 (0.05)                              |
| Gm17 2% salt + 5mM GB        | 0.66 (0.06)                              |
| Gm172% salt+5 mM carnitine   | 0.49 (0.04)                              |
| GM17 2% salt+10 mM carnitine | 0.47 (0.02)                              |
| Gm17 2% salt+5 mM choline    | 0.48 (0.04)                              |
| GM17 2% salt+10 mM choline   | 0.58 (0.08)                              |

<sup>1</sup>Numbers are the average of 3 wells, numbers in parenthesis are the standard deviations.

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## **Appendix 2**

### **Antimicrobial properties of garlic oil and pickling spices**

## Abstract

Garlic aquaresin and pickling spices were tested for their antimicrobial properties against six *Lactococcus lactis* strains and five strains of *Listeria monocytogenes*. The antimicrobial activity was assayed in broth and by the agar spot method. The results indicated that pickling spices did not have any activity against the microorganisms tested, neither in broth nor on the agar. Aquaresin garlic had antimicrobial activity against all the bacteria tested, which was potentiated with the addition of salt and/or nisin in the broth. At high concentrations it was bactericidal; at low concentrations bacteriostatic. However, it was bactericidal in broth when used in combination with salt and nisin at pH 5 and 4.

Gas chromatography analysis of the garlic aquaresin showed a variety of sulfur compounds that could be responsible for the antibacterial properties. However, two substances were found in high concentration: dimethyl disulfide and allyl methyl sulfide. When these substances were tested in broth for antibacterial properties, they were shown to be bacteriostatic. The addition of salt did not change the activity.

These results indicate that other substances were responsible for the antibacterial properties of garlic oil, or that a combination of all the substances gave the properties to garlic. However, the use of garlic oil in combination with other hurdles in a food system can enhance its safety.

## Introduction

Spices and garlic have been used through the ages as ingredients in the preparation of food. They also have been known to have medicinal value for a long time, in particular, antimicrobial properties. However, studies done with spices: garlic, ginger, clove, black pepper, and green chillies, showed that only garlic and clove had antimicrobial activity (Arora and Kaur, 1999). Other studies done with oregano, dill, thyme and their essential oils showed that these compounds are able to inactivate microorganisms by membrane perturbing or rupturing mechanism due to their hydrophobic characteristics. However, the concentrations required to achieve cell death are above tolerable taste thresholds (Brul and Coote, 1999).

Garlic has also been reported to reduce blood lipids and to have anticancer effects. The incidence of stomach cancer was lower in individuals with a high intake of allium vegetables (Sivan *et al.*, 1997). Chemical analyses of garlic cloves have shown that they have unusual sulfur-containing compounds of 1-3% (Ankri and Mirelman, 1999). Garlic has been known to inhibit a wide range of microorganisms, including Gram-positive, Gram-negative bacteria, yeast and molds. The antimicrobial properties of garlic are due to a compound known as diallyl thiosulfonate (allicin) (Cavallito *et al.*, 1944). Allicin is not found in intact plants, but it is formed by the action of the enzyme alliin alkyl-sulfenate-lyase on the non protein amino acid S-allylcysteine S-oxide (alliin). In the plant, the substrate and the enzyme are stored in different compartments. Upon bulb injury, the enzyme catalyses the reaction to yield pyruvate, ammonia, and allylsulfenic acid. Two

molecules of allylsulfenic acid react spontaneously to produce allicin. This enzyme has been found in allium plants, such as garlic and onions (Focke *et al.*, 1989). Allicin is shown to be a specific inhibitor of acetyl-CoA synthetases from plants, yeast, mammals, and bacteria (Focke *et al.*, 1989), an inhibitor of sulphhydryl metabolic enzymes, and the antimicrobial properties are attributed to specific interference with S-H groups (Cellini *et al.*, 1996). Allicin is converted very rapidly to diallyl disulfide and other sulfides because of its instability (Naganawa *et al.*, 1996). Methyl and allyl sulfide derivatives of allicin are formed by the steam distillation of mashed garlic to produce garlic oil, which is used in many medicinal products (Ross *et al.*, 2001).

The antibacterial activity is dependent on the preparation used. Many commercial preparations of garlic are available as powder or oil. Commercial oil is diluted approximately 200:1 with vegetable oil, so the final product reflects the level of allicin in fresh crushed garlic (O’Gara *et al.*, 2000). Garlic distilled oils are mainly composed of diallyl disulfides, diallyl trisulfides, diallyl tetrasulfide, and their amount vary with the geographic origin of the plant, and the percentage of the disulfides and trisulfides present (Avato *et al.*, 2000, Tsao and Yin, 2001).

The incidence of antibiotic-resistant bacteria is a big problem. Development of resistance of bacteria to antibiotics is due to acetylation via arylamine N-acetyl transferase (NAT). It has been shown that the sulfur compounds present in garlic can inhibit the activity of arylamine N-acetyl transferase in *Klebsiella* and *Pseudomonas*, and act synergistically with antibiotics to inactivate the bacteria (Tsao and Yin, 2001). NAT enzymes are very

active in *Salmonella* and could be responsible for resistance to isoniazid in *Mycobacterium tuberculosis*. Arylamine N-acetyl transferase catalyses the acetylation of arylamines using Acetyl Co A. Arylamines are carcinogens. However, inactivation of N-acetyltransferase can inhibit the development of gastric and colon cancer or induce the development of breast cancer in animal models (Chen *et al.*, 1999). Therefore, the role of garlic as an anticancer agent remains to be elucidated.

In *Helicobacter pylori*, garlic oil reduced the spiral rods and cell motility, but increased coccoidal forms, clumping and cell debris, which indicated progressive cellular destruction. This suggests that garlic oil could interfere with cell envelope, causing lysis, or with cell metabolism, triggering autolysis (O’Gara *et al.*, 2000).

