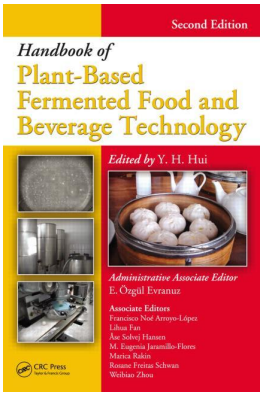


# Handbook of Plant-Based Fermented Food and Beverage Technology

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## Fermentation and Biopreservation of Plant-Based Foods with Lactic Acid Bacteria

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# 3

# *Fermentation and Biopreservation of Plant-Based Foods with Lactic Acid Bacteria*

**Lihua Fan and Lisbeth Truelstrup Hansen**

**CONTENTS**



## 3.1 Introduction

Lactic acid bacteria (LAB) is the term used for a large and rather diverse group of prokaryotes, which have in common the production of lactic acid as their main metabolic end product (Klaenhammer et al. 2005). Traditionally, LAB have been characterized as aerotolerant anaerobes belonging to the Gram-positive bacterial phylum. In the new molecular taxonomy in which organisms are clustered according to their genetic relatedness, the LAB clade is grouped with the firmicutes together with other low %G+C Gram-positive bacteria such as *Clostridium*, *Bacillus*, *Staphylococcus*, *Listeria*, etc. (Teuber and Geis 2006). Use of a combination of traditional and molecular techniques has divided bacteria belonging to the LAB family up into the following genera: *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Melissococcus*, *Tetragenococcus*, *Vagococcus*, *Trichococcus*, *Carnobacterium*, *Desemzia*, *Leuconostoc*, *Weisella*, and *Pediococcus* (Hammes and Hertel 2006).

It has been generally recognized that the tolerance of oxygen varies among strains and that, strictly speaking, LAB have no use for oxygen as they do not carry out oxidative phosphorylation but only use substrate level phosphorylation or fermentation to create energy (Björkroth and Holzapfel 2006). Lately, however, evidence has appeared that some members of LAB including *Lactobacillus sanfranciscensis*

may be using NADH-oxidases or Mn(II) to allow the use of oxygen as an external electron acceptor which in turn significantly increases the energy yield (Jänsch et al. 2011).

LAB naturally occurs in a large variety of natural environments in which soluble carbohydrates, protein breakdown products, and low oxygen tension are found. The production of extracellular polysaccharides by many LAB allow them to adhere well to surfaces and making them excellent biofilm formers (Ruas-Madiedo et al. 2009). In general, LAB are resistant to low pH conditions and also have some resistance to higher osmotic pressures. These characteristics are important when it comes to the industrial use of LAB, in which bacteria play an important role in the preservation and microbial safety of many fermented foods. The basis of LAB's protection of foods is mainly due to their production of organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds such as free fatty acids or phenyllactic acid, bacteriocins, and antibiotics such as reutericyclin (Höltzel et al. 2000). Bacteriocins are ribosomally synthesized, extracellularly released low molecular mass peptides which have a bactericidal or bacteriostatic effect on bacteria, either from the same species or across genera (Klaenhammer 1988; Cotter et al. 2005). Although nisin is the only purified bacteriocin used thus far in industrially processed foods, many bacteriocins produced by various LAB may have potential applications in food products (Helander et al. 1997; Cleveland et al. 2001; Settanni and Corsetti 2008). Increasingly, it is also recognized that LAB have antifungal properties and may be used to eliminate fungal spoilage or the formation of mycotoxins (Rouse et al. 2008; Dalié et al. 2010). Also, through the manipulation of the intrinsic and extrinsic environment, LAB can naturally outcompete unwanted microorganisms such as *Staphylococcus aureus* (Charlier et al. 2009). An example of this outcompetition is seen in the production of sauerkraut, in which one of the production steps consists of the addition of salt to decrease water activity and at the same time to extract moisture and nutrients from the shredded cabbage. The growth of LAB is now selected over the growth of endogenous Gram-negative spoilage bacteria. The subsequent decrease in pH caused by the production of lactic (and acetic) acid by LAB further causes Gram-negative bacteria to stop growing and slowly die off (Adams and Nicolaides 1997). Furthermore, studies have shown that LAB, during the fermentation of plant material such as carrot juice, degrade oxalate, phytate, and tannins, which in combination with the decrease in pH, increase the solubility (bioavailability) of essential metals such as iron, manganese, and zinc but not copper (Bjergqvist et al. 2005).

## 3.2 Plant-Associated LAB

LABs are found naturally occurring in a wide range of environments, including the surface of growing and postharvest plant materials. Naturally, selection means that successful LAB have become adapted to their particular environment. This adaptation can be expressed in different ways, which includes the ability to use the available nutrients, tolerance of the pertinent environmental conditions (intrinsic and extrinsic factors), and possibly the production of antimicrobial compounds to inhibit competitors and secure survival. In choosing a suitable LAB for biopreservation and fermentation purposes, it is most obvious to look for strains/species which are already adapted to grow well under the given plant nutritive conditions.

