

AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____
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Microgard, a commercially-available pasteurized fermented milk, was found inhibitory to gram negative bacteria such as Pseudomonas putida, some well-known food-borne pathogens (e.g. Salmonella, Yersinia and Aeromonas) and some fungi as revealed by an agar incorporation assay method. Gram positive bacteria, however, were not inhibited. Rather, some bacteria in this group were stimulated by Microgard. The inhibitory activity of Microgard was optimum at pH 5.3 and below.

Thermal stability and protease sensitivity were characteristic of Microgard. Addition of albumin or some emulsifying substances antagonized Microgard whereas treatment with proteases at an initial pH of 11.0 enhanced its inhibitory ability. Purified eluates of Microgard from a Sephadex Column (gel filtration) exhibited thermal stability and maximum UV absorbance. Furthermore, they

gave positive results with the Biuret test, indicating the presence of protein type substance(s). Diacetyl, known to be inhibitory for psychotrophic bacteria, was present in Microgard as well as several organic acids.

Filter-sterilized fermentation growth metabolites of Pediococcus spp., Propionibacterium spp., Bifidobacterium longum, Lactobacillus plantarum and a black-pigmented yeast were tested for antimicrobial activity as Microgard had been tested. Results revealed that some of these metabolites were inhibitory to Pseudomonas putida and Listeria monocytogenes, which represented food spoilage and pathogenic bacteria, respectively.

Microbiological Control of Food Spoilage
and Pathogenic Microorganisms
in Refrigerated Foods

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MICROBIOLOGICAL CONTROL OF FOOD SPOILAGE
AND PATHOGENIC MICROORGANISMS
IN REFRIGERATED FOODS

CHAPTER 1

SCREENING MICROGARD AGAINST FOOD SPOILAGE
AND PATHOGENIC MICROORGANISMS

ABSTRACT

Microgard, a commercially-available fermented milk product containing anti-metabolites, was tested for inhibition of several food spoilage and pathogenic microorganisms. Gram negative bacteria such as Pseudomonas, Salmonella, Yersinia and Aeromonas were inhibited by Microgard. Most gram positive bacteria, however, were insensitive to its inhibitory action. Listeria monocytogenes was inhibited under some conditions. Some of the fungi tested were partially inhibited. Microgard functioned optimally at pH 5.30 and below. Several media used to grow certain test organisms reversed the inhibitory action by Microgard. Unknown organisms including lactobacilli, yeasts and Propionibacterium used in certain Microgard applications were characterized biochemically using the Analytab Products, Inc. (API) system.

INTRODUCTION

Much of the food harvested by man is lost before it can be consumed due to spoilage by microorganisms, some of which may be pathogenic. It has been estimated that 20% of all fruits and vegetables are spoiled by bacteria, yeasts and molds (Jay, 1986). Other data suggest that 5% of grains harvested are lost before consumption as a result of mold contamination (Flores-Galarza et al., 1987). Certain diseases may be associated with this spoilage. For example, an estimation of eight to ten million cases of enteric disease annually is attributed to food-borne bacteria including Salmonella, Campylobacter, Yersinia, Aeromonas and Escherechia coli. These diseases cost two billion dollars annually in direct medical costs and lost productivity (Kvenberg et al., 1987).

Little research has been done to develop naturally occurring metabolites produced by selected bacteria which can inhibit the growth of food spoilage and pathogenic organisms. Such naturally-occurring inhibitors could replace the use of chemical preservatives. One of these naturally-occurring metabolites now being used to extend the shelf life of some dairy and other food products is known commercially as Microgard. Microgard was developed through a cooperative research effort between the Department of Microbiology at Oregon State University and Wesman Foods, Inc. in Beaverton, Oregon. It is grade A

skim milk fermented by specific dairy microorganisms (Propionibacterium shermanii) and then pasteurized (Weber et al., 1986). Microgard was shown to be an inhibitor of Pseudomonas putida and certain yeasts and molds when tested in a previous study (Salih, 1985 and Weber et al., 1986). The product is added at 1% to some dairy products, such as cottage cheese, yogurt and salad dressing. Also, non-dairy Microgard is available for use with non-dairy products.

Spoilage and health-hazardous contamination by pathogenic microorganisms may occur even though refrigeration, pasteurization, thermal processing, fermentation and preservatives are widely used to extend the shelf life of many foods. This may be overcome by adding inhibitors to foods which will antagonize the growth and end-product production of food spoilage and pathogenic microorganisms even during refrigeration. Cottage cheese is spoiled mainly by psychotrophic bacteria during refrigeration (Brocklehurst and Lund, 1988); cultures of Streptococcus diacetylactis or Microgard are known to prevent this spoilage (Elliker et al., 1964; Salih, 1985 and Weber et al., 1986).

While the quality of food depends upon many factors such as food handling procedures, economics, scientific knowledge and ordinances and regulations in the food industry (Bishop et al., 1986), the growth of pathogenic bacteria can occur almost independent of these factors. In

the past, refrigeration was believed to be sufficient to inhibit microorganisms. However, recent information has shown that holding foods at 5°C does not restrain the growth of food-borne pathogens such as Clostridium botulinum type E, Yersenia enterocolitica, Listeria monocytogenes, E. coli (Palumbo, 1986) or spoilage yeasts such as Kluyveromyces marxianus (Fleet et al., 1987). Furthermore, gram negative bacteria including Aeromonas hydrophila, Pseudomonas putida, E. coli and Alcaligenes faecalis are easily isolated from foods stored at low temperatures (Phillips et al., 1987).

Different approaches have been undertaken to extend the shelf life and stabilize foods. Buffered acids offered microbiological stability to salads and acid foods (Debevere, 1987), while low salt soy protein hydrolysate extended the shelf life of sausage batter at ambient temperatures (Vallejo-Cordoba et al., 1987). Furthermore, preservation of cheese and bread has been reported by using sorbic acid, dehydroacetic acid and propionic acid (Saito et al., 1987; Ayres et al., 1980), and other workers have mentioned that nisin inhibited the outgrowth and toxin production by Clostridium botulinum in pasteurized cheese products (Somers et al., 1987 and Henning et al., 1986). Up-grade, a fermentation product of whey, was reported to extend the shelf life of bread and other baked goods (Morris, 1985). Likewise, organisms including B. subtilis

(ropy bread), Bacillus cereus, E. coli, Pseudomonas aeruginosa and Staphylococcus aureus were suppressed by parabens and benzoic acid (Freese et al., 1973 and Eklund, 1985). Inhibition of microorganisms in foods by weak acids has been viewed differently by various research workers. While Eklund (1985) stated that inhibition by propionic acid and benzoic acid was due to both undissociated and dissociated species of the acids, other researchers found that the inhibition was caused by only the undissociated molecules (Jay, 1986; Rusul et al., 1987 and Kabara, 1978).

The type or composition of foods influences the nature of microbial spoilage which occurs. For example, spoilage of meat and poultry as well as many heated, moist foods was attributed to psychotropic bacteria like Pseudomonas putida and others (Jay, 1986 and Ayres et al., 1980). Rind rot defect of Swiss cheese was found to be caused by Pseudomonas and Lactobacillus bacteria (Smith et al., 1987).

Several food commodities have been incriminated for being contaminated with pathogens. Enterotoxigenic E. coli, including antibiotic resistant types, were found present in some types of foods including butter (Franco, et al., 1987 and Abbar et al., 1987). Similarly, Doyle et al. (1987) isolated for the first time E. coli O157:H7 from retail fresh meat and poultry. This serotype is noteworthy because it causes hemorrhagic colitis. Listeria

monocytogenes outbreaks have been reported as a result of consumption of pasteurized milk and soft-ripened cheeses (Rosenow et al., 1987). Other workers proved that Yersinia enterocolitica and Salmonella typhimurium could survive on the surface of waxed and plastic containers of refrigerated milk (Stanfield et al., 1987). It was also reported that approximately 50% of pasteurized milk used for cheese manufacture was contaminated with staphylococci. This contamination could be because of either inadequate pasteurization or post-pasteurization contamination (Hirooka et al., 1987).

The nature of spoilage and health-hazardous contamination of food by bacteria can differ according to the causative agent. While psychotrophic organisms produce protease enzymes that survive ultra high temperature (UHT) pasteurization and thus reduce the shelf life of milk, food-borne organisms such as Aeromonas hydrophila and Shigella dysenteriae cause gastrointestinal diseases when the food is consumed (Christen et al., 1987; Palumbo et al., 1987; Smith, 1987 and Cousin, 1982). Other types of foods such as poultry and meat have been shown to be the main reservoir of Salmonella (Kvernberg et al., 1987). Dried fish, however, was reported to be spoiled by fungi including Aspergillus niger, Penicillium expansum and Aspergillus flavus (Wheeler et al., 1986). Inhibition of mycotoxin production by fungi was brought about by

treatment of corn with different concentrations of Monoprop (1 part propionic acid and 1 part Versite) (Shotwell et al., 1981). Another type of food which is spoiled by microorganisms, soy sauce, was treated with monocabrin to inhibit the growth of spoilage yeasts (Shibasaki et al., 1978). Unlike Yersinia enterocolitica, Campylobacta jejuni is not an environmental bacterium but rather is restricted to warm-blooded animals (Jay, 1986). Chickens, turkeys and fresh eggs were reported to be the vehicle to transmit C. jejuni to humans (Jay, 1986).

The objective of the present study was to test the susceptibility of different organisms to Microgard. Microorganisms used represented food spoilage as well as food-borne pathogenic bacteria.

MATERIALS AND METHODS

Microbial Strains and Culture Conditions

A total of 24 microorganisms including gram positive and gram negative bacteria, yeasts and molds were screened. Also, well-known food-borne pathogens were included in this study.

Each culture was activated in the appropriate broth medium under conditions specified by the supplier before being tested. Table 1.1 lists the culture used and from where each was obtained. Maintenance of the inert cultures was in slants or deep agar stabs under refrigeration temperature. Suspensions of molds were prepared according to a method obtained from General Mills, Minneapolis, MN. The surfaces of Potato Dextrose Agar plates were inoculated with the desired test mold and incubated at the appropriate temperature (25-30°C) for 5 days. Then 10 grams of wheat flour was added to each petri plate and the plates were vibrated in a jerky motion to dislodge the spores. The wheat flour did not need to be treated in any way because contaminating mold spores were present in small numbers relative to the inoculated test mold spores. The flour shakings from 10-20 plates were combined in a clean, dry container and stored at room temperature until needed. One gram of the mold spore suspension was added to 99 ml of 0.1% sterile peptone water to give a 1/100 dilution, and then further dilutions were carried out.

Preparation of Growth Media

For each microorganism tested, a selective or recommended medium was used and prepared according to the manufacturer's specifications. Generally speaking, each medium was adjusted to pH 5.30 with 10% tartaric acid before autoclaving at 121°C for 15 minutes. The media used with fungi, on the other hand, were adjusted to pH 4.00 prior to sterilization.

Microgard Preparation

Liquid Microgard was obtained from Wesman Foods, Inc., Beaverton, OR and had a pH of 6.00. Adjustment of pH was carried out with sterile 10% tartaric acid to pH 5.30. When non-dairy Microgard was screened against yeasts and molds, the pH was lowered to 4.00. In some cases, the pH was adjusted to 5.30 before autoclaving. Different concentrations of Microgard such as 0.5, 1, 3 or 5% were incorporated into the sterilized, cool media.

Determination of the Minimum Inhibitory Concentration

(MIC)

A. The Agar Incorporation Method:

The pour plate assay was used to determine the MIC as percentage volume to volume (% v/v) (Fig. 1.1).

The test bacterium (Pseudomonas putida) was activated overnight in Lactose Broth (Difco, USA) at 30°C. Dilution (1/10) in 1% Peptone (Difco) water (sterile) gave a

transmittance of 91.9% at a wavelength of 595 nm when measured using a Perkin-Elmer 35 Spectrophotometer.

Plate Count Agar (Difco) was adjusted to pH 5.30 using 10% tartaric acid before autoclaving. Similarly, Microgard was adjusted to the same pH before being incorporated at different concentrations into the sterile, melted agar. Just before pouring the plates, 2,3,5-Triphenyltetrazolium chloride solution (Sigma) was added to 0.0001% final concentration. This solution was filter sterilized (0.45 μm filter, Millipore) and used as the coloring indicator. At this point, the pH was expected to be 5.30. Plates were then incubated aerobically at 30°C for 24 hours. Growth (red colonies) was evident in controls that did not contain Microgard. A reduced number of colonies in test plates indicated a positive effect of the inhibitor, which was quantitated by calculating percent inhibition (see below).

B. Well Diffusion Assay:

The same growth and medium conditions mentioned above were used except the medium was poured into disposable petri plates (100 x 15 mm, VWR Scientific Inc.). Each plate contained 17 ml of medium. In this case medium was free of Microgard. Bacterial cell suspension (0.1 ml, 1/100 dilution) was spread evenly onto the surface of the solidified medium. Wells were made in the agar by using a sterile straw 0.5 cm in diameter. Each well was filled with 25 microliters (μl) of Microgard.

Influence of pH and Testing Method on the Inhibition

In this experiment, two different techniques for testing were carried out. Both pour plate and well assays were used as mentioned earlier (Hogg et al., 1987; Reddy et al., 1983). In the pour plate assay the medium was adjusted to pH values of 5.0, 5.30, 5.60 and 5.80. In the well assay the medium was adjusted to 5.30 and 5.70. Accordingly, Microgard was adjusted to the same pH as the growth medium.

The test organism used was Pseudomonas putida. After incubation, plates were checked to determine the inhibition.

Preparation of the Inoculum

Each organism tested during this study was activated under conditions which will be described later. The active organism was diluted further using sterile 0.1% peptone water (Debevere, 1987). Dilutions used ranged from $1/10^2$ - $1/10^8$. These dilutions were used in the pour plate assay and prepared according to Standard Methods for the Examination of Dairy Products (1985).

Susceptibility Testing

The minimum inhibitory concentration (MIC) was determined by the agar incorporation procedure (Hogg et al., 1987). Microgard was incorporated into the sterilized medium as percent v/v. The MIC was designated as the

lowest concentration that gave 100% inhibition after 2 days of incubation. At the same time, both the disc assay and the agar well diffusion techniques were applied in some cases to measure the inhibition zone (Brady, 1987 and Reddy, 1983). Microgard-free medium was used as a control. Meanwhile, plates that had neither Microgard nor the test organism were sacrificed in order to measure pH during the incubation period. Similarly, other sets of plates which contained medium plus Microgard only were sacrificed in order to measure the pH. pH measurements were carried out using an X-EL combination Flat Surface Electrode (Corning #476195). Tests for sterility were done using the medium and Microgard separately.

The percentage inhibition was calculated as:

$$\% \text{ inhibition} = \frac{\text{Control count} - \text{Sample count}}{\text{Control count}} \times 100$$

as described in Freese (1973).

Test Microorganisms

The strain, source of each bacterium, activation steps and growth media will be described for each individual organism (Table 1.1). For more details concerning the preparation of media, refer to previous descriptions in Materials and Methods above.

A) Gram Negative Bacteria:

1) Pseudomonas putida:

This bacterium was received from Wesman Foods Inc., Beaverton, OR. It was routinely activated in lactose broth (Difco) at 30°C for approximately 24 hours. The pour plate medium used was Plate Count Agar (Difco) acidified to pH 5.30 before autoclaving. Just before pouring the plates, 2,3,5-Triphenyltetrazolium chloride (filter sterilized) was added to give 0.0001% concentration (Ayres et al., 1980 and Jay, 1986). The plates were incubated at 30°C aerobically.