The objective of this study was:

- To investigate the antibacterial properties of aquaresin garlic and pickling spices used to prepare a deli-style pickle product on selected *Lactococcus lactis* strains and *Listeria monocytogenes*, serotypes 4b, 1/2a, 1/2b.

## Materials and Methods

### Garlic and spices preparation

Aquaresin garlic was obtained from Kalsec, Kalamazoo, MI. This product is a combination of water and oil dispersible resins. It is ideal for the use in brines and other food systems that have high water content. Stock solutions were prepared by diluting it with sterile saline solution. Concentrations were prepared in a range that included the concentration accepted to be used in the product (minimally processed brined refrigerated cucumbers). That concentration was 0.77mg/mL.

Pickling spices of undefined composition due to proprietary rights of the company were obtained from Ba-Tampte Pickle Products, Brooklyn, NY. Spices (50 g) were weighed and stomached with 50 mL of sterile saline solution and filtered. The filtrate was used to test antimicrobial activity.

Antimicrobial activity was assayed in agar by the spot method (Fleming *et al.*, 1975). Briefly, 10 µl of the solution were spotted onto the surface of MRS (Difco, MI) or TSG (Difco, MI) agar, allowed to dry, and overlaid with the test microorganism previously diluted in 0.75% soft agar ( $1 \times 10^6$  CFU/mL). Plates were allowed to dry and incubated at 30 or 37 °C.

In broth system, aquaresin garlic was diluted and added into the broth at different concentrations. Bacteria previously diluted were also added to reach a final concentration

of  $1 \times 10^6$  CFU/mL. Different concentrations of aquaresin garlic were tested (0.1, 0.2, 0.3, 0.7, 1, 2, 3 mg/mL), growth was reported as turbidity of the culture (positive) or clear (negative).

*L. monocytogenes* was grown overnight at 37°C in TSG. Cells were harvested and resuspended in saline solution to reach a concentration of  $1 \times 10^7$  CFU/mL. Cells were inoculated into a microtiter plate to get a final concentration of  $1 \times 10^6$  CFU/mL. Growth rate of the bacteria was also determined in a microplate reader (Bio-Tek instruments, Inc.) at 600 nm optical density, and growth rates were determined by Gompertz model, (Custom software, Breidt, unpublished).

### **Gas Chromatography-Mass Spectrometry**

The Purge & Trap GC-MS system consisted of a CDS 6000 Purge & Trap unit, and a HP5890 II GC with a HP 5972 mass selective detector. A 50x dilution of the sample was purged for 30 minutes at room temperature with the volatiles adsorbed on a Tenax trap. The trap was desorbed at 180° C. for 6 minutes with the volatile components transferred directly to the GC column, a DB-5MS (30m x 0.25mm id., 0.25um film thickness, J&W Scientific). The oven temperature was held at -20°C during volatile desorption, then heated at 10°C min<sup>-1</sup> to 140°C, held for 1 minute, then heated at 40°C min<sup>-1</sup> to 220°C and held for 3.5 min. Helium was delivered to the GC column with the injector set to a constant pressure of 7.7 psi, with an injector temperature of 180°C. The detector and MS interface temperatures were set to 280 ° C. A mass range of 35-225 Da was scanned.

Compounds were identified with HP enhanced ChemStation G1701BA software using the NIST98 spectral library.

Two compounds were present at high concentrations: allyl methyl sulfide and dimethyl disulfide. They were also tested for antimicrobial properties.

Allyl methyl sulfide, dimethyl disulfide were obtained from Aldrich (Aldrich Chemical Co, Mil, WI), and they were used alone or in combination, previously diluted in sterile saline solution.

### **Strain Preparation**

Lactic acid bacteria: *Lactococcus lactis* strains from the USDA culture collection, NCSU, Raleigh, NC were: LA119, LA123, LA138, LA140, LA157, and LA218. The bacteria were grown overnight in GM17 broth (Difco, MI) at 30°C, centrifuged and resuspended in sterile saline solution (0.87%) until use.

Listeria strains: *L. monocytogenes* (B164, 4b) was obtained from USDA culture collection, NCSU, Raleigh, NC, *L. monocytogenes* strains (B181 4b, B182 1/2a, B183 1/2b, B184 4b) were obtained from the culture collection of Sophia Kathariou (NCSU, Raleigh, NC). Listeria was grown overnight in TSG (Difco, MI) broth or GM17 at 37°C, centrifuged and resuspended in sterile saline solution (0.87%) until use.

## Results and Discussion

### Antimicrobial activity of pickling spices and aquaresin garlic

According to the agar spot method, the pickling spices filtrate did not show any antimicrobial activity against the bacteria tested. It did not have activity in broth either. Aquaresin garlic showed antimicrobial activity against all the bacteria tested, indicated by the presence of a zone of inhibition. The activity was dose and strain dependent (Table 1 and 2). It has been shown that enterotoxigenic *E. coli* strains are more easily inhibited by garlic than those that constitute the internal microflora (Sivan *et al.*, 1997). Concentrations of 2mg/mL and 3 mg/mL of garlic oil in the medium inhibited bacterial growth completely at 30°C; counts went down to undetectable levels after 7 hours. Garlic oil in concentrations of 80 mg/mL has been shown to reduce the population of *Klebsiella* and *Pseudomonas* to <2 logs within 6-8 hours respectively (Tsao and Yin, 2001). According to Kalsec's recommendations for aquaresin garlic, the amounts to be used should be between 0.005-0.01%. However, the concentrations showing bactericidal effect are too high to be able to use aquaresin garlic as a seasoning agent in a food system. In broth assay, it was shown to be bacteriostatic at the concentrations allowed in food, bacteria started growing after 6 hours or more depending on the concentration used. In combination with salt, the inoculated bacteria decreased by 99% the first four hours. After that, growth restarted (Fig 1). In the case of *L. lactis* (Fig 2), the addition of salt inhibited the bacteria for at least 3 days. After that, growth restarted but very slowly. The bacteria did not grow at all at 18°C (data not shown). This could indicate that garlic and

salt interfered with mechanisms of adaptation of the cell to grow at low temperatures. *Lactococcus* is a mesophilic bacterium whose optimum temperature for growth is 30°C.