Therefore, which LAB are regularly found in fermented and nonfermented fruit and vegetable products? Literature reports using both culture-dependent and -independent methods mainly associate members of the LAB genera *Leuconostoc, Lactobacillus, Weissella, Pediococcus, Enterococcus*, and *Lactococcus* with fruits and vegetable products (see recent reviews by Björkroth and Holzapfel 2006; Devriese et al. 2006; Hammes and Hertel 2006; and Teuber and Geis 2006). In the past, culture methods have identified homofermentative strains such as *Lactobacillus plantarum* as being very common. Of the heterofermentative LAB, species such as *Leuconostoc mesenteroides* and *Leuconostoc citreum* dominate. However, this view on the plant-associated LAB flora is bound to change as more studies using molecular biology methods investigate the microbial ecology of products.

In one such recent study conducted by Plengvidhya et al. (2007), an rRNA gene intergenic transcribed spacer–polymerase chain reaction method combined with 16S rRNA sequencing was used to identify 686 LAB isolated from four sauerkraut fermentations. Strains normally identified by traditional biochemical

methods including *Leuco. mesenteroides*, *Lb. plantarum*, and *Lactobacillus curvatus* were abundant. However, unexpectedly, they found low numbers of *Pediococcus pentosaceus* and *Lactobacillus brevis*, which are LABs normally expected to play a major role in fermentation. Use of the molecular method revealed the presence of strains such as *Leuco. citreum*, *Leuconostoc argentinum*, *Lactobacillus paraplantarum*, *Lactobacillus coryniformis*, *Weissella* sp., and the newly described *Leuconostoc fallax*, indicating that the LAB flora developing during fermentation of sauerkraut is much more diverse than previously thought. When using 16S rRNA sequencing to analyze the identity of 120 LAB strains isolated during kimchi fermentation, *Leuco. citreum* was found to dominate in the early phase of fermentation whereas *Lactobacillus sakei/Lb. curvatus* and *Lb. brevis* took over in the latter phases (Choi et al. 2003). They also isolated a few *Weissella confusa* strains. A more recent investigation of kimchi fermentations confirmed the presence of these LAB species as well as *Weisella koreensis* and in the overripening stage also *Leuconostoc gelidum* and *Lb. brevis* (Park et al. 2010).

## 3.3 Fermentation of Plant Material

The fermentation of plant material is an ancient preservation method, which likely originated in Asia. Nowadays, the traditional Korean product, kimchi, is the most popular fermented vegetable food in many Asian countries (Cheigh and Park 1994; Lee 1997; Kwon and Kim 2007). The most common fermented vegetable products in Europe and North America (Canada and the United States) are sauerkraut, cucumbers, and olives (Niksic et al. 2005; Mäki 2004; Settanni and Corsetti 2008). Before the advent of modern food preservation technologies such as refrigeration, controlled atmosphere storage, and freezing, the consumption of fermented vegetable products ensured continuous access to essential vitamins including vitamin C, minerals, and dietary fibers for people in Northern climates with just one annual growth season.

### 3.3.1 Kimchi

#### *3.3.1.1 Introduction*

Kimchi is a traditional Korean fermented vegetable product, which is most commonly prepared with Chinese cabbage as the main ingredient in combination with many minor ingredients such as red pepper powder, ginger, garlic, radish, and fish sauce (Cheigh and Park 1994; Kim et al. 2008). Kimchi preparation methods differ depending on the variety of kimchi and the ingredients used, as well as on whether the production takes place domestically or industrially. Also, the types of kimchi differ from region to region because of differences in harvest and weather conditions. The final flavor profile is determined by the ingredients, condiments, the addition of salt and spices, and fermentation. According to a review by Lee (1997), approximately 190 different types of kimchi are produced in Korean households. In general, kimchi tastes best when it is fermented for 2 to 3 weeks at 2°C to 7°C, to reach a pH level of 4.2 and a titratable acidity of 0.6% to 0.8% (Kim et al. 2008).

#### *3.3.1.2 Fermentation Process*

The principal process consists of pretreatment, brining, blending of ingredients, and fermentation steps. For example, the preparation method for Baechu kimchi (cut Chinese cabbage kimchi) is as follows: pretreated Chinese cabbage is cut into cubes of 3 to 5 cm, brined in a salt solution (8–15% w/v) for 2 to 7 hours, rinsed with fresh water, and drained. In a separate process, sliced Oriental radish, carrots, green onion, onion, chopped garlic, ginger, red pepper, salt-pickled seafood, other minor ingredients, and dry salt are combined to make a premixture according to the specific recipe. This premixture is blended well with the treated cabbage. This mixture is then placed in a fermentation vessel sealed with a lid and fermented either for 1 to 3 weeks at low temperatures of 2°C to 10°C or for 1 to 3 days at 20°C to 25°C. After fermentation, kimchi is usually stored at temperatures between 0°C and 8°C and served cold (Cheigh and Park 1994).