2) Pseudomonas aeruginosa ATCC 419:

The same conditions as above were used.

3) Achromobacter delicatulus ATCC 19103:

The same conditions as above were used.

4) Escherichia coli V 517:

The bacterium was activated in Lactose Broth at 30°C for 24 hrs. The medium used was Violet Red Bile Agar (Difco) acidified to 5.30 before autoclaving (Speck, 1984 and Jay, 1986). The plates were incubated at 30°C.

5) Salmonella paratyphi ATCC 9281:

This pathogen was rehydrated in Brain Heart Infusion broth (Difco) and incubated at 37°C for 24 hrs.

Salmonella-Shigella Agar (Difco) was used as the growth medium (Speck, 1984 and Bullerman et al., 1984).

6) Salmonella typhimurium:

This strain was obtained from Oregon State University, Microbiology collection. The same conditions were used as mentioned above for the previous Salmonella.

7) Shiegella dysenteriae E 19b:

The same conditions as for S. typhimurium were used.

8) Campylobacter jejuni ATCC 29428:

Activation was done by rehydrating the lyophilized culture (American Type Culture Collection) in Thioglycollate Medium (BBL, USA) followed by incubation at 37°C for 2 days. Brucella Agar (Difco) was used as the growth medium (Washington, 1985, Ray et al., 1984 and Moran et al., 1987). The plates were incubated at 42°C in a controlled atmosphere (Campy Pak, microaerophilic system).

9) Yersinia enterocolitica ATCC 23715:

The freeze-dried pathogen was suspended in Brain Heart Infusion broth and incubated at 30°C for 2 days. The bacterium was plated out in Mackonky Agar (Difco) and then incubated at 30°C (Washington, 1985 and Bacteriological Analytical Manual, 1984).

10) Aeromonas hydrophila ATCC 7965:

Brain Heart Infusion (BHI) was used and incubation was at 30°C for 24 hrs. American Type Culture Collection recommends Nutrient Agar (Difco) as the growth medium.

B) Gram Positive Bacteria:

1) Lactobacillus spp.:

The same conditions were used with all lactobacilli used in this study. Each bacterium was propagated in MRS broth (Difco) and incubated at 37°C (De Man et al., 1960; Rose et al., 1985). Similarly, every organism was grown in MRS + 2% agar added before autoclaving. Further incubation was done at 37°C under anaerobic conditions by using Gas Pak system (BBL).

The following lactobacilli were used:

Lactobacillus isolate (pickles)

Lactobacillus brevis

Lactobacillus plantarum (Microlife Technics)

Lactobacillus isolates 79A and 79B (Wesman Foods, Inc.)

2) Bacillus cereus var. mycoides:

The lyophilized bacterium was rehydrated in Nutrient Broth (Difco) and incubated at 30°C for 24 hrs. The organism was plated out in Nutrient Agar (Difco) (Bacteriological Analytical Manual, 1984).

3) Staphylococcus aureus:

Propagation of this pathogen was carried out in BHI broth at 37°C. Nutrient Broth supplemented with sodium chloride (final concentration 10%) did not suppress the growth of this organism. Microscopic examination revealed

that this organism was a gram positive coccus in clusters. Mannitol Salt Agar (Difco) was used as the plating medium with incubation at 37°C (Speck, 1984; Holt, 1977 and Tham et al., 1987).

4) Propionibacterium shermanii:

Microgard was prepared using this organism (Wesman strain). Activation was in BHI broth or sodium lactate broth incubated at 30°C for 2-3 days. Thioglycollate medium + 2% agar was used as the growth medium (Vedamuthu et al., 1967 and Malik et al., 1968). The organism was incubated at 30°C in Gas Pak for 3-5 days.

5) Listeria monocytogenes ATCC 7644:

Lyophilized culture was suspended in sterile BHI broth and incubated at 37°C for 18-24 hrs. Dilutions of the organism were routinely done in 0.1% sterile peptone water (1/10² - 1/10⁷). Different media were used as well as addition of some substances:

Brain Heart Infusion (Difco) + 2% agar

Tryptic Soy Agar

Tryptic Soy Agar + 0.2% glucose (Sigma)

Tryptic Soy Agar + 0.1% Tween 80

Blood Agar Base (Difco)

All media were adjusted to pH 5.30 before autoclaving.

Blood agar was recommended by American type Culture Collection. However, acidification of blood to 5.30 was

not feasible. Infusion agar also was used since it has been reported to be used with Listeria (Washington, 1985). In addition, different sensitivity tests were carried out including the agar-incorporation method, the disc assay, the agar well diffusion technique and incorporation of different concentrations of Microgard and the organism in BHI broth (pH 5.30) with incubation at designated temperatures for 5 days. Plate counts were carried out and compared to controls. Two different incubation temperatures (30 or 37°C) were used in these tests. The plate counts were carried out in Tryptic Soy Agar + 0.2% glucose.

C) Fungi:

This group included molds and yeasts which were used as indicator organisms.

1) Test Yeast (Fred Meyer):

The organism was received from Wesman Foods Inc., Beaverton, Oregon. It was maintained on slants of Potato Dextrose Agar (Difco). Malt Extract Broth (Difco) was inoculated with this yeast followed by incubation at 30°C. Potato Dextrose Agar acidified to pH 4.00 was the growth medium (Speck, 1984). Plates were incubated at 30°C for 2 days.

2) Kluyveromyces marxianus var. marxianus:

This was an American Type Culture Collection (ATCC) strain #8554. The same conditions mentioned above were used, however, activation was carried out in YM broth (Difco).

3) Yeast Isolate:

This isolate was supplied by Wesman Foods Inc. It was interesting in that a black pigment was produced by this organism but it grew slowly at 30°C. The organism was plated on in Mycophile Agar (BBL) followed by incubation at 30°C for 4 days.

4) Aspergillus niger:

This mold was isolated from spoiled, sweetened-condensed milk. Spore suspensions were diluted in 0.1% sterile peptone water and grown in Mycophile Agar (Speck M.L., ed., 1984) and incubated at 25°C for 7 days.

5) Penicillium expansum:

The same conditions used for A. niger were used.

Effect of Type of Medium on Inhibition:

In this experiment two bacteria were used to represent spoilage and pathogenic microorganisms. Different media were used with each bacterium. All conditions mentioned earlier in the susceptibility testing were used as well as those in the preparation of the growth media.

1. Pseudomonas putida:

Two media were used as the growth agar, Plate Count Agar (Difco) and Blood Agar Base (Difco). Microgard was incorporated in these two media at the same concentrations.

2. Listeria monocytogenes ATCC 7644:

Several media along with some modifications were used:

Tryptic Soy Agar (Difco)

Tryptic Soy Agar + 0.1% Tween 80

Tryptic Soy Agar + 0.2% glucose

Brain Heart Infusion (Difco) + 2% agar (Difco)

Blood Agar Base (Difco)

The pour plate, well and disc assays were used. The disc diameter was 1/2 inch (Schleicher and Schuell, USA).

Identification of Unknown Organisms

The API system tests (Analytab Products, Inc., Plainview, N.Y.) were applied to biochemically characterize unknown microorganisms. Two strains of Lactobacillus, 79A and 79B (Wesman Foods Inc.), were identified by their carbohydrate utilization profiles. Similarly, a black yeast isolate (Wesman Foods) was tested using the API 20 clinical yeast system.

Finally, a Propionibacterium species isolated from Swiss cheese (Tillamook) was identified through the API system along with litmus milk growth results (Bullerman et al., 1984).

RESULTS

The Influence of pH of the Media and Type of Test on the Inhibition

Figures 1.2 and 1.3 illustrate the results obtained. It appeared that Microgard functioned optimally at pH 5.3 and below in the agar incorporation method; some inhibitory activity (approximately 25%) was lost when the pH was adjusted to 5.70 as compared to inhibition at pH 5.30 in the well assay.

Sensitivity of Gram-negative Bacteria

Table 1.2 summarizes the effect of Microgard on all gram negative organisms tested. Psychotrophs were highly inhibited by low concentrations. This group included Pseudomonas putida, Pseudomonas aeruginosa, Achromobacter delicatulus, Yersinia enterocolitica and Aeromonas hydrophila. Among these organisms, Pseudomonas aeruginosa was the most resistant to low concentrations of Microgard. At these low concentrations 0.5% (v/v) inhibition of Pseudomonas putida during short periods was overcome as the incubation increased.

Food-borne pathogens Salmonella typhimurium, Yersinia enterocolitica, and Aeromonas hydrophila were inhibited by Microgard (Table 1.3). Salmonella typhimurium overcame low concentrations of Microgard (1% and 3%) and thus resisted inhibition when the incubation at 37°C was prolonged (Figs.

1.4a, 1.4b, 1.4c). As a result, the minimum inhibitory concentration (MIC) was higher for this bacterium than the other pathogens. Salmonella paratyphi was more sensitive to Microgard than S. typhimurium even after a longer period of incubation. The pH of the medium went above pH 5.30 as the organism overcame the inhibition.

A relationship existed between inhibition and the number of organisms being tested. While the 10^{-4} dilution of Salmonella typhimurium was strongly inhibited by 1% Microgard, at 10^{-2} this organism was not inhibited under the same conditions. The only bacterium in the gram negative group which was not inhibited by Microgard was E. coli.

Effect of pH on Growth of Test Organisms

It was mentioned earlier that both the agar media and Microgard were adjusted to pH 5.30 when performing the susceptibility tests. Both Campylobacter jejuni and Shigella dysenteriae, however, did not grow at this pH in the Microgard-free media used as controls. Consequently, it was impossible to test any effect of Microgard on these organisms. An interesting finding, however, was that Campylobacter jejuni grew in one of the experiments at this pH but was converted from rods to coccus-shaped bacteria when incubated under microaerophilic conditions. The converted cells were gram positive rather than gram negative and were not inhibited by Microgard. However,

there was a complete absence of growth in the controls when this experiment was repeated using the same conditions.

Influence of the Growth Media

Pseudomonas putida was much more resistant to inhibition by Microgard when grown in Blood Agar Base (pH 5.30). A high concentration of Microgard (3%) was necessary in this medium to achieve complete inhibition. Plate Count Agar (pH 5.30) was superior in allowing inhibition by Microgard against P. putida. These findings appear in Fig. 1.5.

Listeria monocytogenes gave variable inhibition results when different media and testing conditions were used. More details on this will be mentioned in the following section.

Sensitivity of Gram Positive Bacteria

Organisms in this class generally were not susceptible to the antimicrobial ability of Microgard though slight inhibition was observed when these bacteria were diluted to low numbers. Only 28% inhibition was found when 3% Microgard was incorporated in the test medium and the organism (Lactobacillus isolate 79A) diluted to 10^{-8} . Similarly, even Propionibacterium shermanii was slightly inhibited when 5% Microgard was present at a cell dilution of 10^{-8} .

Addition of Microgard to the media used in testing these bacteria caused morphological changes in the colonies in comparison to controls. Those inhibited slightly by Microgard (Lactobacillus 79A and 79B) had smaller colonies. The Lactobacillus isolate from pickles was neither inhibited nor modified in colony size.

In other experiments, gram positive bacteria not inhibited by Microgard were stimulated. This group included Staphylococcus aureus, Bacillus cereus, Lactobacillus brevis and Lactobacillus plantarum.

Listeria monocytogenes

This pathogen gave variable inhibition results in repeated experiments. Inhibition was caused by Microgard when the organisms were tested by the pour plate assay in BHI agar but not in Tryptic Soy Agar (\pm 0.1%, and 0.2% Tween 80 or glucose, respectively) or in Blood Agar Base. Furthermore, when the experiment was repeated in BHI agar under the same conditions stimulation of the Listeria was observed.

Incubation at 30°C or 37°C did not influence the activity of Microgard against Listeria monocytogenes. The pH of the media that contained only Microgard was measured after the incubation period in all experiments and was found to be in the range of 5.2-5.30.

In the case of the Disc assays on BHI agar, no inhibition zones were found around the Discs.

When the organism was inoculated in broth (BHI, pH 5.30) and Microgard added at different concentrations, the count results revealed no inhibition by Microgard. Rather, the bacteria exposed to Microgard had higher plate counts than control tubes without Microgard.

Also, higher counts resulted from tubes incubated at 30°C in contrast to those at 37°C.

Susceptibility of Fungi

Inhibition of Penicillium expansum and the black yeast isolate occurred when each was exposed to Microgard, though not completely (Table 1.4). While 5% Microgard gave 58% inhibition of the black yeast, the same concentration in the agar caused 93% inhibition of P. expansum. On the other hand, Kluyveromyces marxianus, the Fred Meyer yeast isolate and Asperigillus niger were not inhibited under these conditions. However, some inhibition was obtained against K. marxianus when the organism was diluted further and the concentration of Microgard increased to 7%.

Effect of Microgard on the Initial pH of the Growth Media

For all the different kinds of media used in screening tests, no effect on the initial pH was noticed when only Microgard was incorporated without test organisms. Thioglycollate medium containing only Microgard dropped below pH 5.30 after anaerobic conditions (Gas Pak system) at 30°C for 3-4 days. The more Microgard incorporated, the

lower the pH reached. The pH decreased from 5.30 to 4.5 to 4.00 depending on the amount of Microgard added (Fig. 1.6).

It is important to emphasize that no contamination or visible growth was noticed on these plates. Furthermore, microscopic examination revealed the complete absence of cells. Also, this ability to lower the initial pH of this medium during incubation was lost when Microgard (at pH 5.30) was autoclaved at 121°C for 15 minutes before being incorporated.

Identification of Unknown Microorganisms

The fermentation of different carbohydrates by the two strains of Lactobacillus (79A and 79B) were similar (Table 1.5). Strain 79A utilized trehalose but not Raffinose. Lactobacillus viridescense NCDO 1179 possessed the same biochemical properties though it does not utilize lactose.

Biochemical properties of the black yeast isolate are shown in Table 1.6. Rhodotorula rubra has similar characterizations. Adonitol and sorbitol are utilized by R. rubra whereas the black yeast was not able to ferment them. Furthermore, galactose is strongly utilized by Rhodotorula rubra.

Finally, the Propionibacterium cheese isolate reduced litmus milk and the top layer of the tube remained pink after 72 hours of incubation at 30°C. At the same time, curd formation took place. When the incubation period was increased to 96 hours, the color of the whole tube was

white. Similarly, Propionibacterium shermanii ATCC 9616 and P. shermanii (Wesman) gave results identical to the unknown. Biochemical reactions are summarized in Table 1.7.

DISCUSSION

These investigations showed that Microgard exerted greatest antibacterial action on gram negative bacteria. E. coli, however, was not sensitive to Microgard. This may be due to the rigidity of the cell wall of this organism as stated by Mustafa et al. (1969). Similarly, some spheroplasts from E. coli were not inhibited by increasing concentrations of the antimicrobial substance produced by L. plantarum (Anderson, 1986). Jay (1982) also pointed out that E. coli, among all gram negative bacteria tested, was not killed by diacetyl. Gram positive bacteria, on the other hand, were not only insensitive to Microgard but were stimulated in some instances. Variable results were obtained when Microgard was assayed against Listeria monocytogenes. In the disc assay technique, a zone around the disk was noticed, but it was not clear.