The addition of salt in the medium and incubation of *L. monocytogenes* at low temperatures acted synergistically with garlic to inhibit the growth of *Listeria*. In the presence of salt and garlic, no growth occurred at 5°C for 7 weeks; compared to the controls with no garlic or salt, where *Listeria* reached 10<sup>8</sup> CFU/mL after 3 weeks (data not shown). However, after 11 weeks *Listeria* reached 10<sup>8</sup> CFU/mL.

Samples of *Listeria* placed at 18°C reached 10<sup>8</sup> CFU/mL after 5 days of storage demonstrating that the effect is bacteriostatic at low concentrations generally used to prepare foods. Garlic inhibited growth the first 24 hours, in the presence of garlic and salt or garlic and salt and nisin growth was inhibited for 3 days. After that, growth restarted but did not reach the same numbers as the control at 5 days (Fig.3).

When nisin (50 µg/mL) was added into the medium containing garlic and salt, there was no difference (P>0.05) from the ones containing garlic and salt. The effect was also bacteriostatic. However, at 5°C, growth was inhibited for more than 13 weeks, but the counts were constant, meaning that *Listeria* survived the treatment; if there is a condition of abuse temperatures, *Listeria* will grow. At higher concentrations of nisin (100, 200 µg/mL) *Listeria* did not survive at all temperatures tested. When the pH of the medium was reduced with lactic acid to 4 and 5, even at low nisin concentrations, *Listeria* was killed (Fig. 4). Nisin is shown to be more effective at low pHs. *Listeria* cells surviving

the treatment showed a round shape or deformed shape, and a lot of cell debris was observed under scanning electron microscopy (Fig 4). This demonstrates that a combination of preservation methods will be able to destroy *Listeria*, rather than relying in one alone. Issues in nisin cost and nisin resistance of *Listeria* may prevent the use of nisin in food systems.

### **Antimicrobial activity of sulfur compounds present in aquaresin garlic**

When the 2 compounds (dimethyl disulfide and allyl methyl sulfide), present in high proportions in the aquaresin garlic, were tested for their antimicrobial properties, activity was dose dependent and strain dependent. They were bacteriostatic alone or in combination with salt. Similar results were reported by Kyung and Fleming (1997) in the case of dimethyl disulfide. Other investigators reported bactericidal activity of these compounds at very high concentrations, 10 times higher than the concentrations used in this study (Ross *et al.*, 2000). However, when both compounds were included in the medium containing salt, they inhibited the bacteria longer than when used alone. They were bactericidal for strains B164, 4b and B181, 4b (data not shown). Antimicrobial activity of garlic oil has been reported to vary according to the medium used, rich medium (containing proteins and cysteine) can reduce the antimicrobial effect of garlic (Ross *et al.*, 2001). The media used in the present study can be considered rich: GM17 and TSB, this means that the results obtained may be underestimating the antimicrobial effect of garlic and its sulfur compounds.

## Conclusions

- Sensitivity of the bacteria to garlic oil was strain and dose dependent. According to these results, *L. monocytogenes* (B181, 4b) was the most sensitive to garlic oil and its compounds. This indicates that sensitivity of the strain varies and we need to take into account this fact when developing or using antimicrobial substances in a food system.
- The amount of garlic recommended to use in foods has bacteriostatic effect, even when used in combination with other hurdles, such as salt or nisin or pH. Higher concentrations (1-2 mg/mL) are bactericidal. The combination that was shown to be bactericidal was: 0.77 mg/mL aquaresin garlic, 2% salt, 50 µg/mL nisin at pH 5.
- The sulfur compounds tested had bacteriostatic activity alone or in combination. It can be concluded from these results that the antibacterial activity of the aquaresin is given by the synergistic effect of all the sulfur compounds found, even though they were present at lower concentrations than dimethyl disulfide and allyl methyl sulfide.
- Sensitivity of the bacteria to garlic depended also on the temperature used, low temperatures being able to inhibit growth longer than high temperatures. It seems that relying only on one method for preservation is risky. Refrigeration and sanitation are still important to keep a food safe.

Table 1. Antimicrobial activity of aquaresin garlic by the spot method

| Strain           | Undiluted | Dilution  |           |           |
|------------------|-----------|-----------|-----------|-----------|
|                  |           | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| <i>L. lactis</i> |           |           |           |           |
| LA119            | +++       | ++        | +         | -         |
| LA123            | +++       | ++        | +         | -         |
| LA138            | +++       | +/-       | -         | -         |
| LA140            | +++       | +         | -         | -         |
| LA57             | +++       | +         | -         | -         |
| LA218            | +++       | +         | -         | -         |