The fermentation occurs mainly due to LAB and yeast strains, which are naturally present in the diverse microflora of the raw material. The fermentation of kimchi is initiated by *Leuco. mesenteroides* under anaerobic conditions (Cheigh and Park 1994; Lee 1997). This organism differs from other LAB species in that it can tolerate fairly high concentrations of salt and sugar. *Leuco. mesenteroides* initiates growth in vegetables more rapidly over a wide range of temperatures and salt concentrations than any other LAB. Its heterofermentative metabolism produces carbon dioxide and acids which rapidly lower the pH and inhibit the development of undesirable microorganisms. The carbon dioxide produced replaces the oxygen, making the environment anaerobic and suitable for the growth of subsequent species of lactobacillus. Removal of oxygen also helps to preserve the color of fermented vegetables and stabilizes the ascorbic acid (vitamin C) that is present in the vegetables. As the pH drops to between 4.6 and 4.9, *Leuco. mesenteroides* becomes relatively inhibited, but the fermentation continues with other LAB such as *Streptococcus* (now classified as *Enterococcus*) *faecalis*, *Lb. brevis*, *Pediococcus cerevisiae*, and *Lb. plantarum*. Lee et al. (2005) analyzed the kimchi microflora using denaturing gradient gel electrophoresis, a 16S rRNA gene–based culture-independent molecular technique, and found that *W. confusa*, *Leuco. citreum*, *Lb. sakei*, and *Lb. curvatus* were the main microorganisms responsible for kimchi fermentation. Other techniques, which have been used to analyze the microbial community dynamics during kimchi fermentation, include genome-probing microarray for quantitative, high-throughput monitoring (Bae et al. 2005), polymerase chain reaction–enzyme-linked immunosorbent assay for the screening of many bacterial isolates from fermented vegetables (Tamminen et al. 2004) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis profiles of whole cell proteins for the identification of LAB in kimchi (Tae-Woon et al. 2003).

The most important factors that affect kimchi fermentation are the endogenous plant microorganisms, salt concentration, fermentable carbohydrates, other available nutrients, presence of inhibitory compounds as well as oxygen, pH, and temperature. The effect of salt concentration, temperature (Lee et al. 2008), and pH is mostly due to the influence of these factors on the rate and extent of lactic acid fermentation. The optimum fermentation time to obtain the most acceptable quality of fermented kimchi with different salt concentration and temperature combinations has been investigated by Mheen and Kwon (1984). They found that the optimum period of fermentation for Baechu kimchi is 1 to 2 days at 30°C with a 3.5% salt concentration, and 2 days with a 5% salt concentration and the same temperature. At 14°C, 5 to 12 days are required if combined with 3.5% salt, and this increases to 10 to 18 days if 5.0% salt is used.

The use of well-defined starter cultures is of considerable commercial interest as it can improve the control and predictability of the fermentation process (Chang and Chang 2010). Choi et al. (2003) isolated the dominant *Leuco. citreum* IH22 in their polyphasic study of the LAB community in kimchi fermenting at 15°C and successfully used this strain to obtain a faster and more consistent fermentation to yield a product with better organoleptic properties than the control kimchi.

#### *3.3.1.3 Microbiological Characteristics*

The kimchi fermentation pattern is similar to that found in other vegetable lactic acid fermentations; however, due to the many types of kimchi products, comparatively greater variance is found in the associated LAB flora than in other products. Also, because the raw ingredients for kimchi are traditionally not blanched before use, the epiphytic LAB as well as the rest of the other microflora associated with the plant material in the field will be retained to possibly influence the fermentation.

As with other vegetable fermentations, the brining step is very important for kimchi fermentation. Brining extracts the water and nutrients from the raw materials by osmotic activity and suppresses the growth of some undesirable bacteria that may spoil the kimchi. At the same time, it makes conditions relatively favorable for LAB by increasing the salt content in the Chinese cabbage or radish. The LAB count increases approximately fourfold after brining of the Chinese cabbage (Kim et al. 1987; Cheigh and Park 1994). Also, after the brining and rinsing/washing treatments, the counts of bacteria, yeasts, and molds are greatly decreased.

The fermentation containers are usually covered with lids to provide anaerobic conditions and thus minimize the growth of aerobic microorganisms. *Leuco. mesenteroides* is the predominant LAB in the

early fermentation stages but, because of the decrease in pH, is gradually replaced by other LAB such as *E. faecalis*, *Lb. brevis*, *P. cerevisiae*, and *Lb. plantarum*. There is a considerable overlap of LAB species. The growth of each species depends on its initial number in the raw material (Chinese cabbage and other ingredients), the concentrations of salt and sugar, the absence of oxygen, and the fermentation temperature. *Lb. plantarum* is commonly present in the greatest numbers after the initial fermentation and results in maximum acidity at the later stages. Aerobic yeasts and molds may appear on the surface of improperly covered vegetables at the later fermentation stages. The appearance of undesirable and spoiled kimchi, which is characterized by off-flavors and softened texture, is possibly due to excessive aerobic growth of molds and film-forming yeasts (Cheigh and Park 1994). Softening and excessive acidification of kimchi at the overripening stages during fermentation and storage are the most serious problems. It should be pointed out that once prepared, kimchi is ready-to-eat at any stage of the fermentation depending on the individual consumer's preferential taste. Because there is no sterilizing process during kimchi processing, microbial quality and safety are very important issues for the shelf life of kimchi (Kwon and Kim 2007).