Test results in assaying bacterial inhibitors can be affected by several factors. Jay (1982) demonstrated that diacetyl was not effective using the disc assay procedure and incubation at 37°C, in contrast to inhibition obtained at 10°C or 20°C. Vancomycin was inhibitory by the agar-incorporation method but not in the conductivity test against the same organism (Hogg et al., 1987). Likewise, E. coli and Pseudomonas aeruginosa derived from liquid cultures were significantly less sensitive towards an antimicrobial agent (chlorhexidine diacetate) than those

from solid cultures (Al-Hiti et al., 1983). The antimicrobial activity of specific compounds can be reversed by several types of chemicals such as starch, albumin, glucose, acetate and ash minerals (Schales, 1951; Jay, 1982; Mustafa et al., 1969; Ismaeel et al., 1986; Shibasaki et al. 1978 and Kabara, 1978). This observation may explain the absence of inhibition by Microgard in blood agar base inoculated with P. putida.

Psychotrophic organisms including P. putida, P. aeruginosa and Aeromonas hydrophila were more sensitive to Microgard than Salmonella typhimurium. Consequently, low concentrations suppressed the growth of psychotrophs over a longer incubation period.

S. typhimurium, on the other hand, exhibited insensitivity toward low concentrations of Microgard as the incubation increased. In this regard, Staphylococcus aureus has been reported to initiate growth after being inhibited by metabolites of Lactobacillus plantarum (Anderson, 1986). In our investigation, the pH of agar media with Microgard was higher than 5.30 (initial pH) whenever organisms were insensitive to Microgard after a long period of incubation. However, this was not the case with gram positive bacteria, especially Lactobacillus spp. which were not initially inhibited by Microgard and in this case the pH fell below 5.00. As a result, Microgard appears more specific against gram negative organisms while

Nisin is inhibitory against gram positive bacteria (Susanne et al., 1986). While P. aeruginosa is known to be resistant to some antibiotics (Mehta et al., 1983), it was successfully inhibited by Microgard.

In terms of the most effective pH for inhibition by Microgard, it functioned optimally at pH 5.30 or below. This property renders Microgard useful as a shelf life extender for acid-type foods. Furthermore, Microgard was still active though to a lesser degree at pH 5.70 in the well diffusion assay. Also, in comparison with other antimicrobial substances, diacetyl and propionate have been reported to be much more inhibitory at pH values below 7.00 (Jay, 1982 and Freese et al., 1973). Ascorbic acid, however, was found bacteriocidal for Campylobacter jejuni at pH 7.00 (Juven et al., 1986).

It is worthwhile to mention that Microgard was assayed against several organisms using different selective and nutritious media; Microgard may be less inhibitory under such conditions. It has been documented in the literature that diacetyl is most effective as an inhibitor in plate count broth but not in BHI or cooked-meat medium (Jay, 1982). Also, diacetyl was found to be inhibitory against Clostridium spp. in thioglycollate broth (selective for anaerobes) but not inhibitory in plate count broth (Jay, 1982). In addition, Kabara (1978) stated that the

antimicrobial concentration may be increased by a factor of ten in the presence of serum.

In this study, both Campylobacter jejuni and Shigella dysenteriae did not grow in the absence of Microgard (controls) at pH 5.30, thus making it impossible to determine the percent of inhibition by Microgard. Similarly, C. jejuni did not grow in nutrient broth acidified to pH 5.3 by hydrochloric acid or in yogurt at pH 4.2-5.30 (Cuk et al., 1987 and Juven et al., 1986). Also, it was found that Shigella dysenteriae 1 was inhibited by Salmonella-Shigella agar (Speck, 1984).

An interesting finding was that C. jejuni cells grew in Brucella agar under microaerophilic conditions at 42°C where they underwent conversion from rods to cocci. This occurred even though the pH of the Brucella agar was adjusted to 5.30 before incubation. This may be due to a slight increase in the initial pH which allowed the growth of this particular bacterium. This conversion from rods to cocci was also noted by Moran et al. (1987a and 1987b). It was further noted that the cocci were not inhibited by Microgard and this may be related to the slight increase in the initial pH of the medium. It would be of interest in the future to study the influence of pH and Microgard on the conversion from rods to cocci and to determine whether or not these new forms are more resistant to any antimicrobial agents.

Microgard was not able to retard the growth of all gram positive bacteria tested under this study. In fact, Microgard allowed luxurious growth when assayed against Staphylococcus aureus, Lactobacillus brevis and Bacillus cereus. This was not surprising since Microgard contains nutrients which may stimulate growth of bacteria resistant to Microgard. Also, in some cases inhibition by Microgard seemed to be dependent on the dilution of the gram positive bacteria used in the assay. Less inhibition was obtained as the bacteria were diluted further.

Concerning the Microgard-producing bacterium Propionibacterium shermanii, the organism itself was slightly inhibited by Microgard and this inhibition was also dilution dependent. It has been claimed that bacteriocin production and immunity are encoded on a specific plasmid in Pediococcus (Gonzalez et al., 1987). Furthermore, the accumulation of fermentation end-products caused a decrease in both growth rate and end product elaboration (Herrero, 1983; Blance et al., 1987 and Blance et al., 1987).

The fungi (yeasts and molds) tested were either insensitive or moderately inhibited by Microgard. Several workers have reported that yeast growth results in the production of hydrolyzing enzymes and degradation of inhibitory substances, such as propionate, under some conditions (Fleet et al., 1987; Suihko et al., 1984; Miyakoshi et al., 1987 and Magau et al., 1986). Also, the

kind of fungus can influence the activity of any inhibitor. For example, Torulopsis holmii was able to overcome propionate and sorbate inhibition whereas Saccharomyces uvarum was inhibited by propionate rather than acetate or lactate (Suikko et al., 1984 and Moon, 1983).

Another interesting finding was that Microgard incorporated in thioglycollate medium caused a decrease in the initial pH and this decrease was proportional to the concentration added to the growth medium.

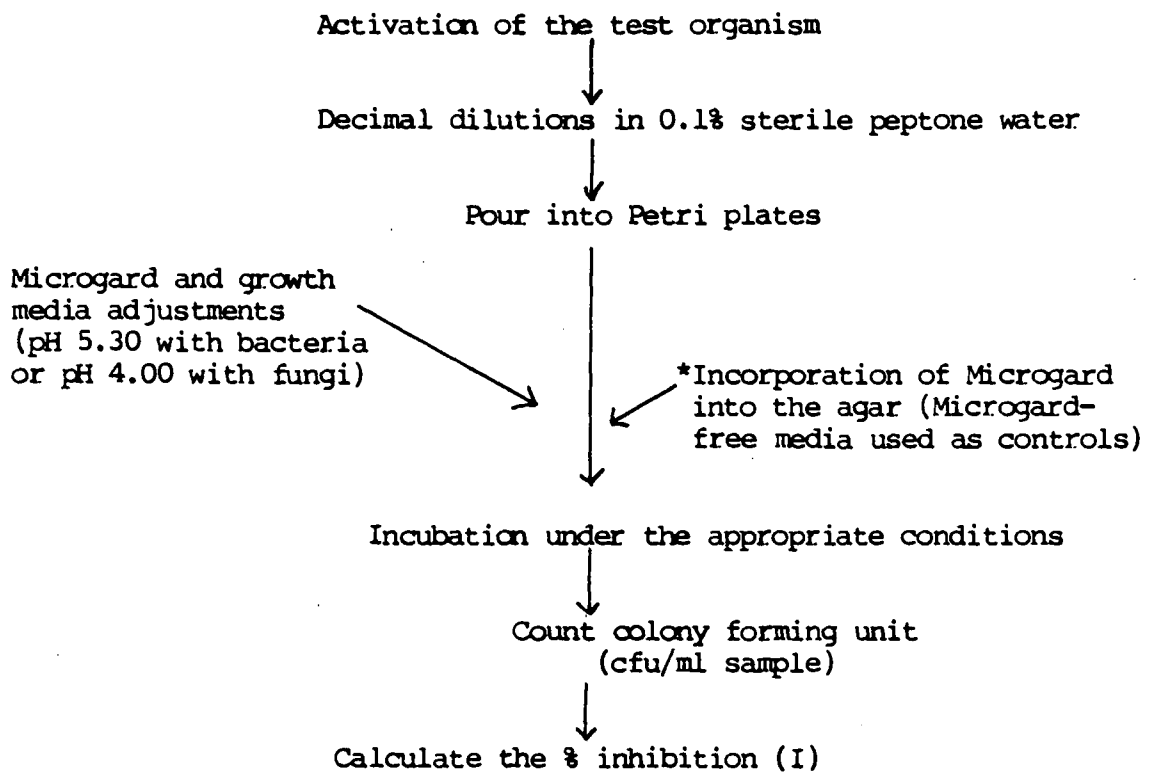
Extracellular proteolytic enzymes have been reported to be produced by Propionibacterium acnes in a growth broth (Ingram et al., 1983). So, it is possible that Propionibacterium shermanii produces proteases that are specific in the thioglycollate medium. Schwert et al., (1948) stated that an esterase activity, causing the lowering of the pH, was characteristic of trypsin and this may explain the lowering of the pH of thioglycollate medium with the addition of Microgard.

Propionibacterium cheese isolate gave the same results as those of Propionibacterium shermanii as noted by growth in litmus milk. This isolate was able to metabolize trehalose and esculin in the API system, and the use of trehalose to distinguish between P. freudenreichii subsp. Shermanii (P. shermanii) and P. thoenii was valid (Britz et al., 1980). Esculin hydrolysis has been used to divide the Propionibacteria into two groups (Holt, 1977). Most

recently, a method for differentiating between several species of these bacteria based on electrophoresis of the proteins in cell-free extracts was describe by Baer (1987).

Future research is needed to evaluate the addition of Streptococcus lactis extracts or nisin to Microgard. This will offer potent inhibition of gram negatives and gram positives by both Microgard and nisin, respectively, but in one system. Nisin has been found to be a potent inhibitor of L. monocytogenes (Benkerroum, personal communication).

Fig. 1.1. Outline of the pouring assay (agar incorporation method).



Microgard (MG) Assay

%MG ^a	Cfu/ml 10 ⁻²	%I ^b
0	TNTC	0
1 ^c	0	100
3	0	100
5	0	100

a %MG incorporated in the agar medium

$$b \text{ \%I} = \left(\frac{\text{SPC } 0\% \text{ MG} - \text{SPC } X\% \text{ MG}}{\text{SPC } 0\% \text{ MG}} \right) 100$$

c MIC = lowest full % MG causing 100% I at 10⁻² dilution of test organism.

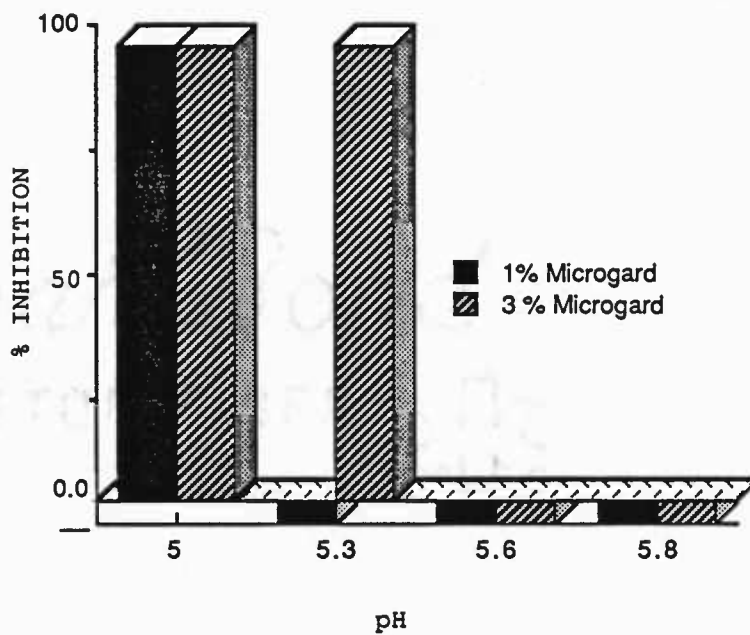


Fig. 1.2. Correlation between inhibition of *P. putida* (1/10² dilution) by Microgard and the pH of the agar medium during the agar incorporation method.

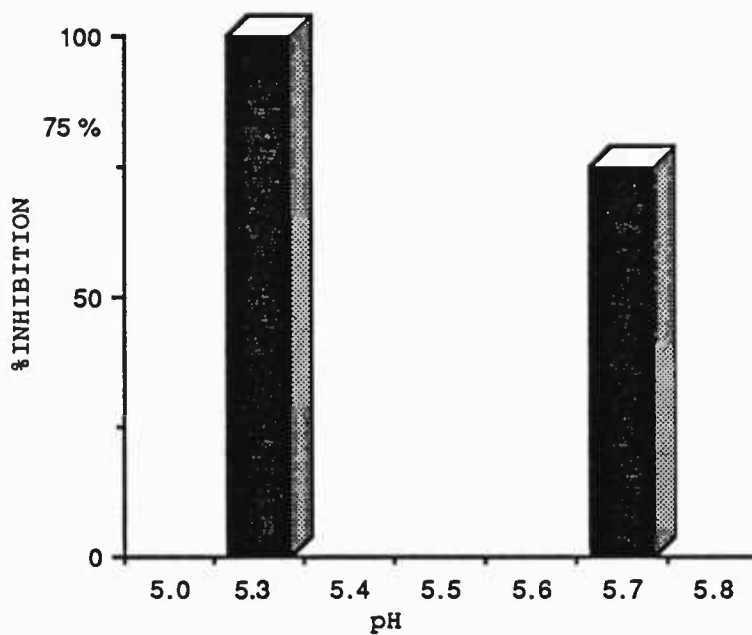


Fig. 1.3 Sensitivity of *P. putida* towards Microgard at two pH values determined by the inhibition zone during the agar well diffusion assay.

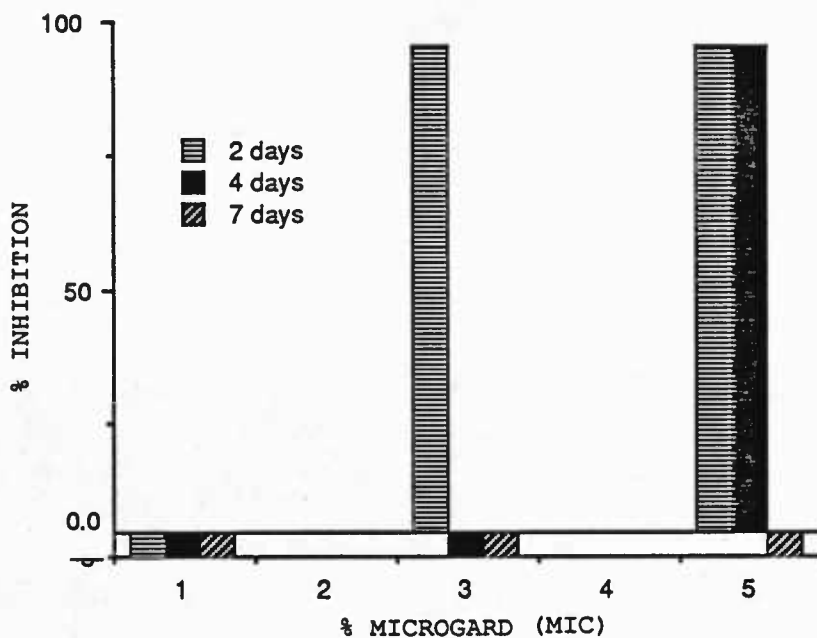


Fig. 1.4a. Influence of the incubation period of *Salmonella typhimurium* ($1/10^2$ dilution) on the minimum inhibitory concentrations (MIC) of Microgard during the agar incorporation method.