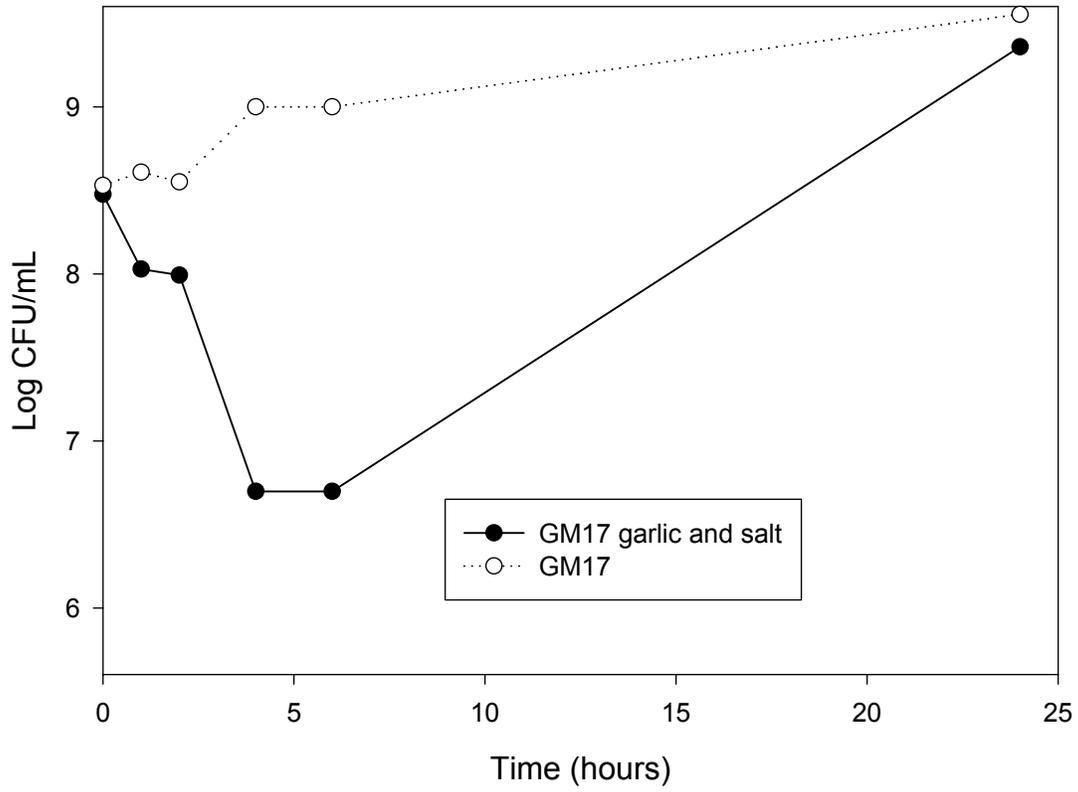
Aquaresin garlic was diluted with 0.85% NaCl and spotted on the surface of MRS agar. + diameter of the zone of inhibition.

Table 2. Antimicrobial activity of aquaresin garlic in broth (1mg/mL)

| Strain                  | 0 day | 1 day | 2 days | 3 days |
|-------------------------|-------|-------|--------|--------|
| <i>L. monocytogenes</i> |       |       |        |        |
| B164, 4b                | -     | -     | ++     | +++    |
| B181, 4b                | -     | -     | -      | -      |
| B182, 1/2a              | -     | -     | ++     | +++    |
| B183, 1/2b              | -     | -     | +/-    | ++     |
| B184, 4b                | -     | -     | ++     | +++    |

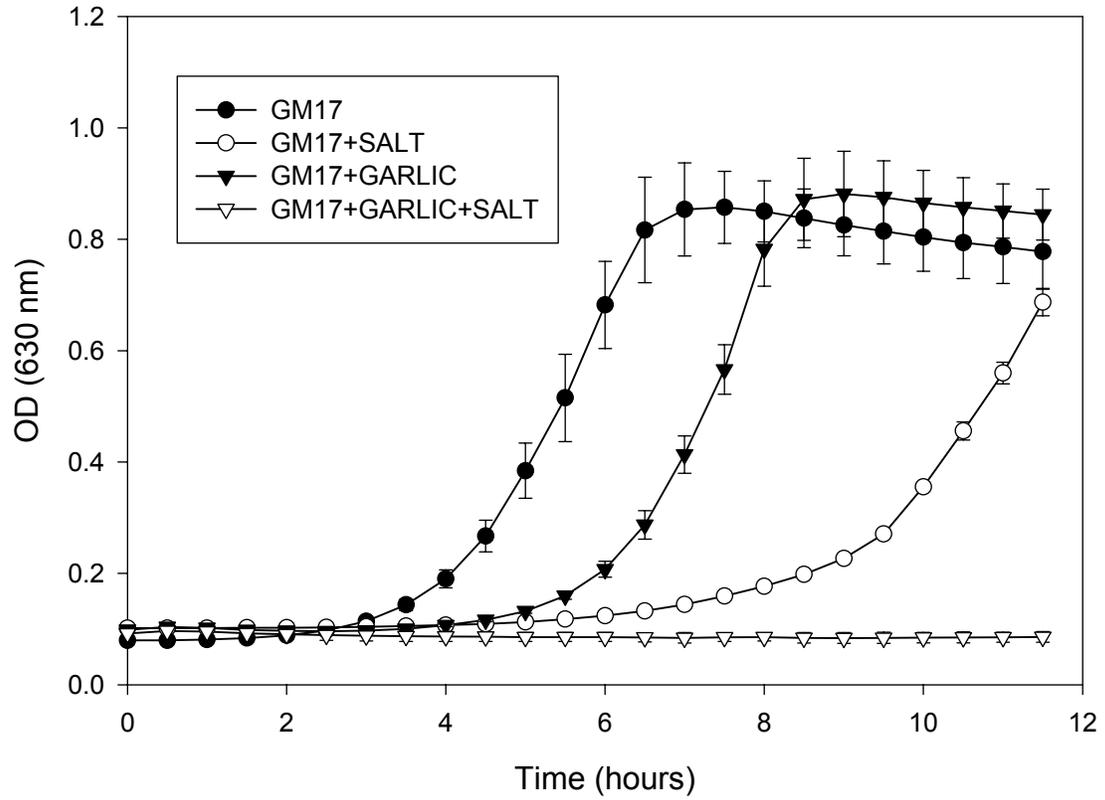
Tubes were incubated at 37°C and check for growth. Turbidity of the culture was reported as growth.