### 3.3.2 Sauerkraut

#### *3.3.2.1 Characteristics of LAB in Sauerkraut Fermentation*

The LAB that dominate Western vegetable fermentations, such as sauerkraut, mainly belong to the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. Homofermentative strains of lactobacilli produce 85% lactic acid from glucose, whereas heterofermentative strains produce lactic acid, carbon dioxide, ethanol, and acetic acid in equimolar amounts. The strains of *Lb. sakei* and *Lb. curvatus* are phenotypically closely related, and they are considered beneficial in sauerkraut fermentation (Mäki 2004).

Previous studies using traditional biochemical identification methods to study the ecology of commercial sauerkraut fermentations revealed that the obligate heterofermenter, *Leuco. mesenteroides*; the facultative homofermenter, *Lb. plantarum*; the homofermenter, *Ped. pentosaceus*; and the obligate heterofermenter, *Lb. brevis* were the primary microorganisms responsible for fermentation. However, as previously mentioned, using DNA fingerprinting techniques, Plengvidhya et al. (2007) identified more species of LAB to be present in sauerkraut fermentation including *Leuco. citreum*, *Leuco. argentinum*, *Leuco. fallax*, *Lb. paraplantarum*, *Lb. coryniformis*, and *Weissella* sp.

In addition to the desirable LAB, there are undesirable microorganisms present on cabbage which can interfere with the sauerkraut production process. The quality of the final product depends largely on how well the undesirable organisms are controlled during the fermentation process. Some spoilage organisms use the protein as an energy source, producing unpleasant odors and flavors.

#### *3.3.2.2 Fermentation Process*

Shredded cabbage is placed in a container and salt is added. Mechanical pressure can be applied to expel the cabbage juice, which contains fermentable sugars and other nutrients suitable for LAB activity. The heterofermentative gas-producing cocci (*Leuco. mesenteroides*) start the initial fermentation. When the acidity (lactic acid) reaches 0.25% to 0.3%, the activity of this group of LAB slows down, although their enzymes continue to function. The fermentation is subsequently continued by the lactobacilli (*Lb. plantarum*, *Lb. cucumeris*, and *Lb. brevis*) until the acidity level increases to 1.5% to 2%. The high salt concentration and low temperature inhibit these bacteria to some extent. Finally, *Lb. pentoaceticus* continues the fermentation, bringing the acidity to 2% to 2.5% to complete the fermentation (Vaughn 1985; Daeschel et al. 1987).

The end products from the sauerkraut fermentation are lactic acid along with smaller amounts of acetic and propionic acids, a mixture of gases (of which carbon dioxide is the principal gas), small amounts of alcohol, and a mixture of aromatic esters. The acids, in combination with alcohol, form esters which contribute to the characteristic flavor of sauerkraut. The acidity helps in controlling the growth of spoilage organisms and contributes to the extended shelf life of the product.

#### 3.3.2.3 Temperature and Salt

Temperature control is one of the most important factors in the sauerkraut production process. The optimum temperature for sauerkraut fermentation is approximately 21°C. A temperature range of 18°C to 22°C is most desirable for initiating fermentation due to the growth and metabolism of *Leuco. mesenteroides*. In contrast, temperatures higher than 22°C favor the growth of *Lactobacillus* species.

Salt also plays an important role in initiating the sauerkraut production process and affects the quality of the final product (Viander et al. 2003; Holzapfel et al. 2003). Salt is added to withdraw juice from the cabbage, thus creating a more favorable environment for the development of the desired LAB flora. Generally, salt is added to a final concentration of 2.0% to 2.5%. At this concentration, lactobacilli are slightly inhibited, but cocci are not affected. It is essential to use pure sodium chloride because salts with added alkali may neutralize the acid.

#### 3.3.2.4 Starter Cultures

Instead of relying on the endogenous LAB flora and inherent variability between different harvest areas, cultivars, processing environments, etc., the use of starter cultures can improve the fermentation process to ensure the safety and health aspects of the products. More importantly, the use of starter cultures results in a more controllable and consistent quality sauerkraut and helps to speed up the fermentation process. Starter cultures also helps ensure that the correct level of acidity is reached in the fermented product such that undesirable (Gram-negative) microorganisms are inhibited. It is possible to add starter culture such as *Lactococcus lactis* without adverse effects on final product quality. These starters only survive for a short time but initiate the acidification process; therefore, they do not disturb the natural sequence of microorganisms. The racemase-deficient *Lactobacillus bavaricus* strains are also used as starter cultures in sauerkraut (Mäki 2004). Johanningsmeier and colleagues (2005) successfully used *Leuco. mesenteroides* with or without malo-lactic activity to ferment sauerkraut. However, although the addition of *Leuco. mesenteroides* in the early stages of fermentation can provide attractive flavors to the final product (Breidt 2004), the sequence of subsequent LAB growth may become altered to possibly result in a final product that is incompletely fermented. The correct sequence of organisms is essential in achieving a stable product with typical sauerkraut flavor and aroma. It should be noted that the juice from previous sauerkraut fermentations was traditionally used as a starter culture for subsequent fermentations. The efficacy of using old spent juice depends largely on the types of organisms present in the juice and its acidity (Battcock and Azam-Ali 1998).