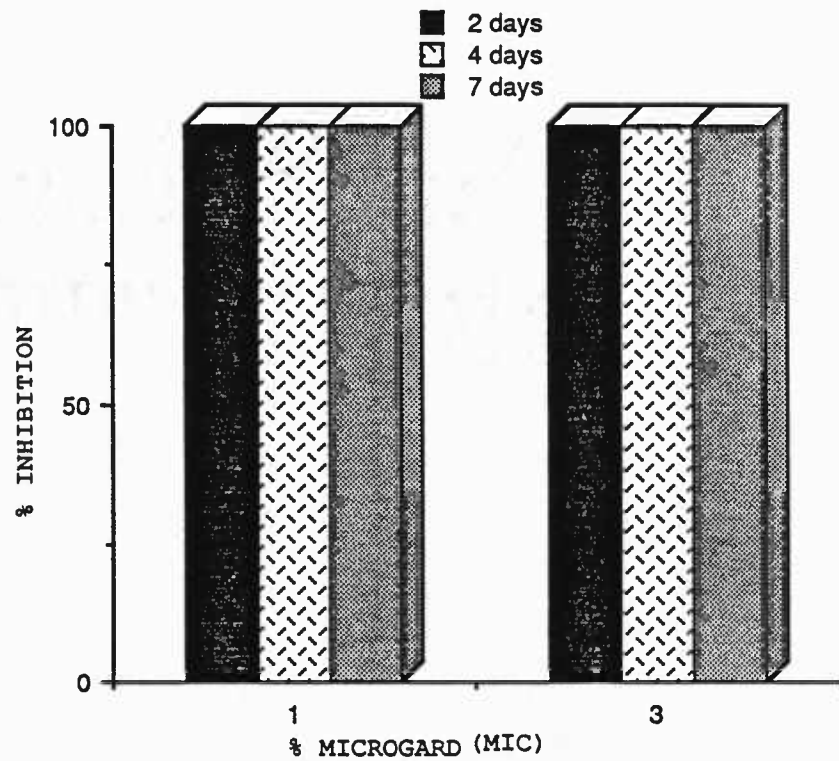


Fig. 1.4c. Influence of the incubation period of Achromobacter delicatalus on the minimum inhibitory concentration of Microgard as revealed by the agar incorporation assay.

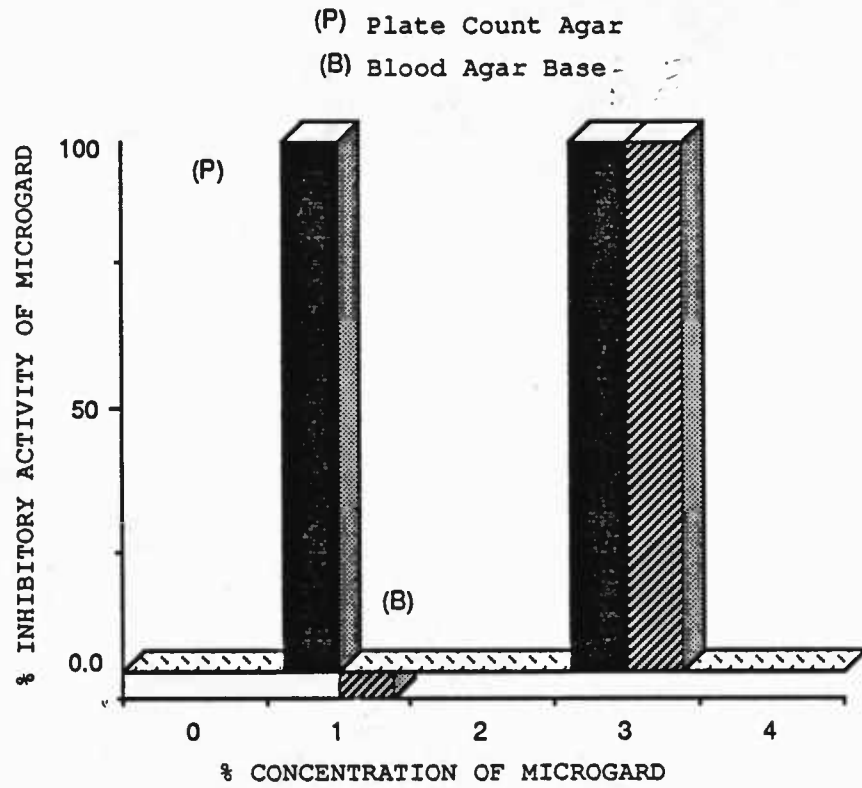


Fig. 1.5. The antagonistic effect of the growth medium on the inhibition by Microgard assayed against *P. putida* during the agar incorporation method.

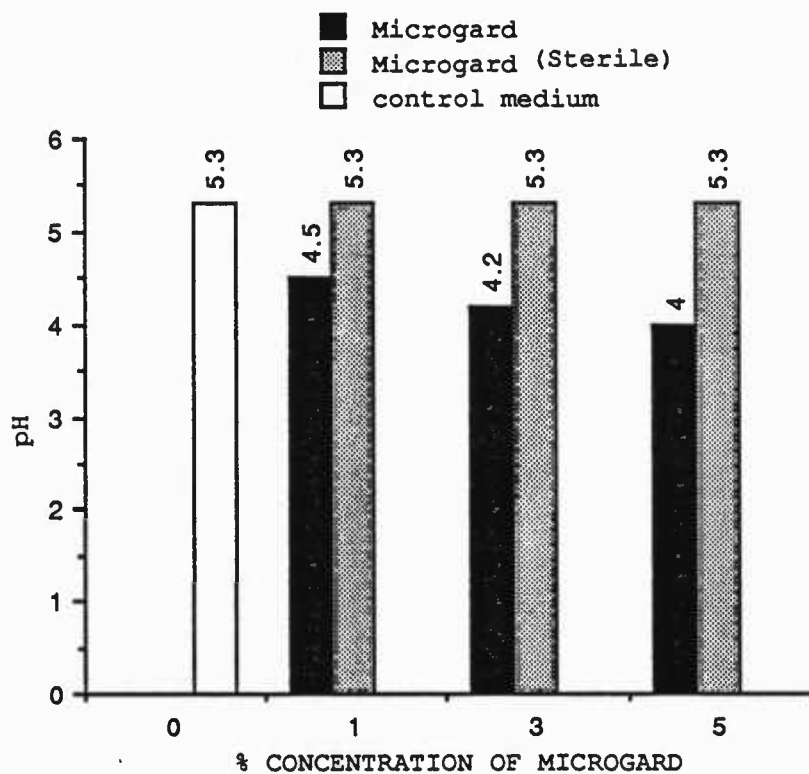


Fig. 1.6. Effect of Microgard addition on the pH of Sodium Thioglycollate Medium (initial pH 5.3) incubated anaerobically at 30 degree C. for 4 days.

Table 1.1. Microorganisms and growth media used in this study.

Organism	Strain or source	*Growth Media
A. Gram Negative		
1. <u>Pseudomonas putida</u>	Wesman	Plate count agar
2. <u>Pseudomonas aeruginosa</u>	ATCC 419	Plate count agar
3. <u>Achromobacter delicatulus</u>	ATCC 19103	Plate count agar
4. <u>Escherichia coli</u>	V 517	Violet red bile agar
5. <u>Salmonella paratyphi</u>	ATCC 9281	Salmonella-shieggella agar
6. <u>Salmonella typhimurium</u>	OSU	Salmonella-shieggella agar
7. <u>Shieggella dysenteriae</u>	E 19b	Salmonella-shieggella agar
8. <u>Campylobacter jejuni</u>	ATCC 29428	Brucella agar
9. <u>Yersinia enterocolitica</u>	ATCC 23715	Mackonkey agar
10. <u>Aeromonas hydrophila</u>	ATCC 7965	Nutrient agar
B. Gram Positive		
1. <u>Lactobacillus isolate</u>	Pickles	MRS agar
2. <u>Lactobacillus 79A</u>	Wesman	MRS agar
3. <u>Lactobacillus 79B</u>	Wesman	MRS agar
4. <u>Lactobacillus brevis</u>	Lab collection	MRS agar
5. <u>Lactobacillus plantarum</u>	Microlife Tech.	MRS agar
6. <u>Bacillus cereus var. mycoides</u>	Lab collection	Nutrient agar
7. <u>Staphylococcus aureus</u>	Lab collection	Manitol salt agar
8. <u>Listeria monocytogenes</u>	ATCC 7644	BHI, TSA, blood agar base
9. <u>Propionibacterium shermanii</u>	Wesman 9616	Thioglycollate agar
C. Fungi		
1. Yeast (Fred Meyer)	Wesman	Potato dextrose agar
2. <u>Kluyveromyces marxianus</u>	ATCC 8554	Potato dextrose agar
3. Black yeast	Wesman	Mycophile agar
4. <u>Aspergillus niger</u>	Sweet-condensed milk	Mycophile agar
5. <u>Penicillium expansum</u>	Lab collection	Mycophile agar

* More details in Materials and Methods.

Table 1.2. Antagonistic activity of Microgard against different microorganisms as revealed during the agar pour plate assay (agar incorporation assay).

Test Organism	Sensitivity
A. Gram Negative	
1. <u>P. putida</u>	++
2. <u>P. aeruginosa</u>	++
3. <u>Achromobacter delicatulus</u>	++
4. <u>E. coli</u>	-
5. <u>Salmonella paratyphi</u>	++
6. <u>S. typhimurium</u>	++
7. <u>Shiegella dysenteriae</u>	NG
8. <u>Campylobacter jejuni</u>	NG
9. <u>Yersinia enterocolitica</u>	++
10. <u>Aeromonas hydrophila</u>	++
B. Gram Positive	
1. <u>Lactobacillus</u> isolate (Pickles)	-
2. <u>Lactobacillus</u> 79A	-
3. <u>Lactobacillus</u> 79B	-
4. <u>L. brevis</u>	--
5. <u>L. plantarum</u>	--
6. <u>Bacillus cereus</u>	--
7. <u>Staphylococcus aureus</u>	--
8. <u>Propionibacterium shermanii</u>	-
9. <u>Listeria monocytogenes</u>	(?)
C. Fungi	
1. Yeast isolate (Fred Meyer)	--
2. <u>Kluyveromyces marxianus</u>	+
3. Black yeast	+
4. <u>Asperigillus niger</u>	-
5. <u>Penicillium expansum</u>	+

NG = no growth in control plates (pH 5.30)
 ++ = complete inhibition (no growth)
 + = partial inhibition
 -- = stimulation effect
 (?) = variable results
 - = no inhibition

Table 1.3. Minimum inhibitory concentration (MIC) of Microgard against various gram negative bacteria as revealed by the agar incorporation assay.

Strain	*Cfu/ml	MIC (% v/v)
1. <u>Pseudomonas putida</u>	6 x 10 ⁷	1
2. <u>P. aeruginosa</u>	6.8 x 10 ⁷	3
3. <u>Achromobacter delicatulus</u>	5 x 10 ⁶	1
4. <u>Salmonella paratyphi</u>	2.5 x 10 ⁷	1
5. <u>S. typhimurium</u>	4 x 10 ⁷	3
6. <u>Yersinia enterocolitica</u>	1.5 x 10 ⁷	1
7. <u>Aeromonas hydrophila</u>	6.6 x 10 ⁶	1

* Colony forming unit per ml

Table 1.4. The antagonistic effect (% inhibition) of Microgard against susceptible fungi as revealed by the agar incorporation assay.

Organism	Dilution	% Microgard	% Inhibition
1. <u>Pencillium expansum</u>	1/10 ³	5	93
2. <u>K. marxianus</u>	1/10 ⁶	5	37
3. Black yeast (Wesman)	1/10 ³	5	58

Table 1.5. The API biochemical reactions of Labactobacilli strains 79A and 79B.

Carbohydrate	Assimilation Ability	
	79A	79B
Glycerol	-	-
Erythritol	-	-
D-arabinose	-	-
L-arabinose	-	-
Ribose	-	-
D-xylose	-	-
L-xylose	-	-
Adonitol	-	-
β -methyl xyloside	-	-
Galactose	-	-
Glucose	+	+
Fructose	+	+
Mannose	+	+
Sorbose	-	-
Rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
Mannitol	-	-
Sorbitol	-	-
α -methyl glucoside	-	-
α -methyl mannoside	-	-
N-acetyl glucose amine	+	+
Amygdalin	-	-
Arbutin	-	-
Esculin	-	-
Salicin	-	-

Table 1.5 (continued)

Carbohydrate	Assimilation Ability	
	79A	79B
Cellebiose	-	-
Maltose	+	+
Lactose	+	+
Melibiose	+	+
Saccharose (Sucrose)	+	+
Trehalose	+	-
Inulin	-	-
Melezitose	-	-
Raffinose	-	+
Starch	-	-
Glycogen	-	-
Xylitol	-	-
Genitibiose	-	-
D-Turanose	-	-
D-Lyxose	-	-
D-Tagatose	-	-
D-Fucose	-	-
L-Fucose	-	-
D-Arabitol	-	-
Gluconate	-	-
2-ketogluconate	-	-
5-ketogluconate	-	-

Table 1.6. The biochemical characteristics of the black yeast tested with API 20C.

Carbohydrate	Utilization Test
Glucose	+
Glycerol	+
2Keto gluconate	-
Arabinose	+
Xylose	+
Adonitol	-
Xylitol	+
Galactose	+
Inositol	-
Sorbitol (Glucitol)	-
Methyl-D-glycoside	-
N-Acetyl D glucose amine	-
Cellobiose	+
Lactose	-
Maltose	+
Sucrose	+
Trehalose	+
Melezitose	+
Raffinose	+

Table 1.7. Carbohydrate assimilations by Propionibacterium isolated from Swiss cheese as revealed by the test system.

*Carbohydrate	Utilization
Glycerol	++
Erythritol	++
Ribose	+
Adonitol	+
Galactose	+
Glucose	+
Fructose	+
Mannose	+
Inositol	+
Arbatin	<u>+</u>
Esculin	+
Maltose	+
Lactose	++
Saccharose	+
Trehalose	+
D-Turanose	<u>+</u>
Gluconate	<u>+</u>

* Those substrates not utilized are listed in Table 1.5.
 ++ utilized rapidly after 24 hrs. incubation at 30°C
 + utilized rapidly after 2 days incubation

REFERENCES

- Abbar, F.M., and M.T. Mohamed. 1987. Occurrence of enteropathogenic E. coli serotypes in butter. *J. Food Protec.* 50(10): 829-831.
- Al-Hiti, M.M.A., and P. Gilbert. 1983. A note on inoculum reproducibility: A comparison between solid and liquid culture. *J. Appl. Bact.* 55: 173-175.
- American Public Health Association. 1985. Standard methods for the examination of dairy products. 15th ed. American Public Health Assoc., New York.
- Anderson, Rolf. 1986. Inhibition of Staphylococcus aureus and spheroplasts of gram-negative bacteria by an antagonistic compound produced by a strain of Lactobacillus plantarum. *Int. J. Food Microbiology* 3: 149-160.
- Ayres, J.C., J.G. Mundt, and W.E. Sandine. 1980. *Microbiology of foods*. W.H. Freeman and Company, San Francisco.
- Baer, A. 1987. Identification and differentiation of propionibacteria by electrophoresis of their proteins. *Milchwissenschaft* 42(7): 431-433.
- Benkerroum, Noredine. 1988. Personal communication. Microbiology Dept., Oregon State University, Corvallis, OR.
- Bishop, J.R., and C.H. White. 1986. Assessment of dairy product quality and potential shelf-life: A review. *J. of Food Protec.* 49(9): 739-753.
- Blanc, P., and G. Gowa. 1987a. Kinetics of inhibition in propionic acid fermentation. *Bioprocess Engineering* 2: 175-179.
- Blanc, P., G.M. Faup, and G. Goma. 1987b. Production of acetic and propionic acids from household refuse enzymatic hydrolysate by Propionibacterium acidipropionici. *Biomass* 12: 49-55.
- Brady, M.S., and S.E. Katz. 1987. Simplified plate diffusion system for microbial assays of antibiotics. *J. Assoc. of Anal. Chem.* 70(4): 641-646.
- Britz, T.J., and P.L. Steyn. 1980. Comparative studies on propionic acid bacteria. *Phytophylactica* 12: 89-103.