Fig 1. Effect of aquaresin garlic on *L. lactis* in GM17 at 30 °C



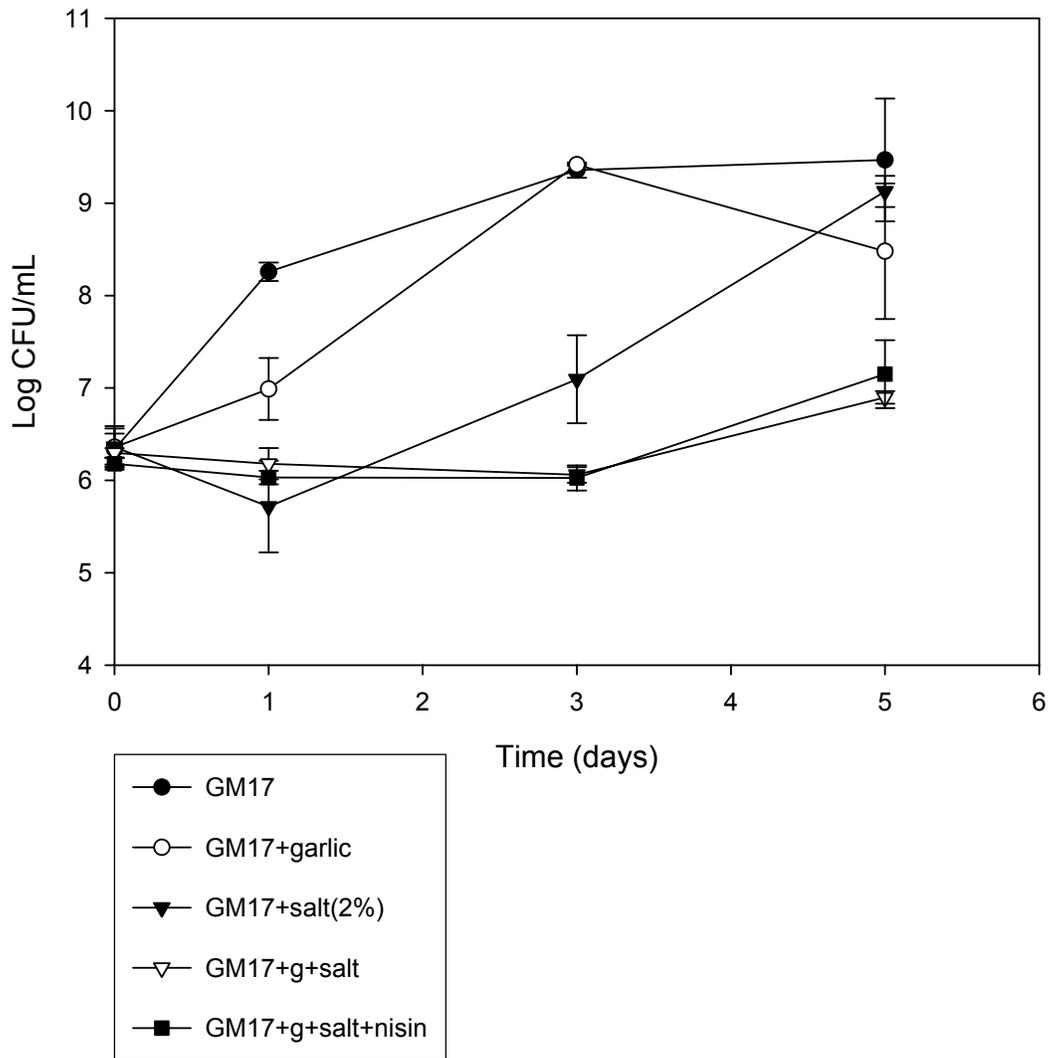
Numbers are the average of 2 samples. Salt was 2%, garlic concentration 0.77 mg/mL

Figure 2. Effect of garlic aquaresin and salt on the growth of *L. lactis* at 30°C



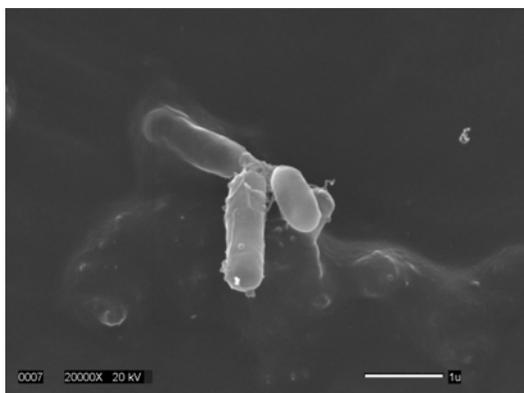
Each point is the average of 3 wells. Garlic concentration was 0.77 mg/mL, salt was 2%. Error bars show the standard deviations.

Figure 3. Effect of garlic, nisin and salt on *L. monocytogenes* at 18°C, pH 6.6

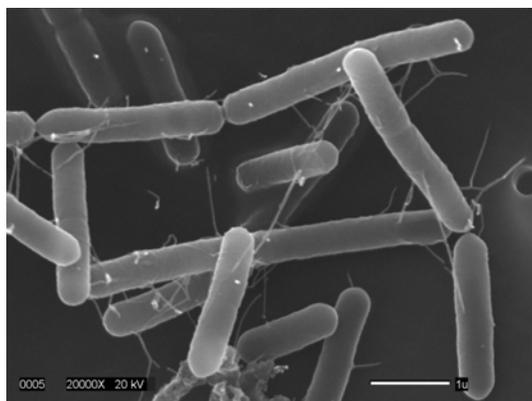


Numbers are the average of 3 samples. Nisin 50 µg/mL, garlic concentration 0.77 mg/mL. Error bars show the standard deviations.

Figure 4. Scanning Electron Microscopy of *Listeria monocytogenes* in the presence of garlic, salt and nisin at pH 5.