#### 3.3.2.5 Spoilage and Defects in the Sauerkraut Process

Most spoilage of sauerkraut is due to aerobic soil microorganisms which break down the protein to produce undesirable flavor and texture changes. The growth of these aerobes can be controlled by the fermentation rate and control of temperature and salt concentrations.

Whenever the normal sequence of bacterial growth is altered, it usually results in a soft product. *Lactobacillus* sp. seem to have a greater ability than the cocci to break down cabbage tissue, thereby causing the softening. High fermentation temperatures and reduced salt concentrations favor the growth of *Lactobacillus* sp. If the salt concentration is too low initially, the *Lactobacillus* sp. will grow rapidly at the beginning and affect the normal sequence of fermentation.

Several factors can favor the growth of spoilage organisms. For example, an uneven distribution of salt and an insufficient level of cabbage juice to cover the sauerkraut during the fermentation can allow undesirable aerobic bacteria and yeasts to grow on the surface of the sauerkraut, causing production of off-flavors and discoloration. In addition, if the fermentation temperature is too high, growth of undesirable pigmented microorganisms may result in the development of dark spots (Battcock and Azam-Ali 1998).

Pink sauerkraut is caused by a group of yeasts which produce an intense red pigment in the juice and on the surface of the cabbage (Battcock and Azam-Ali 1998) if the salt is unevenly distributed or added to an excessive concentration, allowing yeast to grow while inhibiting the LAB. Savard et al. (2002) isolated and identified two spoilage yeasts, *Saccharomyces bayanus* and *Saccharomyces unisporus*, from

spoiled fermented vegetables. In addition, the shelf life of unpasteurized fermented vegetables is influenced by the presence of fermentative yeasts which may grow during storage if the sugar catabolism by LAB is incomplete (Fleming et al. 1985).

### 3.3.3 Comparison of Kimchi and Sauerkraut

Although the Western and Eastern fermented vegetable products seem to share many commonalities, there are some differences. For example, the preferred end point of fermentation is different for kimchi and sauerkraut (Lee 1997). In the Oriental tradition, the best-tasting kimchi is attained before overgrowth of *Lb. brevis* and *Lb. plantarum* happens, with an optimal product pH of 4.5. The overgrowth of *Lb. brevis* and *Lb. plantarum* affects the quality of kimchi due to their acidification of product pH values to lower than 4.5. In contrast, sauerkraut production depends on these organisms and the fermentation is specifically manipulated to promote their growth by using elective salt and temperature combinations. The optimal range of salt concentration of sauerkraut is 0.7% to 3.0%, whereas it is 3.0% to 5.0% for kimchi (Lee 1997; Holzapfel et al. 2003).

Several strains of bacteriocin-producing microorganisms have been isolated from kimchi and sauerkraut (Choi et al. 1999; Ohmomo et al. 2000; Settanni and Corsetti 2008). For example, *Enterococcus faecium* strains found in kimchi have been reported to have a broad spectrum of bacteriocin activities, and several *Lactobacillus* spp. have also been shown to have an antimicrobial effect. The organic acids, bacteriocin(s) produced during fermentation, and the antimicrobial activity of the ingredients together contribute to the control of undesirable microflora, including pathogenic microorganisms, without costly preservation treatments and packaging (Lee 1997).

## 3.4 Biopreservation of Vegetable Products

The idea of using biopreservation with innocuous commensal bacteria to inhibit foodborne microbial pathogens and spoilage organisms is not new but has mainly been explored in meat and fish products (Calo-Mata et al. 2008; Castellano et al. 2008; Lücke 2000). This purposeful use of microorganisms and their metabolites aims to extend the shelf life and improve the safety of foods (Ross et al. 2002). It is, in particular, biopreservation by use of active LAB cultures and their metabolites including bacteriocins that has gained considerable interest both in terms of their antibacterial (Chen and Hoover 2003; Bhattacharyya and Bhattacharjee 2007) and antifungal effects (Schnürer and Magnusson 2005).

As fresh-cut, ready-to-eat fruit and vegetable products are becoming more popular with consumers, the food industry needs to seek out ways of managing the associated microbial hazards and quality problems. Biopreservation with LAB of plant origin may present an attractive alternative to chemical washes and preservatives (Schnürer and Magnusson 2005; Allende et al. 2007; Settanni and Corsetti 2008). It is generally recognized that LAB best suitable for biopreservation should originate from the same type of food material as they are intended for use in (Vescovo et al. 1995, 1996). In a search for plant LABs with biopreservative properties, Trias and colleagues (2008) isolated 523 LAB strains from more than 700 samples of fruits and vegetables. After an initial screening, they selected 18 antagonistic strains for further characterization. Challenge experiments with *Leuco. mesenteroides*, *Leuco. citreum*, *Lb. plantarum*, *Lact. lactis*, and *Weisella cibaria* on apples and lettuce showed that four *Leuconostoc* sp. strains completely inhibited *Listeria monocytogenes* and reduced the growth of *Escherichia coli* and *Salmonella typhimurium*. Rouse and colleagues (2008) investigated the antifungal properties of plant-derived *W. confusa*, *W. cibaria*, *Lb. plantarum*, and *Ped. pentosaceus* strains and found activity against a range of spoilage fungi. As their work was performed in Spain and Ireland, respectively, we were wondering if antagonistic LAB could also be isolated from local produce from our area in Atlantic Canada and used for biopreservation.