- Brocklehurst, T.F., and B.M. Lund. 1988. The effect of pH on the initiation growth of cottage cheese spoilage bacteria. *Int. J. Food Microbiology* 6(1): 43-49.
- Bullerman, L.B. and J.H. Rupnow. 1984. A laboratory manual for food microbiology. Institute of Agriculture and National Resources, Lincoln, Nebraska.
- Christen, G.L., and M. Senica. 1987. Casein, fluorescein isothiocyanate as a substrate for measuring protease production by psychotrophic bacteria. *J. Food Protec.* 50(9): 744-749.
- Cousin, M.A. 1982. Presence and activity of psychotrophic microorganisms in milk and dairy products: A review. *J. Food Protec.* 45(2): 172-207.
- Cuk, Z., A. Annan-Prah, M. Janc, and J. Zajc-Satler. 1987. Yoghurt: An unlikely source of Campylobacter jejuni/coli. *J. Appl. Bacteriology* 63: 202-205.
- Debevere, J.M. 1987. The use of buffered acidulant systems to improve the microbiological stability of acid foods. *Food Microbiology* 4: 105-114.
- DeMan, J.C., M. Rogosa, and M.E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriology* 231: 130-135.
- Doyle, M.P. and J.L. Schoeni. 1987. Isolation of E. coli 0157:H7 from retail fresh meat and poultry. *Appl. and Envir. Microbiology* 50(10): 2394-2396.
- Eklund, T. 1985. Inhibition of microbial growth at different pH levels by benzoic and propionic acids and esters of p-hydroxybenzoic acid. *Int. J. Food Micro.* 2: 159-167.
- Elliker, P.R., W.E. Sandine, B.A. Hauser, and W.K. Moseley. 1964. Influence of culturing cottage cheese dressing with different organisms on flavor and keeping quality. *J. Dairy Science* 47: 680.
- Fleet, G.H., and M.A. Mian. 1987. The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiology* 4: 145-155.
- Flores-Galaza, R.A., Bonita A. Glatz, and coworkers. 1985. Preservation of high-moisture corn by microbial fermentation. *J. Food Protec.* 48(5): 407-411.

- Food and Drug Administration. 1984. Bacteriological analytical manual (BAM). 6th ed. U.S. Food and Drug Administration, Washington, D.C.
- Franco, Bernadette D.G.M., B.E.C. Guth, and L.R. Trabuls. 1987. Enterotoxigenic E. coli isolated from foods in Sao Paulo, Brazil. J. Fd. Pr. 50(10): 832-834.
- Freese, E., C.W. Sheu, and E. Galliers. 1973. Function of lipophilic acids as antimicrobial food additives. Nature 241: 321-325.
- Gonzalez, C.F., and Blair S. Kunka. 1987. Plasmid associated bacteriocin production and sucrose fermentation in Pediococcus acidilactici. Appl. and Env. Micro. 53(10): 2534-2538.
- Henning, Susanne, R.M. Hammes, and W.P. Hammes. 1986. Studies on the mode of action of nisin. Int. J. of Food Micro. 3:121-134.
- Herrero, A. 1983. End-product inhibition in anaerobic fermentations. Trends in Biotechnology 1(2): 49-53.
- Hirooka, E.Y. Sonia, P.C. Desalzburg, and M.S. Bergdall. 1987. Production of staphylococcal enterotoxin A and thermonuclease in cream pies. J. Food Protec. 50(11): 952-955.
- Hogg, G.M., M.F. Patterson, and J.G. Barr. 1987. Correlation between antibiotic sensitivity testing by conventional and conductivity measurements. J. Appl. Bact. 62: 189-195.
- Holt, J.G., ed. 1977. The shorter Bergey's manual of determinative bacteriology. 8th ed. Williams and Wilkins, Baltimore.
- Ingram, E., K.T. Holland, G. Gowland, and W.J. Cunliffe. 1983. Studies of the extracellular proteolytic activity produced by Propionibacterium acnes. J. Appl. Bact. 54: 263-271.
- Ismaeel, N., J.R. Furr and A.D. Russell. 1986. Reversal of the surface effects of chlorhexidine diacetate on cells of Providencia stuartii. J. Appl. Bact. 61: 373-381.
- Jay, James M. 1982. Antimicrobial properties of diacetyl. Appl. and Env. Micro. 44(3): 525-532.

- Jay, J.M. 1986. Modern food microbiology. Van Nostrand Reinhold Company, New York.
- Juven, B.J., and J. Kanner. 1986. Effect of ascorbic, isoascorbic and dehydroascorbic acids on the growth and survival of Campylobacter jejuni. J. of Appl. Bact. 61: 339-345.
- Kabara, Jon J. 1978. Fatty acids and derivatives as antimicrobial agents: A review. Chapter 1 In The pharmacological effects of lipids. Jon J. Kabara, ed. Am. Oil Chem. Soc., Champaign, Ill.
- Kvenberg, J.E., and D.L. Archer. 1987. Economic impact of colonization control of food-born disease. Food Technology July: 77-81.
- Magan, N., and J. Lacey. 1986. Water relations and metabolism of propionate in two yeasts from hay. J. Appl. Bact. 60: 169-173.
- Malik, A.C., G.W. Reinbold, E.R. Vedamuthu. 1968. An evaluation of the taxonomy of propionibacterium. Can. J. Micro. 14: 1185-1191.
- Mehta, A.M., K.A. Patel, and P.J. Dave. 1983. Isolation and purification of an inhibitory protein from Lactobacillus acidophilus AC1. Microbios. 37: 43.
- Miyakoshi, S., H.U. Chiyami, and coworkers. 1987. Distribution of the methylcitric acid cycle and B-oxidation pathway for propionate catabolism in fungi. Agric. Biol. Chem. 51(9): 2381-2387.
- Moon, N.J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bact. 55: 453-460.
- Moran, A.P., and M.E. Upton. 1987a. Factors affecting production of coccoid forms by Campylobacter jejuni in solid media during incubation. J. of Appl. Bact. 62: 527-537.
- Moran, A.P., and M.E. Upton. 1987b. Effect of medium supplements, illumination and superoxide dismutase on the production of coccoid forms of Campylobacter jejuni ATCC 29428. J. Appl. Bact. 62(1): 43-51.
- Morris, C.E. 1985. Derived from whey, it's a milk replacer. Food Engineering March: 63.

- Moustafa, H.H., and E.B. Collins. 1969. Effect of selected food additives on growth of Psuedomonas fragi. J. Dairy Science 52(3): 335-340.
- Palumbo, Samuel A. 1986. Is refrigeration enough to restrain foodborne pathogens? J. Food Protec. 49(12): 1003-1009.
- Phillips, J.D., and M.W. Griffiths. 1987. The relations between temperature and growth of bacteria in dairy products. Food Microbiology 4(2): 173-185.
- Ray, Bibek, and C. Johnson. 1984. Sensitivity of cold-stressed Campylobacter jejuni to solid and liquid selective environments. Food Microbiology 1: 173-176.
- Reddy, N.S., and B. Ranganathan. 1983. Preliminary studies on antimicrobial activity of Streptococcus lactis subsp. diacetylactis. J. Food Protec. 46(3): 222-225.
- Rose, Sally A. 1985. A note on yeast growth in media used for the cultivation of Lactobacilli. J. Appl. Bact. 59: 153-156.
- Rosenow, E.M., and E.H. Marth. 1987. Listeria, listeriosis and dairy foods: A review. Cultured Dairy Prod. Journal 22(4): 13-17.
- Rusul, Gulam, F.E. El Gazzar, and E.H. Marth. 1987. Growth and aflatoxin production by Aspergillus parasiticus NRRL 2999 in the presence of acetic or propionic acid at different initial pH values. J. Food. Protec. 50(11): 909-914.
- Saito, Isao, H. Oshima, N. Kawamura, K. Uno, and M. Yamada. 1987. Determination of sorbic, dehydroacetic, and propionic acids in cheese by liquid chromatography and gas chromatography. J. Assoc. of Anal. Chem. 70(3): 507-509.
- Salih, M.A. 1985. Studies on growth metabolites produced by Propionibacterium shermanii. Ph.D. thesis. Microbiology Dept., Oregon State University.
- Schales, O. 1951. Effect of albumin on the antibacterial activity of diketones. Arch. Biochem. Biophys. 34: 56-63.
- Schwartz, G.W., H.N.S. Kaufman, and J.E. Snoke. 1948. The specific esterase activity of trypsin. J. Biol. Chem. 172: 221-239.

- Shibasaki, I., and N. Kato. 1978. Combined effects on antibacterial activity of fatty acids and their esters against gram-negative bacteria. Chapter 2 In The pharmacological effects of lipids. Jon J. Kabara, ed. Am. Oil Chem. Soc., Champaign, IL.
- Shotwell, O.L., G.A. Bennett, W.F. Kwolek, and C.W. Hesseltine. 1984. Effect of propionic acid on aflatoxin levels of stored Georgia 1981 Dent corn samples. J.A.O.C.S. 61(3): 526-528.
- Smith, D.A., E.M. Mikolajcik, and J.B. Lindamood. 1987. Causative organisms and chemical nature of the Swiss cheese rind rot defect. Cultured Dairy Prod. Journal 22(4): 9-12.
- Somers, E.B., and S.L. Taylor. 1987. Antibotulinal effectiveness of nisin in pasteurized process cheese spreads. J. Food Protec. 50(10): 842-848.
- Speck, M. L., ed. 1984. Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D.C.
- Stanfield, J.T., C.R. Wilson, W.H. Andrews, and G.J. Jackson. 1987. Potential role of refrigerated milk packaging in the transmission of listeriosis and salmonellosis. J. Food Protec. 50(9): 730-732.
- Suihko, M.L., and V. Makinen. 1984. Tolerance of acetate, propionate and sorbate by Saccharomyces cerevisiae and Torulopsis holmii. Food Microbiology 1: 105-110.
- Tham, Wilhelm, and L.J. Hajdu. 1987. Comparison of 6 media for the isolation of Staphylococcus aureus from foods. Food Microbiology 4(2): 133-146.
- Vallejo-Cordoba, B., S.N., W.D. Powrie, and T. Beveridge. 1987. Extended shelf life of frankfurters and fish frankfurter-analogs with added soy protein hydrolysates. J. Food Science 52(5): 1133-1136.
- Vedamuthu, E.R., and G.W. Reinbold. 1967. The use of candle oats jar incubation for the enumeration, characterization and taxonomic study of propionibacteria. Milchwissenschaft 22(7): 428-431.
- Washington, J.A., ed. 1985. Laboratory procedures in clinical microbiology. Springer-Verlag, New York.

- Weber, G.H., and W.A. Broich. 1986. Shelf life extension of cultured dairy foods. *Cultured Dairy Products Journal* November: 19-23.
- Wheeler, K.A., A.D. Hocking, J.I. Pitt, and A.M. Anggawatl. 1986. Fungi associated with Indonesian dried fish. *Food Microbiology* 3(4): 351-357.

CHAPTER 2
CHARACTERIZATIONS OF MICROGARD

ABSTRACT

Gas chromatographic as well as high pressure liquid chromatographic analyses of Microgard revealed the presence of propionic acid, diacetyl, acetic acid and lactic acid. Proteases such as -chymotrypsin antagonized Microgard activity, while albumin and certain emulsifiers destroyed the inhibitory activity. Exposure to proteases at high pH levels (11) enhanced the inhibitory ability. Dialysis removed active substance(s) of Microgard, whereas boiling and autoclaving did not destroy these materials. Sephadex column results revealed that the molecular weight of the inhibitory principle(s) was about 700 daltons and those substances were heat stable and protein in nature.

Purified active preparations from Sephadex exhibited a maximum absorbance at 210 nm; these preparations dramatically lost inhibitory ability upon cold storage (2-5°C) for 2 months. While Microgard exerted a bacteriocidal effect on P. putida at high concentration (3 to 5%), a bacteriostatic effect was obtained at low levels (.5 - 1%). Contamination and growth of yeasts abolished the inhibitory activity.

INTRODUCTION

Propionibacteria are important in the development of flavor and eye formation in Swiss cheese and to prevent the growth of spoilage fungi in high-moisture corn and raw plant materials (Langsrud et al., 1973; Flores-Galarza et al., 1985 and Gavrilova et al., 1987). Growth metabolites of propionibacteria include propionic acid, acetic acid and carbon dioxide (Shaw et al., 1923; Hettinga et al., 1972; Wood et al., 1936; Hitchner, 1934, and Blanc et al., 1987). Propionic acid has GRAS (generally recognized as safe) status as a food additive and has been found non-mutagenic in rats (Basler et al., 1987).

Numerous workers have reported on the associative growth, during propionic acid production, of specific lactic acid bacteria and Propionibacteria (Sherman et al., 1921; Schutz et al., 1987; Bodie et al., 1987a and Bodie et al., 1987b). While propionic acid production by propionibacteria decreases when the concentration of whey and oxygen tension increase in autoclaved media (Anderson et al., 1986 and Cove et al., 1987), autoclaving the carbohydrate with other media components enhances growth of this bacterium (Langsrud, 1973).

Succinate and volatile compounds, including sesquiterpenes, were found to be produced by starter propionibacteria (Dumont et al., 1978; Crow et al., 1986 and Crow, 1987). Thomas (1987), however, pointed out that

production of acetate was due to non-starter bacteria. Other end products such as vitamin B12 and diacetyl were found to be produced by propionibacteria during fermentation (Lee, 1970; Perez-Mendoza et al., 1983; Ro et al., 1979 and Marwaha et al., 1983).

Growth metabolites of Propionibacteria shermanii were reported to extend the shelf life of some dairy and food products, and the metabolites were not believed to be protein in nature (Salih, 1985). However, an antiviral peptide (propionin) has been extracted from Propionibacteria freudenerichii (Ramanathan et al., 1966).

The antagonistic ability of lactic acid bacteria for other microorganisms can be attributed to acid production and associated pH reduction, hydrogen peroxide, nutrient depletion, decrease in reduction-oxidation potential (E_h), and production of carbon dioxide and antibiotics (Anderson, 1986). Antibiotics such as nisin, a polypeptide produced by Streptococcus lactis, may act to interfere with membrane functions (Hennig et al., 1986). Products of an anaerobic metabolism, on the other hand, interact with many parts of the cell (Herrero, 1983 and Blanc et al., 1987). Also, it has been found that the production of bacteriocin and immunity are encoded on a specific plasmid (Gonzalez et al., 1987 and Reeves, 1972).

Examples of bacteria which produce antimicrobial substances of a protein nature include Propionibacterium

spp. (Ramanathan et al., 1966), Streptococcus spp. (Oxford, 1944; Reddy et al., 1983; Branen et al., 1975 and Lana, 1987) (note: the lacti streptococci now are members of the Lactococcus genus [Sandine, 1988]), Leuconostoc citrovorum (Branen et al., 1975), and Lactobacillus spp. (Anderson, 1986; Serot et al., 1987; Guern et al., 1987; Anderson et al., 1987; Mehta et al., 1983; Brink et al., 1987 and Schillinger et al., 1987).