Inoculated cells  $1 \times 10^6$  CFU/mL. In the presence of nisin, garlic, salt at pH5



Inoculated cells  $1 \times 10^6$  CFU/mL Control in GM17 and salt

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## **Appendix 3**

**Conjugation of *Lactococcus lactis* strains isolated from Minimally Processed Brined Refrigerated Cucumbers and *Lactococcus lactis* strains nisin-producers**

## Abstract

Two *Lactococcus lactis* strains isolated from Minimally Processed Brined Refrigerated Cucumbers (MPBRC) were chosen as recipients of the nisin phenotype; they metabolize raffinose, and this can be used as natural marker. The donors were nisin-producer strains of *Lactococcus lactis* (ATCC 11454) and *L. lactis* (LA218) isolated from sauerkraut fermentation that carries pGKC. The method for gene transfer was conjugation; mating was done varying the proportions of donors and recipients on the surface of glucose milk agar plates, filter method and direct plating on to selective agar. Incubation of the plates was done at 18°C and 30°C for 18 and 6 hours, respectively. According to these results, conjugation occurred at 18°C and not at 30°C, and only between the recipients and the ATCC type strain. No transconjugants were recovered from the mating of LA218 and the recipients. The transconjugants from 11454 could inhibit the indicator strain (*Lactococcus lactis cremoris*), indicating the ability to produce nisin, compared with the original parents, which did not show any activity. However, when they were grown in broth, nisin activity was not detected.

PCR targeting the nisin genes revealed that the transconjugants did not have the genes involved in nisin production and secretion, but they had the genes involved in nisin immunity. Surprisingly, the recipients did have these genes too. This could mean that the strains already developed a system to avoid nisin production by deleting these genes but keeping the ones for immunity to survive in an environment where nisin producers could be found.

## Introduction

The development of starter cultures capable of in situ production of bacteriocins during fermentation has shown potential in the control of spoilage and pathogenic microorganisms in foods (Scannel *et al.*, 2001). Conjugation has been described widely in the lactococci, and has been exploited for the development of improved starter strains, notably by the introduction of plasmids carrying bacteriophage resistance determinants (Gason *et al.*, 1995, Klaenhammer, 1987).

Nisin is a bacteriocin effective against *Listeria monocytogenes* and other Gram-positive bacteria. Nisin biosynthesis, regulation and immunity depend on 11 genes, which are located on a large conjugative transposon in the chromosome. A/ZBTCIFEG gene cluster consists of 2 operons, and requires the presence of nisin for transcription (Quiao *et al.*, 1996, Ra *et al.*, 1996). The conjugative genes present on the nisin transposons make it possible to transfer the nisin gene cluster to other bacteria in a non recombinant fashion (Li and O'Sullivan, 2002). Transmission of the genes transforms non-producers into nisin producing strains; complete loss of the transposon converts a producer in a non-producer (Gason, 1984). However, some strains of *L. lactis* are not able to transfer genes, and others cannot accept the transposon (De Vuyst, 1994). *Enterococcus* transconjugants were immune to nisin but unable to produce it (Broadbent *et al.*, 1995). Nisin expression in a food grade manner in other LAB is essential to successful incorporation of these LAB in food systems (Li and O'Sullivan, 2002).

The objective of this study was:

- To make *Lactococcus* strains isolated from MPBRC to produce nisin through conjugation. A natural method to transfer genes without considering the bacteria a GMO (genetically modified organism).

## Material and Methods

### Bacterial strains

**Donors:** *L. lactis*, ATCC 11454 (NCK6540), raffinose<sup>-</sup>, nisin<sup>+</sup> obtained from T. Klaenhammer (NCSU, Raleigh, NC), *L. lactis* (LA 218) (USDA culture collection), raffinose<sup>-</sup>, nisin<sup>+</sup>, carrying plasmid pGKC, which confers chloramphenicol resistance (cm<sup>+</sup>).

**Recipients:** *L. lactis*, LR10, LR11, raffinose<sup>+</sup>, nisin<sup>-</sup> isolated from minimally processed brined refrigerated cucumbers.

Donors were grown in sucrose M17 broth (SM17) (Difco, MI) with 5 µg/mL of chloramphenicol (cm) for LA218, and recipients in raffinose M17 (RM17) at 30°C. Cultures were inoculated in the broth by inoculating a loop of frozen culture SM17 or RM17 broth and incubating overnight. The cultures were transferred (1%) into new SM17 or RM17 (10 mL) broth and incubated for 3-4 hours until OD of donor was 0.3 and recipients 0.4 at 630 nm OD. The donor cells were washed 3 times with sterile saline

solution and resuspended in 1 mL of saline solution. The recipient cells were washed one time and placed in ice until use.

### **Media for conjugation**

Milk agar medium was prepared with 5% skim milk powder and 1% glucose, 15 g of agar per liter of medium.

Brom cresol purple agar (BCP) with different carbons sources (sucrose, or raffinose, or glucose) and 40 mg/L of brom cresol purple agar was used as the indicator agar.

Selective media were prepared by adding chloramphenicol 5µg/mL or nisin 50µg/mL to raffinose indicator agar (RIA).

### **Conjugal Matings**

Conjugal matings were carried out on milk agar plates as described by Steele and McKay (1986). The filter method, described by Gason (1984), was also tested, and the direct plate conjugation (DPC), described by Broadbent and Condo (1991). Donors and recipients were mixed in the following ratios: 1:1, 1:2, 1:3, 1:4, and 100 µL were spotted on the center of glucose milk agar plates. Controls (donor alone or recipient alone) were also prepared. Plates were incubated at 18 °C overnight or at 30°C for 6 hours.

The cells were harvested from the plate with a sterile cotton tip and placed in saline solution. Serial dilutions were prepared to plate on non-selective agar (GM17), raffinose indicator agar, raffinose indicator agar + 50 µg/mL of nisin, RIA cm, and RM17cm.

Plates were incubated at 18°C for 72 hours or at 30°C for 48 hours, and counted.

### **Transconjugants**

Colonies growing on RIA with nisin, RIA cm, and RM17cm were picked with a tooth pick and plated on GM17. After 6 hours of incubation they were overlaid with the indicator microorganism *L. lactis cremoris*, (LA120). Colonies giving a clear zone of inhibition were isolated and grown in broth to test for nisin production. They were also tested for raffinose metabolism.