### 3.4.1 Case Study

As biopreservation of fresh or fresh-cut products using bacteriocinogenic LAB isolated from vegetables presents an innovative approach to improve the safety and quality of the products, we examined fourteen

different local vegetable products for content of native LAB (Sharpe 2009). After isolation on LAB-elective agars and tentative identification, cell-free supernatants (CFS) from 92 isolates were screened for antimicrobial activity using the agar diffusion bioassay with minor modifications (Herreros et al. 2005). This resulted in the isolation of eight bacteriocinogenic LAB isolates, which retained inhibitory activity against both indicator bacteria, *Listeria innocua* (ATCC 33090) and *Lb. sakei* subsp. *sakei* (ATCC 15521), after neutralization of pH and elimination of $H_2O_2$ in CFS. The proteinaceous nature of the bacteriocin(s) was further confirmed by incubating the CFS with proteolytic enzymes (1 mg/mL α-chymotrypsin [C4129], protease [P4630], or trypsin [T8802] from Sigma-Aldrich Corporation, St. Louis, MO) at 37°C for 2 hours as this treatment led to the disappearance of the inhibition zones. The eight strains were later identified using 16S rRNA gene sequencing following the method of Abnous et al. (2009) as one *Lact. lactis* ssp. *lactis* and seven *E. faecium* strains (Table 3.1).

We further tested the eight strains for their antibacterial effectiveness against common produce pathogens and spoilage organisms at 5°C and 20°C using agar diffusion bioassays as well as the antifungal effect against fungal spore growth using a microdilution plate method slightly modified from work done by Lavermicocca et al. (2003). We found that the bacteriocin-like substances (BLS) produced by the eight strains showed a significant antimicrobial effect against *Li. innocua* (Figure 3.1), however, only their production of organic acids and $H_2O_2$ showed strong antimicrobial effects against *Pseudomonas fluorescens* (A7B), *Erwinia carotovora* (ATCC 15713), *Bacillus cereus* (ATCC 14579), *Penicillium expansum* (Pex 03–10.1), *Botrytis cinerea* (B94-b), *Monilinia fructicola* (Mof 03-25), and weak effects against *Leuco. mesenteroides.* The antifungal effect of *E. faecium* and *Lact. lactis* on *Pen. expansum*, *Bo. cinerea*, and *M. fructicola* during incubation at 5°C and 20°C was mainly due to the content of organic acids and $H_2O_2$ in the LAB CFS, as shown in Figures 3.2 and 3.3.

Furthermore, we applied *E. faecium* (13.2) and *Lact. lactis* (7.17) onto fresh-cut salads to investigate their effect on the growth of the natural microflora and *Li. innocua* inoculated on salads. Results showed that the addition of the LAB isolates significantly reduced the loads of naturally occurring *Listeria* sp. ($P = 0.033$), yeasts ($P = 0.011$), *Pseudomonas* sp. ($P = 0.010$), and coliforms ($P = 0.011$) in comparison with the controls. *E. faecium* and *Lact. lactis* also significantly reduced the growth of *Li. innocua* on fresh-cut salads ($P = 0.005$) during a 10-day storage trial at 5°C. Scanning electron microscopy revealed a significantly reduced presence of *Li. innocua* on the surface of fresh-cut salads after the addition of the bacteriocinogenic LAB (Figure 3.4).

In conclusion, our results showed that it is possible to readily isolate antagonistic LAB from vegetable products from Nova Scotia. The BLS producing *E. faecium* and *Lact. lactis* were demonstrated to have the potential to be used as protective cultures in fresh-cut produce to control spoilage and pathogenic microorganisms, thereby improving product shelf life and safety. Although the production of organic acids and $H_2O_2$ by the LAB was important for the inhibition of all test organisms, *Li. innocua* was inhibited solely by the BLS compounds produced by the LAB strains. Future characterization of the bacteriocins produced by the LAB strains and their mechanism of action may result in a better understanding of their implications in food systems, perhaps allowing for more targeted use to inhibit specific

**TABLE 3.1**

Origin and Identification of the Eight BLS Producing LAB Using 16S rRNA Gene Sequencing

| Isolates | Ribosomal Database Project Identification | Accession No. | Similarity (%) | Source |
|---|---|---|---|---|
| 7.12 | *E. faecium* | GQ370527 | 99.2 | Mung bean sprouts |
| 7.14 | *E. faecium* | DQ471797 | 98.6 | Mung bean sprouts |
| 7.17 | *Lact. lactis* subsp. *lactis* | FJ749490 | 99.0 | Mung bean sprouts |
| 12.6 | *E. faecium* | DQ471797 | 99.4 | Swiss chard |
| 12.8 | *E. faecium* | DQ471797 | 97.8 | Swiss chard |
| 12.9 | *E. faecium* | DQ471797 | 100 | Swiss chard |
| 13.1 | *E. faecium* | DQ471797 | 98.8 | Mini seedless cucumbers |
| 13.2 | *E. faecium* | DQ672262 | 99.2 | Mini seedless cucumbers |

*Source:* http://rdp.cme.msu.edu/.