Non-protein antimicrobial substances were found to be produced by Streptococcus thermophilus (Pulusani et al., 1979), Lactobacillus bulgaricus (Abdel-Bar et al., 1987), and Lactobacillus reuteri (Axelsson et al., 1987). Furthermore, Lactobacillus acidophilus produces an antimicrobial substance which has not been characterized (Kim, 1987); this bacterium also can degrade cholesterol (Welch, 1987). Lactic acid bacteria of the Pediococcus genus were found to produce antibacterial growth end products (Fleming et al., 1975; Buhnia et al., 1987; Raccach, 1987; Nout et al., 1987; Gonzalez et al., 1987 and Hoover et al., 1988).

While not a lactic acid bacterium, Brevibacterium linens is capable of producing metabolites which inhibit the germination of fungal spores (Beattie and Torrey, 1986). Also, yeasts and molds (fungi) elaborate growth end-products which are inhibitory (Nakagaw et al., 1982 and Bilinski et al., 1985).

The present study was carried out to characterize the inhibitory metabolites (end products) found in a product known commercially as Microgard. It was believed desirable to learn more about the nature of this product.

MATERIALS AND METHODS

pH Measurements

Different lots and types of Microgard (dairy and non-dairy) were received from Wesman Foods, Inc. The pH was measured with a Corning 125 pH meter.

Percent of Solids in Microgard

Liquid Microgard was dispensed in small aluminum plates, each of which contained 5 milliliters (ml). Drying was carried out in a vacuum oven at 100°C until constant weights were obtained (3 hours). The percent of moisture was calculated as an average of three plates.

The Effect of Dialysis

Liquid Microgard (5 ml) at pH 6.2 was aseptically placed inside dialysis tubing (VWR Cat. #25225-204 size #8) which was previously boiled in water for 20 minutes. Care was taken by wearing gloves to prevent contamination by enzymes. Dialysis was carried out against sterile, distilled water in a 4000 ml flask for 24 hours, with continuous stirring at ambient temperature. Microgard control was used without dialysis. After dialysis, the pH of both control and dialyzed Microgard were adjusted to 5.30 with 10% sterile tartaric acid. Microgard was then

bioassayed against P. putida (as described in Chapter 1) after incorporating 1 or 3% of it into the agar media.

Thermal Stability

1) Boiling:

Liquid Microgard was boiled in a water bath at different initial pH values (3.0, 4.8, 5.3, 5.9, and 6.7). Some bottles containing Microgard were sacrificed in order to measure the temperature inside the bottles and to make sure that exactly 5 minutes had elapsed. The content of the bottles was adjusted to pH 5.30 and assayed against P. putida. Control Microgard was used without boiling.

2) Autoclaving:

Microgard was placed in pre-sterilized bottles (100 ml capacity) which had screw caps. Autoclaving was done at 121°C (250°F) for 15 minutes. Microgard was adjusted to different pH levels before autoclaving; these were 1.4, 1.5, 2.0, 5.3, 6.2 and 11.0. The sensitivity test was done as mentioned above for boiling. Agar containing plates were incubated aerobically at 30°C for 42-45 hours and examined for growth (red colonies).

King Test

Diacetyl detection in Microgard was performed according to the procedure of King (1948). Nonfat dry milk (11% solid) and distilled water were used as negative

controls. Diacetyl solutions (1%, 0.1% and 0.01%) served as positive controls. Samples of Microgard were used for diacetyl detection with or without casein precipitation to pH 4.6 with 10% tartaric acid and filter sterilization (0.45 μ m filters). The development of a cherry red color in the test tubes was recorded as positive for diacetyl.

Gas Chromatography Separation

A GC packed column (Hewlett-Packard 5710A) was used. The column was 80/120 Carbopack BAW 6.6% Carbowax 20 M. The glass column had an O.D. of 1/4 inch and was 6 feet x 2 mm I.D.

The temperature of the oven was raised from 90°C to 200°C at 4°C/1 min. Nitrogen gas was used at a flow rate of 20 ml/1 min. Temperatures of the flame ionization detector (FID) and the injector were maintained at 250°C. Amounts of volatile substances were quantitated using a 3390A Hewlett-Packard integrator.

Microgard samples (acidified to pH 4.50 with 10% tartaric acid to precipitate casein) were centrifuged twice at 10,000xg for 15 minutes at 5°C (Beckman J2-21 centrifuge). Then filter sterilization was done through 0.45 μ m filters (Millipore). Standard solutions of different volatile substances were used to calibrate the column before applying the sample. The column was flushed to remove any residues of volatiles before applying the sample. Then a

sample of 10 μ l was injected in the column and 50 ppm sec-butanol was used as an internal standard.

Citric Acid Analysis

The enzymatic method for citric acid described in Catalogue #139076 (Boehringer Mannheim Biochemical Company, Indianapolis, IN) was used. Both non-autoclaved and autoclaved Microgard were used after centrifugation and filter sterilization.

Hydrogen Peroxide and the Benzidine Test

Catalase (Sigma) was added to Microgard to trace any gas formation (bubbles). Both 35% hydrogen peroxide and distilled water were used as a positive and a negative control, respectively. The Benzidine test (Deibel et al., 1960) was performed by plating Propionibacterium shermanii (Microgard starters, Wesman) in Thioglycollate Medium (2% agar) followed by incubation in Gas Pak (anaerobic conditions) at 30°C for 4 days. The growth medium served as a negative control, while P. putida was grown in BHI agar and was the positive control.

Determination of Organic Acids in Microgard

Microgard preparations for high performance liquid chromatography (HPLC) were carried out as described above in preparing samples for GC analysis.

A 10 μ l sample of Microgard (1:10 dilution) was injected into the HPLC (Beckman model 332 gradient liquid chromatograph system, Bio Rad Aminex HPX-87H, 300 x 7.8 mm. column and a Bio Rad Guard pack Microguard cation H⁺). Sulfuric acid (0.010 N) was used as an eluent. Eluent absorbance was measured at 210 nanometers ($\lambda = 210$ nm) and an absorbance range of 0.01 was used with a Beckman 163 variable wavelength detector.

Amounts of organic acids were quantified using an integrator (Hewlett-Packard 3390A), which was calibrated by different standard solutions of known organic acids and then multiplied by the dilution factor (10).

Dairy vs. Non-Dairy Microgard

Both dairy and non-dairy Microgard lots were bioassayed against K. marxianus ATCC 8554 using the agar incorporation method (Chapter I).

Comparison between Liquid and Spray-Dried Microgard

The agar incorporation method also was used to compare the inhibition by liquid and dehydrated Microgard against Achromobacter delicatulus ATCC 19103. Dry Microgard was rehydrated aseptically in the agar medium to a solid content of 9.5% as for liquid Microgard.

Concentrations of 1% and 3% of liquid Microgard were used and compared to dry Microgard at the same

concentrations. In the case of dry Microgard, the pH of the agar medium was adjusted aseptically with 10% sterile tartaric acid to pH 5.30 after Microgard addition.

Protease Sensitivity

Microgard was subjected to the proteolytic actions of α -chymotrypsin, trypsin, pepsin or protease type III. All enzymes were Sigma products. The pH of Microgard was adjusted to pH 6.9-7.1 by 1 N sterile sodium hydroxide, except with pepsin when the pH of Microgard was lowered to 3.00 by concentrated hydrochloric acid (J.T. Baker). Each enzyme was added at 0.1% (w/v) to Microgard. Enzyme-free Microgard was used as a control. Both controls and samples were incubated at 37°C for 15 hours. Then the pH was raised to 6.50 before boiling in a water bath for 5 minutes (Kato et al., 1987 and Ahern et al., 1985). After inactivation of the enzymes by boiling, the samples and controls were kept in a water bath at ambient temperature (25°C), and then the pH was adjusted to 5.30, after which bioassays were performed against P. putida using the agar incorporation method (Chapter 1).

Deamidation by Proteases

Microgard (pH 6.2) was adjusted to pH 11.00 with 3 N sodium hydroxide. α -chymotrypsin or trypsin was added to a final concentration of 0.04% (w/v). Control Microgard

(free of enzyme) was used under the same conditions. This is called deamidation (Kato et al., 1987).

Both enzyme-treated Microgard and controls were incubated at 37°C overnight. Then the pHs were lowered to 6.8 followed by boiling for 5 minutes. After boiling and cooling, the pH values were adjusted to 5.30 and samples were assayed biologically against P. putida (agar incorporation method).

Whenever proteolytic enzymes were used with Microgard, a boiled enzyme solution in water was assayed similarly against the test organism.

The Antagonistic Effects of Additives

Bovine albumin (Sigma) solution (10% in 2% sodium chloride) was filter sterilized through a 0.45 μ m filter (Millipore) to eliminate contamination. This solution was added to the sterilized Plate Count Agar (pH 5.30) to give a final concentration of 0.01% (v/v).

The agar method was used by incorporating 1% or 3% Microgard in the medium for bioassay against P. putida. Controls (free of either albumin or Microgard) were used under the same conditions. Incubation of the plates, both samples and controls, was performed at 30°C for 3 days. Also, the effect on Microgard of 1% Tween 80 and 1% lecithin (Lecigram 5750 Vivon Chem. Company) in the agar medium was tested against the test organism (P. putida).

Finally, ethylenediaminetetraacetic acid (EDTA), Sigma grade, was added to 0.05% concentration (w/v) in the agar medium and assayed similarly against P. putida. Medium free of Tween and lecithin or EDTA was used as controls as well as Microgard-free medium.

Addition of Tween 80 and lecithin or EDTA was carried out before the agar medium was autoclaved. Incubation at 30°C was carried out for 9 days when the effect of Tween or EDTA was tested.

Purification and Determination of the Molecular Weight

The inhibitory principle(s) was separated from Microgard through a Sephadex G-10 (Sigma) column. The column was 20 x 2.5 cm. and the flow rate was 2 ml/minute. Sodium chloride (0.2 M) was used to equilibrate the column and also as the eluent at ambient temperature. The Sephadex column was standardized with Bacitracin (Sigma #0125, molecular weight 1422.7), sleep inducing peptide (Sigma #1762, MW 848.8) and tyrosine (Sigma #2006, MW 217.7). Each standard solution was made by dissolving 2.5 mg of the respective substance in one ml deionized water. Then two ml of each standard were loaded into the column and eluted with 0.2 M sodium chloride, followed by collecting fractions, each of which was five ml. The absorbance (A) of each collected fraction was determined at 280 nm using a Beckman DU-40 spectrophotometer; the highest

absorbance (a peak) was considered as the excluded standard. A standard curve was constructed by plotting fraction number against the logarithm of the molecular weight (Whitaker, 1963).

Samples of Microgard were prepared by acidification to pH 4.5 with 10% tartaric acid to precipitate casein and the clear supernatant was filter-sterilized through 1.2 μm and 0.45 μm filters (Millipore) several times. A sample of 2 ml was loaded on the pre-washed column and eluted with the same eluent. Collection of fractions (total 22) was done manually and each fraction contained 5 ml. The absorbance of each fraction was measured at 280 nm.

Each fraction (1-22) was adjusted to pH 5.30 with either 10% tartaric acid or 1 N sodium chloride. Then filter sterilization (0.45 μm filter) was carried out to eliminate cross contamination. Finally, each fraction was bioassayed against P. putida by using the disc procedure (Chapter I).

Characterizations of the Biologically Active Fractions

1) Molish Test:

The fraction (#10) active against P. putida as well as the non-active fractions (3 and 17) were examined for the presence of carbohydrate according to the procedure of Pulusani et al. (1979). Lactose and glucose solutions (1%)

were used as positive controls, whereas distilled water served as a negative control.

2) Biuret Test:

Tests for the presence of protein or peptides were carried out using fractions #9 and #10 (active fractions) and non-active ones (3 and 21) as well as the eluent (0.2 M NaCl). The procedure reported in the literature was used (Oser, 1965). Albumin 0.1% solution was a positive control whereas glutamic acid served as a negative control. Sample volume (3 ml) was mixed with an equal amount of 10% sodium hydroxide and thoroughly mixed by a vortex mixer. Then drops of 0.5% cupric sulfate (Sigma) were gradually added until development of a blue-violet color.

3) Protein Content:

A Beckman DU-40 spectrophotometer was used along with nucleic acid Soft-Pac module (Part #533126), Warburg and Christain program (program 10), to determine the amount of protein (parts per million, $\mu\text{g}/\text{ml}$) in all fractions obtained from Microgard passing through the Sephadex column.

4) Thermal Stability:

The bioactive fraction (#9) at pH 5.30 was assayed against P. putida using the disc method before and after autoclaving at 121.1°C (250°F) for 15 minutes. The active fraction was placed in a screw-capped vial before autoclaving.

5) Quantitation of Volatile Substances:

GC analysis was carried out to determine the amount of volatile ingredients, particularly propionic and acetic acids. Fraction #7 was randomly selected to represent the bioactive fraction, whereas fractions 2, 14 and 21 were non-active fractions.

6) Visible and Ultraviolet Spectrum:

Active fractions obtained from Sephadex column were scanned at 200-700 nm using a Beckman DU-40 spectrophotometer in order to obtain maximum absorbance.

7) The Effect of Storage:

Purified fractions of Microgard obtained through Sephadex G-10, bioactive and non-active fractions, were stored at refrigeration temperature in tightly-closed vials for 2 months at initial pH 5.3.

After cold storage, the active fractions, particularly #6 and #7, were assayed against P. putida using the disc assay method.

8) Rechromatogram of the Active Fractions:

The bioactive fraction #10, obtained from Sephadex column (see purification and MW determination) was rechromatogrammed using the same column of Sephadex G-10; 2 ml of this fraction were loaded on the column and eluted with 0.2 M sodium chloride. Fractions (5 ml) were

collected manually (total 20) and examined for inhibitory activity against P. putida (disc assay method).

The Mechanism of Inhibition by Microgard

Microgard was added to an active broth culture of P. putida at 5% concentration (v/v). The broth contained (per liter: 5 g tryptone, 2.5 g yeast extract, and 1.0 g glucose), adjusted to pH 5.30 with 10% tartaric acid before autoclaving. Meanwhile, Microgard was adjusted to pH 5.30 and subjected to autoclaving before being added to the broth cultures. Dilutions of the test organism (P. putida), 1/10, 1/10² and 1/10⁴, were prepared in the broth. Microgard-free broth and bacteria-free broth (containing Microgard) were used as controls. The broth tubes were incubated at 30°C for 44 hours. Then each tube of broth was diluted 10 times by using fresh broth (pH 5.30) which did not contain Microgard, followed by incubation at 30°C for 3 days. After incubation, each broth was diluted again (10 times) by fresh broth (pH 7.00), and incubation was carried out for 20 days. The concentration of Microgard in the last case was approximately 0.05% and the initial pH was 6.8.

RESULTS

Percent Solids and pH

Microgard (liquid) was found to contain 9.50% solids as determined by the vacuum oven drying. The pH of Microgard was 5.9 to 6.20 upon receipt from Wesman Foods.

Dialysis Effects

Microgard subjected to dialysis lost its inhibitory ability against P. putida, even when relatively high concentrations of the dialyzed Microgard were used (3% v/v) in the growth medium. Control Microgard (not dialyzed) retained a strong suppressor effect against the test organism.

Thermal Stability

As shown in Table 2.1, Microgard withstood severe heat treatment (both boiling and autoclaving) at several pH values and still retained inhibitory action for P. putida.

Diacetyl Identification

King test results revealed that diacetyl was present in Microgard as evidenced by the development of a light cherry red color in the sample tubes.

GC Analysis of Microgard

Several volatile substances including diacetyl, acetoin and acetaldehyde, were recovered from Microgard, as indicated by GC analysis. Table 2.2 summarizes the kind and amount of these compounds present. Figure 2.1 shows a typical chromatogram.