### **PCR characterization of transconjugants**

Isolated colonies were grown in GM17 broth. Cultures were frozen in GM17 with 20% glycerol and kept frozen at -80°C. One mL of the overnight culture was used for DNA isolation according to PROMEGA kit, and PCR targeting the nisin genes was performed. Primers targeting the *nis A* gene as described by Moschetti *et al.*, 1999 were used to characterize the isolates:

*nisL*: (5'→3') CGAGCATAATAAACGGC

*nisR*: (5'→3') GGATAGTATCCATGTCTGAAC

Additional primers were designed to target *nisABTCl*, *nisIPRK* and *nisFEG* genes by using the program: Primer3 Output (version 0.2) ([www-genomewi.mit.edu](http://www-genomewi.mit.edu)).

Primers were obtained from Sigma-Genosys (The Woodlands, Texas, USA). PCR was performed using a Robocycler Gradient 96 (Stratagene, La Joya, CA). PCR parameters were as follows:

1 cycle 92°C: 2 min, 30 cycles at 92°C: 30 sec, 55°C: 45 sec, 72: 1 minute, 1 cycle 72: 3 min.

### **Nisin determination**

One mL of a turbid culture was centrifuged, and the supernatant was adjusted to pH 7, filter sterilized, and tested for nisin activity by boiling at 100°C for 2 minutes. A control was included (broth without pH adjustment). The boiled supernatant was spotted on the surface of an MRS agar plate, allowed to dry and overlaid with the indicator strain. Plates were incubated at 30°C for 24 hours. A positive/negative control was also included in each test.

### **Results and discussion**

The three methods of conjugation tested gave different results using the same donor and recipient strains. Among the methods tested, the one that gave transconjugants was the milk agar method. No yellow colonies (transconjugants) were obtained on RM17 cm/RIA cm (chloramphenicol), indicating that no conjugation had occurred between strains LA218 and LR10/LR11. Colonies were white, indicating that the bacteria growing were unable to metabolize raffinose, compared to the control plate (raffinose+) which gave yellow colonies. This means that the strains were not compatible. Visual observation of the donor grown in broth did not show any characteristic described for strains able to conjugate. However, transconjugants were obtained from the mating of the ATCC type strain 11454. The culture in broth showed some kind of clumping or clot formation while

growing. This characteristic had been described for strains able to conjugate (*CluA*). *CluA* is involved in the establishment of cell to cell contact and can induce a constitutive cell aggregation phenotype (Gason, *et al.*, 1995). The clumping is due to the expression of a protein that allows cell to cell contact interaction; in this way bacteria can interchange genetic material. Yellow colonies growing on the indicator agar with nisin were considered the transconjugants. The control plates from the recipients did not show any growth, indicating that the colonies growing on this agar acquired nisin resistance, and therefore nisin biosynthesis ability. The transconjugants obtained are summarized in Table 1.

Results were positive for nisin production when observed on agar plates (Fig. 1). However, the results for nisin tests were negative when the bacteria were grown in broth (Fig. 2). No nisin was detected, even after induction of the nisin promoter by addition of 50 IU/mL of nisin (Li and O'Sullivan, 2002).

PCR targeting the nisin genes gave no product for the *nis* ABTCI, but gave product for the *nis* FEG genes in the recipients and the transconjugants. This could mean that the recipients may have had the genes for the nisin biosynthesis, and they may have lost the biosynthesis genes, but not the immunity genes.

## Conclusions

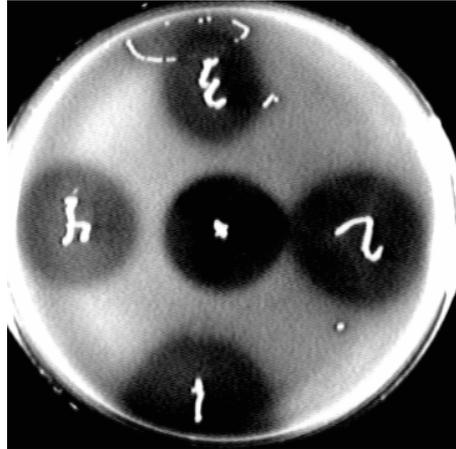
- Conjugation of LA218 and LR10/LR11 was not possible, meaning that these strains are incompatible, or LA218 has no ability for conjugation.
- Conjugation of these *L. lactis* strains (ATCC 11454 and LR10/LR11) was unsuccessful. This could be due to a mechanism of rejection developed by the recipients, or a deletion of the nisin biosynthesis genes.

Table 1. Conjugation of *L. lactis* strains

| Mating of lactocci (donor x recipient) | Phenotype          | Transfer frequency<br>(Transconjugants/donor CFU) |
|--|--------------------|---|
| ATCC 11454 x LR10                      | Raffinose+, nisin+ | $2.4 \times 10^{-8}$                              |
| ATCC 11454 x LR11                      | Raffinose+, nisin+ | $5.5 \times 10^{-8}$                              |
| LA218 x LR10                           | ND                 | ND  |
| LA218 x LR11                           | ND                 | ND  |

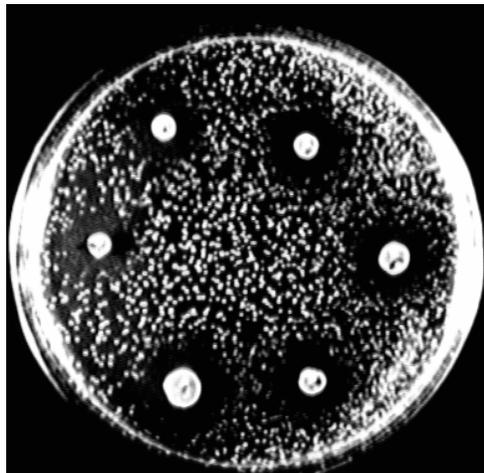
Mating was done on milk agar plates. Experiments were performed 3 times for each donor and recipient combination. ND (not determined, no TC obtained). Yellow colonies growing on RIAnisin plates were tested for nisin production by the lawn method.