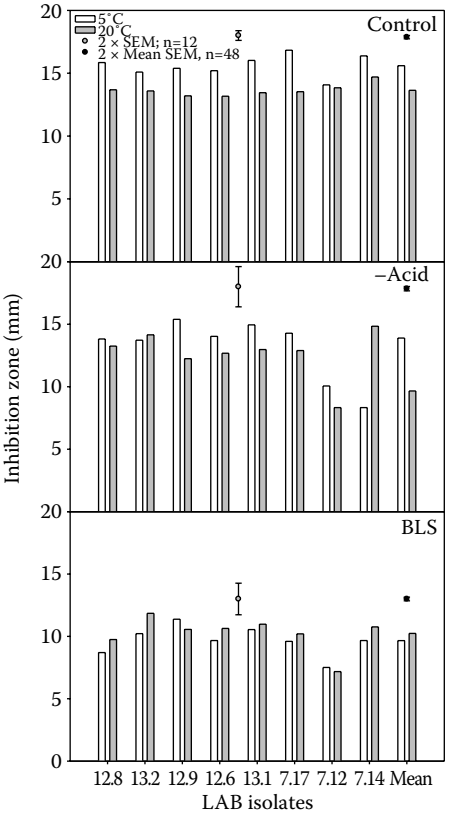


**FIGURE 3.1** Inhibitory effect of the eight LAB isolates on *Li. innocua* at 5°C and 20°C using the agar diffusion bioassay. Control, CFS without any treatment; –Acid, CFS with pH neutralized to 6.0; and BLS, CFS with pH neutralized to 6.0 and $H_2O_2$ eliminated with catalase treatment. The vertical bar represents the standard error of the mean for comparison of means within the figure.

food pathogens such as *Li. monocytogenes*. Finally, because of the virulence of some *Enterococcus* sp. (Devriese et al. 2006), it will be necessary to ascertain that these plant *E. faecium* strains are not virulent before being used in biopreservation or food fermentations (Yoon et al. 2008).

## 3.5 Conclusions and Future Research

The use of naturally occurring LAB to ferment vegetable-based foods is a very old tradition with roots in many cultures worldwide (Steinkraus 1997). Previous times' need for extending the shelf life of vegetables through fermentation has largely disappeared because of the arrival of modern food storage technologies; however, the popularity of fermented vegetable products remains (Liu et al. 2011).

With the advent of DNA-based community analysis techniques, our view of the microbial dynamics during fermentation and biopreservation is likely to change as more research groups apply these techniques to different products from different geographical areas. We expect that further investigations using deep sequencing techniques or pyrosequencing, as exemplified by the recent publication of Sakamoto and colleagues (2011), will provide new ecological insights allowing us to better understand and control the microflora. Also, better knowledge of the microbial ecology should enable us to select better starter cultures, which produce antimicrobial compounds with antagonistic activities against yeasts and spoilage bacteria (Choi et al. 1999), and refine the technology for controlled fermentation and preservation of commercially fermented kimchi and sauerkraut products.

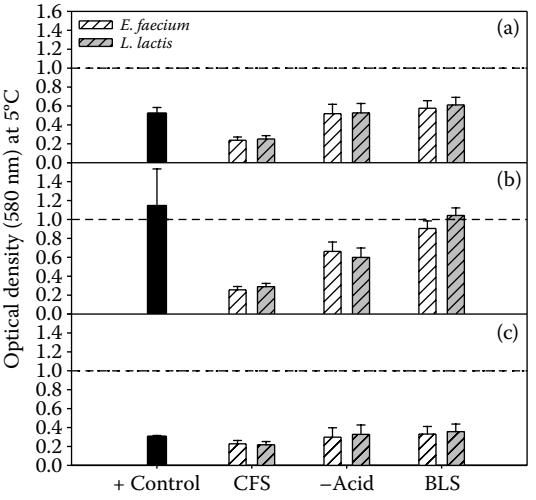


**FIGURE 3.2** The growth of fungal spores (a, *Bo. cinerea*; b, *Pen. expansum*; c, *M. fructicola*) after incubation at 5°C for 96 hours without or with LAB CFS from *E. faecium* and *Lact. lactis*. Positive control, fungal spores in de Man Rogosa Sharpe (MRS) broth without the addition of LAB CFS. CFS (control), fungal spores incubated with untreated CFS. –Acid, fungal spores in CFS after pH neutralization. BLS, fungal spores in CFS after pH neutralization and $H_2O_2$ elimination. The negative control $OD_{580\,nm}$ values (MRS with dead fungal spores) ranged from 0.2 to 0.3.