HPLC Analysis

Table 2.3 summarizes the amount of several organic acids present in Microgard. Lactic, citric, acetic and propionic acids were found by HPLC analysis.

HPLC analysis revealed a ratio of 1:3.5 between propionic and acetic acids. Neither succinic acid nor pyruvic acid were present in Microgard. Also, unknown compounds were eluted at retention times (minutes) 4.81, 8.37, 12.34, 15.27 and 16.96 (Fig. 2.2).

Hydrogen Peroxide and the Antioxidant Enzymes

The possibility of inhibitory activity by hydrogen peroxide present in Microgard was ruled out, since addition of catalase enzyme did not cause bubble formation.

However, addition of hydrogen peroxide (35% food grade) to an active broth culture of Propionibacterium shermanii (Microgard starter obtained from Wesman Foods), caused strong bubble formation in the broth. The Benzidine

test gave negative results with this organism, indicating that pseudocatalase caused the reaction.

Inhibition by Dairy and Non-Dairy Microgard

The same level of inhibitory potency was exerted by both dairy or non-dairy Microgard against the test organism (K. marxianus). Figure 2.3 illustrates the obtained results.

Liquid vs. Spray-dried Microgard

Generally speaking, both kinds of Microgard gave the same inhibition against the test organism (Achromobacter delicatalus), particularly when high concentration of the respective Microgard was applied. However, the form of dry Microgard at 1% (w/v) was not able to continue inhibiting the test organism after 2 days of incubation, as seen in Figure 2.4.

The Effect of Proteolytic Enzymes

All enzymes used except trypsin were able to abolish the inhibitory activity of Microgard. Complete degradation of the active principle(s) in Microgard, however, required several treatments by the respective enzymes (e.g. α -chymotrypsin) and exposure for longer time. Trypsin, on the other hand, did not cause any antagonistic effect on Microgard (Table 2.4).

Deamidation by Proteases

As shown in Figure 2.5, exposures to either trypsin or α -chymotrypsin caused enhancement of inhibition by Microgard. However, incubation of Microgard at pH 11.00 (controls) at 37°C for 24 hours seemed to antagonize the active component(s) in Microgard. High concentration of the Microgard control used (3%) gave the same inhibition when compared to the enzyme-treated samples.

The Effect of Albumin, Emulsifiers and EDTA

Both albumin and Tween 80 as well as lecithin caused inactivation of Microgard and rendered it unable to suppress the growth of P. putida, especially when a low concentration of Microgard was used (1%). However, this antagonistic effect could be reversed by adding an initially high concentration of Microgard, viz. 3%. EDTA addition, on the other hand, had no effect on Microgard activity. Table 2.5 summarizes the obtained results.

Molecular Weight Determination

As shown in Figure 2.6, the active principle(s) in Microgard which gave inhibition zones against P. putida was eluted from Sephadex G-10 column (exclusion limit was 700) in fractions #5 through #13. Fraction #5 was inhibitory to P. putida but gave a quite small inhibition zone. In comparison with standard solutions eluted from the same

column (Figure 2.5), the active species of Microgard had a molecular weight of approximately 700 daltons.

Characterizations of the Purified Materials:

1) Presence of Carbohydrate:

The Molish test gave positive results with the bioactive fractions indicating the presence of a carbohydrate moiety.

2) Biuret Test:

The purified materials which were active biologically against the test organism were of protein nature as indicated by a positive result in this test (development of light violet-blue color).

3) Protein Concentration:

Figure 2.8 illustrates the amount of proteins in all fractions. Three peaks were obtained at fractions 5, 10 and 13, with #10 having the highest protein concentration (168 $\mu\text{g/ml}$). The bioactive fractions against P. putida were between peak #1 and #3 (fractions 5 and 13).

4) Thermal Treatment:

Surprisingly, the bioactive fraction which gave an inhibition zone against P. putida before autoclaving gave almost the same diameter zone after being sterilized.

5) Volatile Acids:

As shown in Figure 2.9, both propionic and acetic acids were recovered from the fractions tested by GC. Fraction #7, which had most of the activity, had the highest amounts of both acids. However, a solution containing the same amount of both acids (pH 5.3) was not able to give any inhibition when compared to bioactive fraction #7.

6) UV Spectrum:

The bioactive purified substances exhibited a UV spectrum in the wavelength range of 210-300 nm. A maximum peak was observed at 210 nm. However, no absorbance was noticed in the visible range (Fig. 2.10).

7) The Impact of Storage:

Column purified active fractions lost, dramatically, their inhibitory ability against the test organism upon storage at 2 to 5°C for 2 months. Commercial, unpurified Microgard was stable under these conditions. Meanwhile, the purified substance did not exhibit UV spectrum after storage at 2-5°C (Fig. 2.10).

8) Purification of the Bioactive Fraction:

Figure 2.11 shows the elution profile of fractions which were obtained from further purification of fraction #10 (see molecular weight determination). Fraction #6 was

active against P. putida, suggesting that the molecular weight of the bioactive purified species was around 700 daltons. Both propionic and acetic acids (GC analysis) were recovered from fraction #6 at concentrations of 21.00 ppm and 4.10 ppm, respectively.

The Lethality of Microgard

The test organism, Pseudomonas putida, was killed by Microgard and no growth was established even after diluting Microgard and raising the pH above 5.30. However, luxurious growth was found in control tubes. Yet Microgard concentration in culture broth at 0.5 and 1% final concentration (initial pH 5.30) seemed to exert only a bacteriostatic effect.

DISCUSSION

Microgard lost its inhibitory activity when subjected to dialysis, suggesting the presence of low molecular weight inhibitors as suggested in the work of Ramanathan et al. (1966). Furthermore, this thermal stability also indicated small sizes for these components. Similarly, other antimicrobial products produced by certain lactic acid bacteria were found to withstand thermal treatments (Buhnia et al., 1987; Oxford, 1944; Abdel-Bar et al., 1987; Pulusani et al., 1979; Kim, 1987 and Branen et al., 1975).

In contrast, end products of other lactic acid bacteria inhibitory to some food spoilage and pathogenic microorganisms have been reported to be inactivated by heat treatments (Gonzalez et al., 1987 and Anderson, 1986).

Flavor compounds such as diacetyl and acetaldehyde were present in Microgard. Diacetyl is significant because of its ability to inhibit gram negative bacteria, and its production by propionibacteria, along with other volatiles, was reported by Lee et al. (1970) and Hettinga et al. (1972). The amount of diacetyl, however, was quite low, and this could be attributed to either long storage, diacetyl reductase activity, or an unfavorable fermentation temperature as stated by Lee et al. (1970) and Walker et al. (1987).

Propionic, acetic and lactic acids were present in Microgard, which could participate in the inhibitory

ability against the microorganisms tested during this study. However, the pKa values of these weak acids fall below pH 5.30 (Jay, 1986 and Rusul et al., 1987), suggesting a marginal inhibitory effect of these acids at this particular pH. The ratio between propionic acid and acetic acid as revealed by HPLC was 1:3.5. Other research workers have reported a ratio of 2:1 (Shaw et al., 1923; Crow et al., 1986; Blanc et al., 1987 and Schutz et al., 1987). Also, a propionic acid fermentation with mixed cultures or 100% carbon dioxide has been reported to raise the ratio to 3:1 (Bodie et al., 1987 and Crow, 1987). Furthermore, the citric acid cycle intermediate succinic acid was not recovered from Microgard, which is not in agreement with the findings of Crow (1987).

Different kinds of Microgard which came from dairy and non-dairy fermentable substrates gave the same inhibitory potency. However, components of growth media can be of critical importance in producing antimicrobial agents using lactic acid bacteria as demonstrated by Branen et al. (1975) and Reddy et al. (1983).

The starter culture for Microgard preparation, P. shermanii ATCC 9616, surprisingly did not give a positive result with the Benzidine test since Propionibacterium species are known to be catalase positive. Peroxidase and catalase have been demonstrated to be produced by Propionibacterium shermanii (Vorob'eva et al., 1987 and

Hitchner, 1934). This disagreement could be attributed to the different sources of each bacterium. Furthermore, a superoxide dismutase enzyme was reported to be produced by P. shermanii (Pritchard et al., 1977).

Microgard was inactivated by proteolytic enzymes, which revealed the protein nature of the inhibitory substances. However, in a previous study, conducted by Salih (1985), opposite findings were reported. Nonetheless, Microgard was not destroyed by trypsin, and results indicated the presence of anti-trypsin activity in Microgard since both trypsin-treated and the control samples reached the same pH (4.80) after incubation. Such activity has been reported in some food systems (Konishi et al., 1985).

Instead of being destroyed, treatments of Microgard with protease enzymes at a higher pH value resulted in enhancement of the inhibitory ability of Microgard. Similar results were found in other food systems in which the wetting and emulsifying abilities of proteins were increased by treatments with proteases at higher pH levels where deamidation occurs (Kato et al., 1987 and Shih, 1987). Also, activation of Clostridium botulinum toxins was demonstrated using proteolytic enzymes (Duff et al., 1956, Tjaberg, 1974 and Sakaguchi et al., 1966).

Emulsifying agents such as Tween 80 or lecithin interfered with the inhibitory activity of Microgard. But

this action could be overcome by using a higher concentration of Microgard. Similar results were reported in the literature regarding nisin; cell wall phospholipids of susceptible organisms are believed to be the target of nisin (Henning et al., 1986).

Likewise, bovine albumin caused at least a 2-fold increase in the concentration of Microgard needed in order to get a 100 percent inhibition of the test organism. The fact that albumin and other substances antagonize certain antibacterial substances has been reported in the literature (Schales, 1951; Shibasaki et al., 1978 and Kabara, 1978). Nisin, however, was not inactivated by globulin (Henning et al., 1986). Furthermore, it was demonstrated in the gel filtration study of Fairclough et al. (1966) and Hummel et al. (1962) that albumin strongly binds tryptophan. Also, bovine albumin was demonstrated to have emulsifying properties (Haque et al., 1988).

EDTA, a food additive inhibitory to bacterial growth (Bulgarelli et al., 1985), did not interfere with the antimicrobial ability of Microgard.

Purified preparations of Microgard (MW 700) eluted from Sephadex G-10 lost activity when they were stored at refrigeration temperature for 2 months. In contrast, Abdel-Bar et al. (1987) found that an antimicrobial agent, produced from Lactobacillus bulgaricus, was stable at 6°C for 18 days but it was not protein in nature. Furthermore,

active purified fractions obtained from Sephadex exhibited a maximum absorbance at 210 nm and gave a positive Biruet test, indicating the presence of peptide bonds or proteins.

Finally, a bacteriocidal effect was offered when higher concentrations (3 to 5%) of Microgard were used. However, when low concentrations of the microbial inhibitor were used, the test organisms (P. putida) were able to initiate growth following initial inhibition. In this regard, diacetyl was also shown to rely on its concentration for its mode of inhibitory action against gram negative and positive bacteria (Jay, 1982). Also, propionibacteria were only partially inhibited by whey filtrate in agar plates but were completely inhibited in broth (Langsrud et al., 1973).

Future research work is needed to investigate the specific structures in microbial cells which are inhibitor targets of Microgard. Also, the ability of Microgard to lower the pH of some protein-containing systems should be addressed by further research.

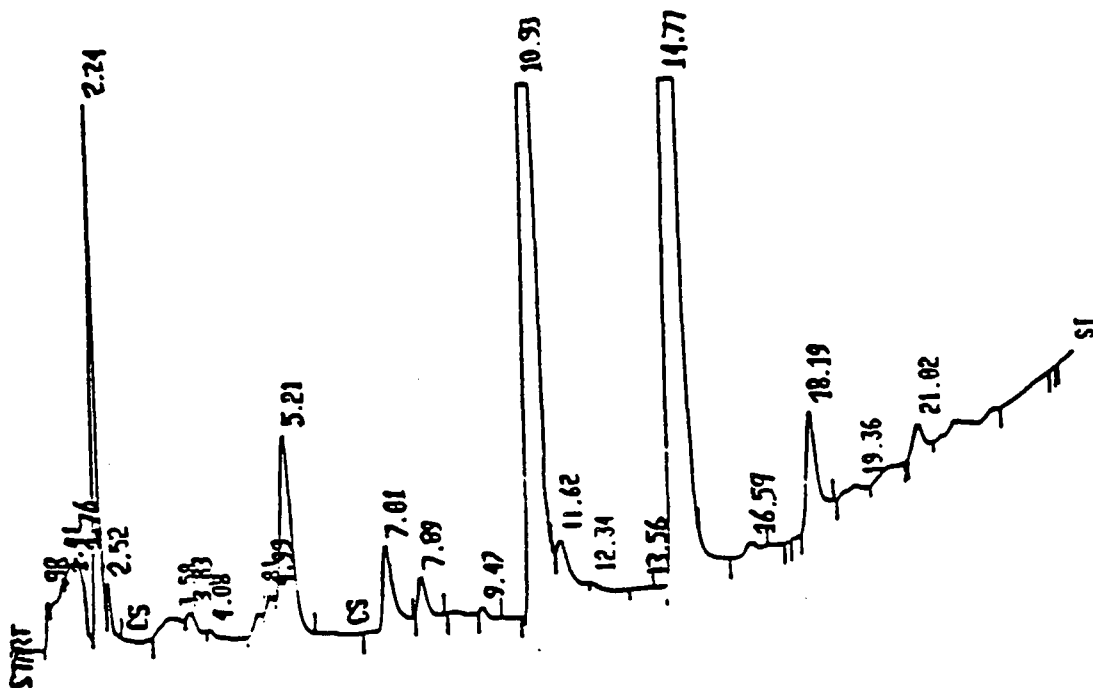


Fig. 2.1. GC analysis of volatiles present in Microgard. Peak identities are as follows: Retention time (min), RT=0.98, acetaldehyde; RT=3.58, ethylacetate; RT=3.83, diacetyl; RT=4.08, isopentanal; RT=4.99, propanol; RT=9.47, acetoin; RT=10.93, acetic acid; RT=11.62, n-amyl alcohol; RT=14.77, propanoic acid; RT=18.19, unknown and RT=21.02, unknown.

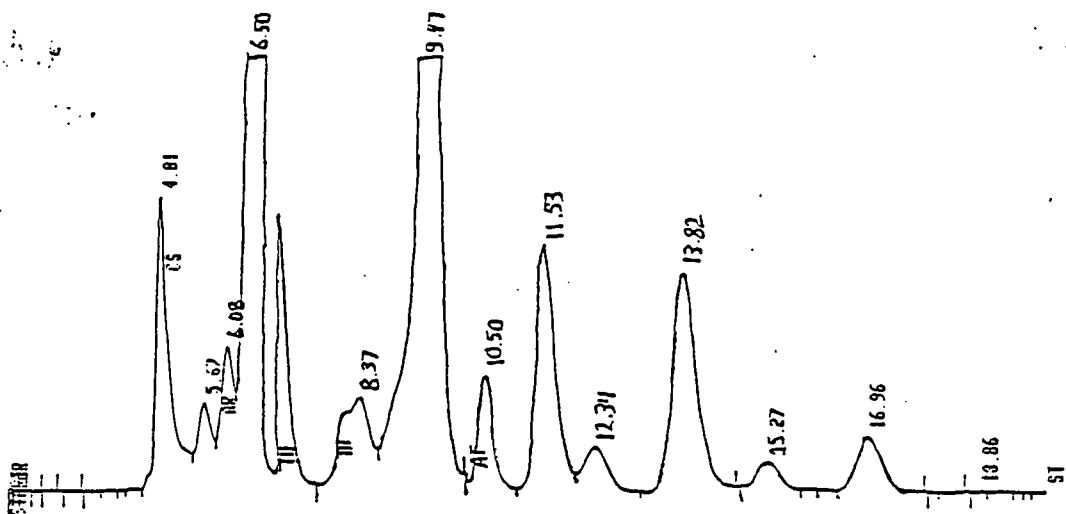


Fig. 2.2 . HPLC chromatogram of Microgard showing the different organic acids found. Retention time (min.), RT=6.08, citric acid; RT=6.50, tartaric acid (added); RT=8.37, unknown; RT=9.47, lactic acid; RT=10.50, internal standard (formic acid); RT=11.53, acetic acid; RT=12.34, unknown; RT=13.82, propionic acid; RT=15.27, unknown and RT=16.96, unknown.