Figure 1. Test for nisin production. Transconjugants overlaid with *L. lactis cremoris*



Middle- Donor (ATCC 11454), 1, 2, 3, 4- Transconjugants. Recipients gave negative result.

Figure 2. Test for nisin production. Transconjugants grown in broth.



TC grown in broth. 2 $\mu$ L of a turbid culture placed on the agar and placed at 30°C for 6 hours. After that overlaid with the indicator strain.

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## **Appendix 4**

### **Minimally Processed Brined Refrigerated Cucumbers, Product Development**

## Abstract

Blanched washed cucumbers (size 2B, 3.5-3.8 cm in diameter) were used to prepare the product. Cucumbers were placed into 46 oz glass jars, and different amounts of aquaresin garlic were added: 1g, 1.5 g, 2 g, 5 g. Pickling spices were irradiated and added in a constant amount in all the treatments (5g). Cold sterile 4% NaCl (5°C) was added immediately after blanching. Control jars were prepared with washed cucumbers and crushed garlic cloves to mimic the product from the supermarket. Jars were placed at 5°C for 5 days. Jars were opened for sensory panel evaluation.

According to taste panelists, the concentration of 1 g of aquaresin garlic was similar to the control (fresh garlic). In general, the evaluation gave a low score in all cases (1-5 scale) because of the fresh cucumber color, flavor and texture.

The recipe chosen to run all the experiments was as follows: 1 g of aquaresin garlic, 5 g of pickling spices, 4% sterile brine, and 680 g of blanched cucumbers.

## Introduction

Refrigerated pickles have gained popularity over the past few decades (approximately 20 percent of all pickles sales) because they undergo minimal processing, keeping their crispness and freshness. Refrigerated pickles may or may not be fermented before refrigeration. They may or may not be acidified and have preservatives added, and are held at room temperature for a few days, and then refrigerated to slow down the fermentation. Refrigerated pickles are available in many varieties, including kosher dills, genuine dills, half-sour, overnight, and sweet pickles, and are available whole or cut into halves, spears, slices, chips or relish, or are sliced lengthwise for sandwiches (Etchells *et al.*, 1976).

A type of non-fermented, non-acidified, no-preservatives-added pickle has to be refrigerated during its whole storage life. This type of product represents 5% of the refrigerated pickles in the retail store (Fleming, 2003). The first step in producing refrigerated pickles is choosing high quality fresh cucumbers. They are graded, washed and packed within hours after they are picked. Cucumbers are chosen for their size, firmness, color, texture and flavor. They are put into jars, covered with a seasoned solution (4% brine, equilibrated to 2%) and vacuum sealed. The product may contain some vinegar (acetic acid), and may include spices, such as fresh garlic cloves (crushed) and dill. The filled jars are immediately refrigerated (Etchells *et al.*, 1976). They are held in refrigeration for a few weeks, so the cucumbers can absorb the seasonings. This curing

step is important in developing the perfect refrigerated pickle. Also, it is the step that officially changes the cucumber to a pickle.

Refrigeration gives these pickles a distinct, fresh, raw cucumber flavor. The production and storage of this item under refrigeration prevents the pickles from spoiling. The pickles are ready for shipping when the concentration of salt within the pickle equals the concentration of the brine. The finished pickles have a distinct, fresh flavor (very much like a deli pickle), are very crisp, and maintain their fresh green cucumber color desired by an important segment of consumers.

The objectives of this study were:

- To use blanched cucumbers, sterile pickling spices, sterile aquaresin garlic to determine the amounts needed to mimic the product from the supermarket.
- To be able to use the product for studies by adding a biocontrol agent in the product and be able to follow it during the course of storage.

## **Materials and Methods**

### **Deli-type pickle product**

Cucumbers (3.5-3.8 cm diam) were washed and blanched for 15 seconds at 80°C (Breidt et al., 2000) and immediately cooled with 4% cold brine (5°C) containing 1, 2, 3, 4, 5 g of sterile garlic oil (Aquaresin garlic, Kalsec, Kalamazoo, MI), and 5 g of irradiated pickling spices (13 KGy). Jars were placed at 5°C for 5 days and then opened to run a taste panel. Control jars were also prepared to mimic the product from the supermarket, the jars had washed cucumbers with fresh crushed garlic clove, and 5 g of pickling spices.

### **Evaluation sheet**

The panel consisted of 13 volunteers, and they were asked to fill the following questionnaire and score each sample in a scale of 1-5.

## NARPS RATING SHEET

Name \_\_\_\_\_

Date \_\_\_\_\_

### *EVALUATION*

**Rate the samples as follows:**

**Very similar  
Similar  
Different**

**How Do you compare samples 1, 2, 3 to sample 5?**

**1-5**

**2-5**

**3-5**

**How Do you compare sample 4 to sample 5?**

**How Dou you compare sample 5 to sample 6?**

**Did you like the product, rate in a scale 1-5 (1 dislike, 5 like very much)?**

## Results and Discussion

In general, all the samples were evaluated very low in the scale of 1-5. Panelists were disappointed with the product because their expectations were of a pickle type taste, not a fresh raw cucumber color, texture and flavor. However, after being instructed on the product characteristics, they agreed to point out that the name of the product was misleading, and that they shouldn't be called pickles. For that reason, the author of this thesis changed the name to Minimally Processed Brined Refrigerated Cucumbers for technical purposes, and for marketing purposes to FRESCU (Fresh refrigerated spicy cucumbers).

Rating of the product was low because it did not have the pickle flavor. Panelists agreed to say that the pickle had a raw, spicy cucumber flavor.

The formulation used in all the studies that mimic the product from the supermarket was as follows:

Cucumbers (3.5-3.8 cm diameter) were washed and blanched for 15 seconds at 80°C, 4% cold brine containing 1 g of sterile garlic oil, and 5 g of irradiated pickling spices (Fig. 1).



Figure 1. Minimally Processed Brined refrigerated Cucumbers (MPBRC)

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