Flavor is an important aspect of the organoleptic quality of kimchi and sauerkraut, and yet the flavor compounds and their relationship to individual LAB strains have not been well elucidated. Research in this area should look at both the flavor chemistry as well as the functional genomics related to the dominant LAB strains during fermentation. The role of the microorganisms and enzymes involved in the softening of tissue of fermented products should also be investigated.

A better understanding of the microbiota may aid in the development of low salt fermentations, which may have altered microbial and sensory characteristics (Breidt 2004; Plengvidhya et al. 2007). However,

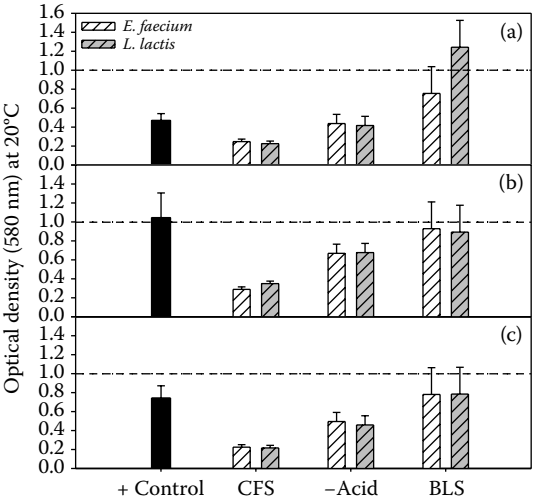


**FIGURE 3.3** The growth of fungal spores (a, *Bo. cinerea*; b, *Pen. expansum*; c, *M. fructicola*) after incubation at 20°C for 40 hours without or with LAB CFS from *E. faecium* and *Lact. lactis*. Positive control, fungal spores in MRS broth without the addition of LAB CFS. CFS (control), fungal spores incubated with untreated CFS. –Acid, fungal spores in CFS after pH neutralization. BLS, fungal spores in CFS after pH neutralization and $H_2O_2$ elimination. The negative control (MRS with dead fungal spores) ranged in $OD_{580\,nm}$ values from 0.2 to 0.3.

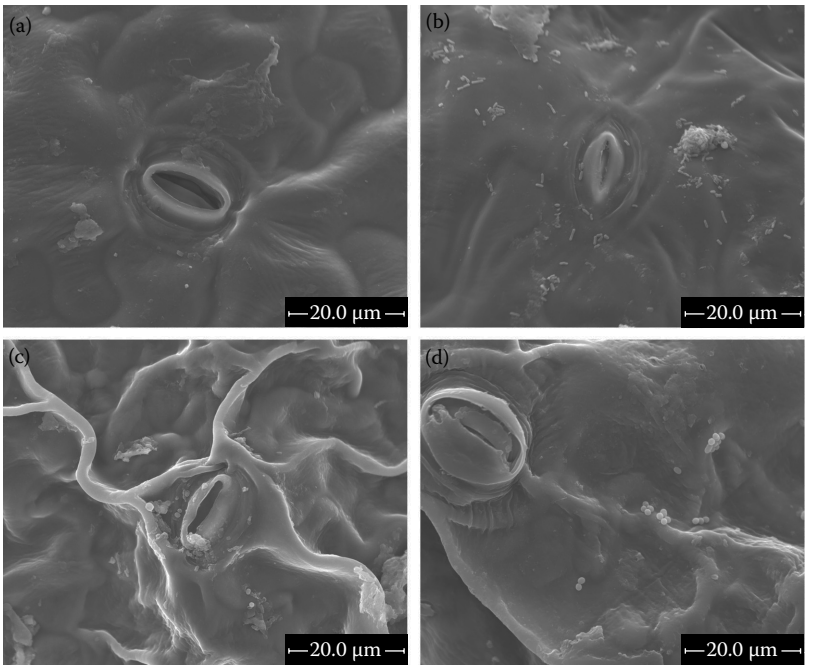


**FIGURE 3.4** Scanning electron micrographs of lettuce leaves from fresh-cut salad samples in the *Listeria* challenge test. Salad samples were observed after storage for 10 days at 5°C. (a) Control sample. (b) Samples inoculated with *Li. innocua* at $5.0 \times 10^3$ cfu/g and no LAB inoculation. (c) *Lact. lactis* and *Li. innocua*–inoculated salad sample. (d) *E. faecium* and *Li. innocua*–inoculated salad sample.

more importantly, it is necessary to assure that the safety of the products is not jeopardized in fermented products with low salt contents or biopreserved products with no salt. The removal of salt may allow for the growth of Gram-negative and Gram-positive pathogens unless the LAB cultures effectively inhibit these.

The properties of the fermenting or bioprotective LAB cultures also need to be investigated. One could envision that cultures be genome sequenced to (a) ascertain the absence of virulence genes or pathogenicity islands, something which is particularly relevant for *Enterococcus* strains; and (b) to affirm particular functional characteristics such as extracellular polysaccharide formation, degradation of specific carbohydrates, bacteriocin production, or probiotic activities. This characterization may allow us to better explore probiotic and anticarcinogenic activities, improve the nutritive properties of kimchi and sauerkraut products, and inhibit foodborne pathogens.

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