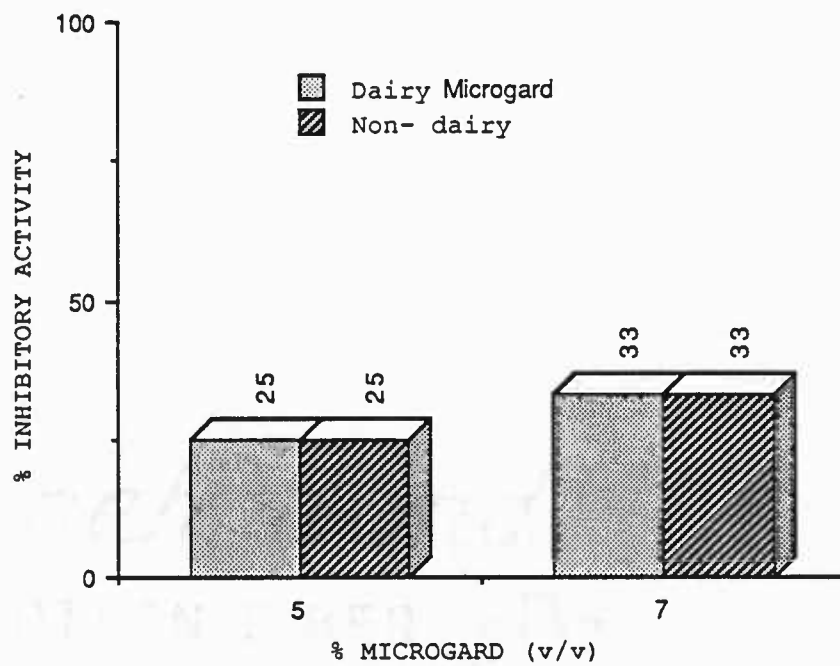


Fig. 2.3. Inhibition of *K. marxianus* ATCC 8554 by either dairy or non-dairy Microgard as revealed by the agar incorporation assay.

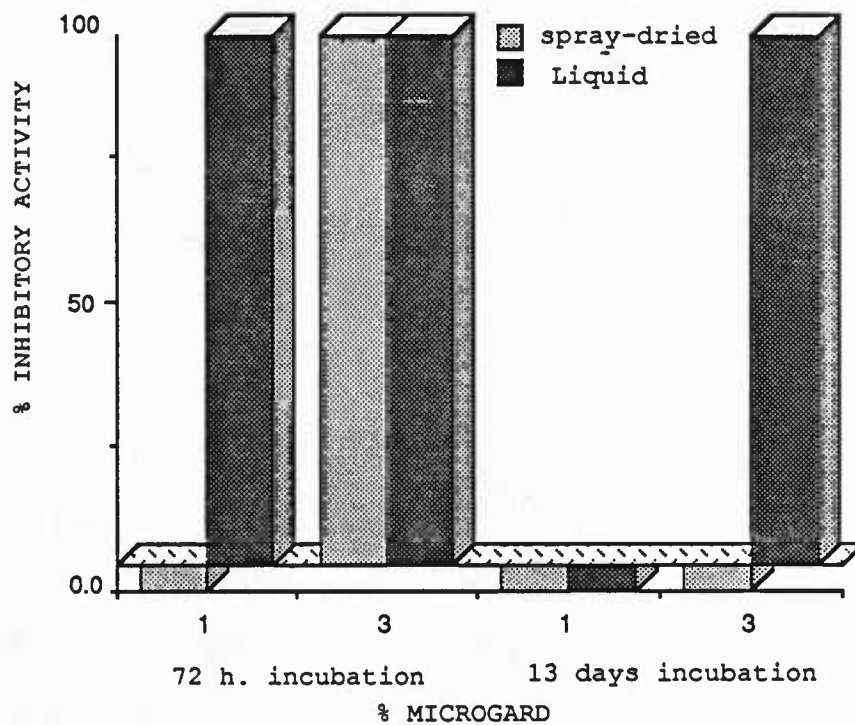


Fig. 2.4. Comparison between inhibition of liquid and spray-dried Microgard at two incubation periods against *Achromobacter delicatulus* ($1/10^2$ dilution) during the agar incorporation assay at 30 degrees C.

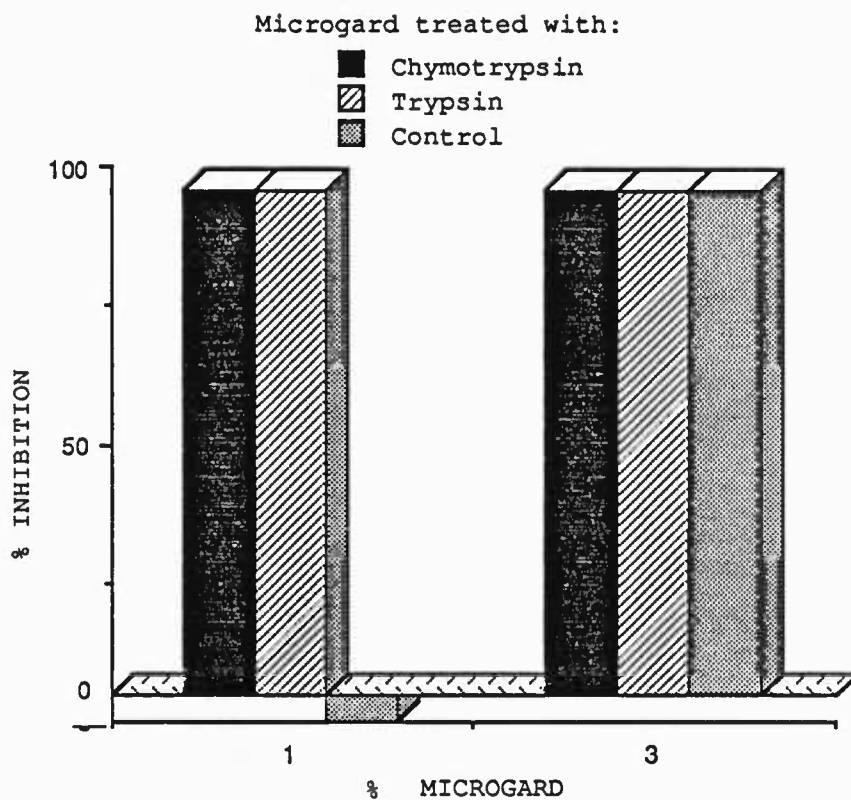


Fig. 2.5. Enhancement of inhibition of Microgard against *P. putida* ($1/10^2$ dilution) by proteases as revealed by the agar incorporation method.

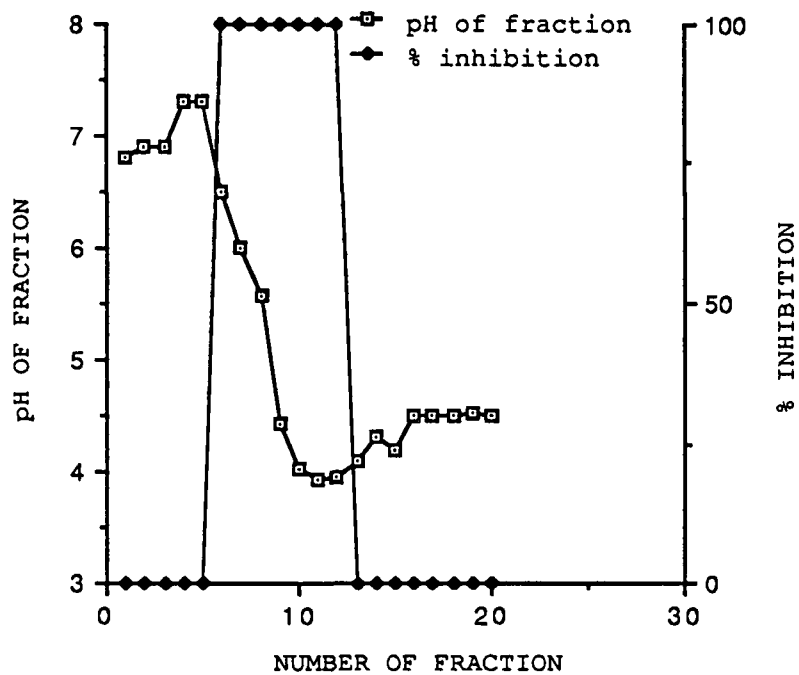


Fig. 2.6. Elution profile of Microgard from Sephadex G-10 column illustrating the bioactive fractions against *P. putida* in the disc assay and the pH of each fraction.

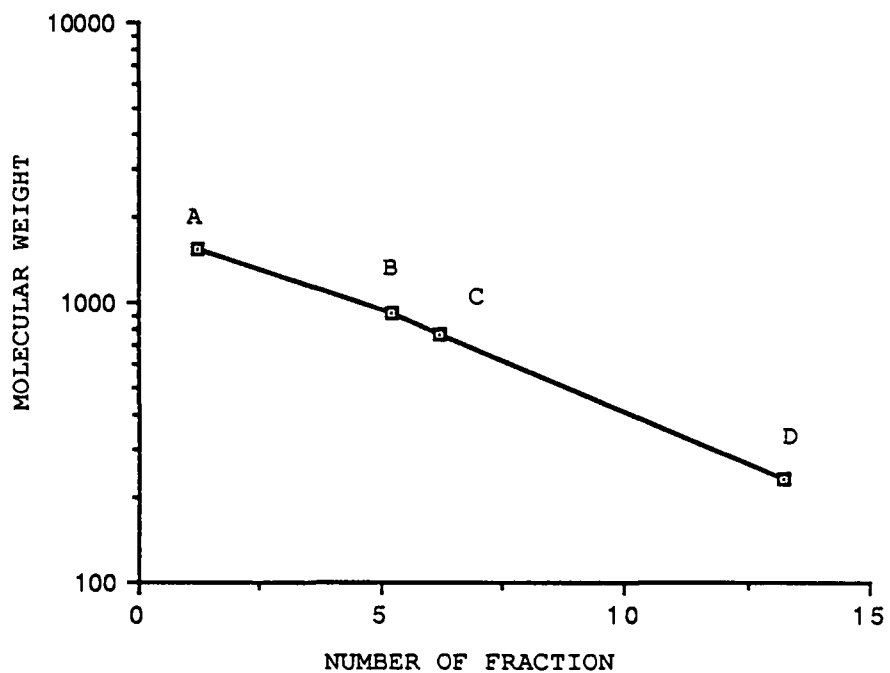


Fig. 2.7. Molecular weight determination of inhibitor(s) from Microgard eluted from Sephadex G-10. A - Bacitracin (MW 1422); B - peptide (MW 848); C - inhibitory fraction(s) from Microgard; and D - tyrosin (MW 217.7).

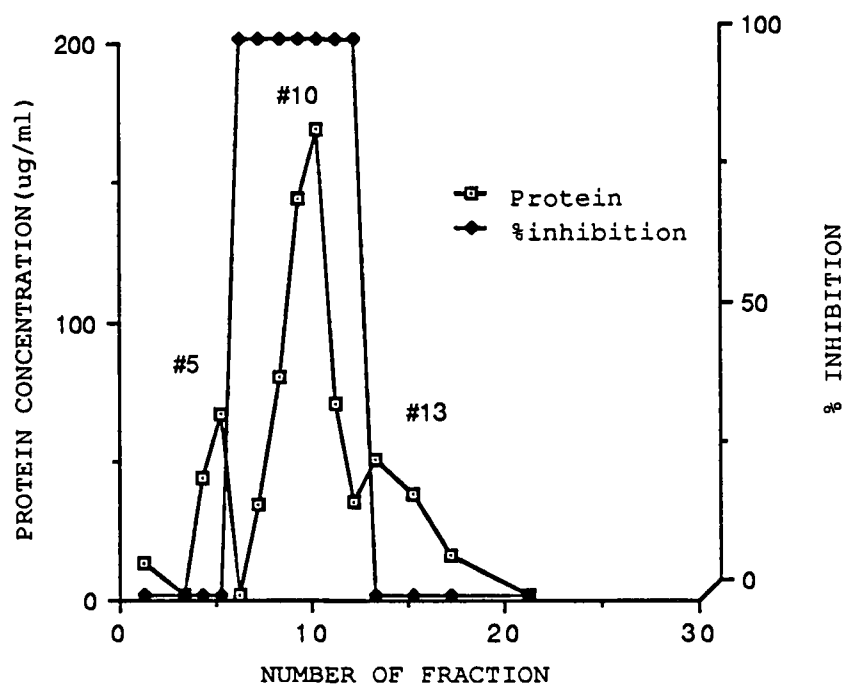


Fig. 2.8. Amounts of protein present in Microgard fractions eluted from Sephadex G-10 and their relations to the bioactive fractions against *P. putida* as revealed by the disc assay.

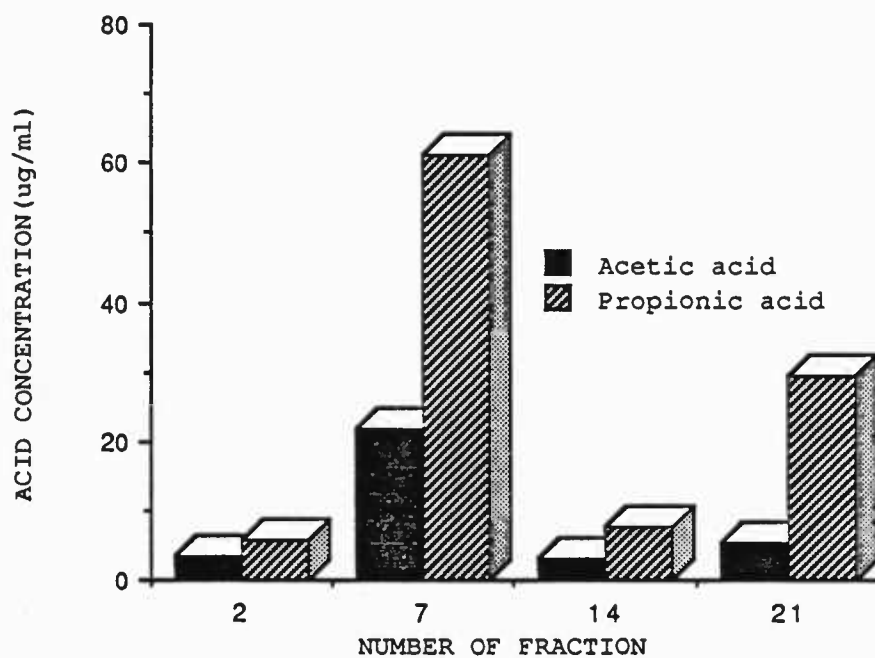


Fig. 2.9. GC analysis of volatile acids in active fraction #7 and non-active fractions obtained from Microgard fractionation through Sephadex G-10.

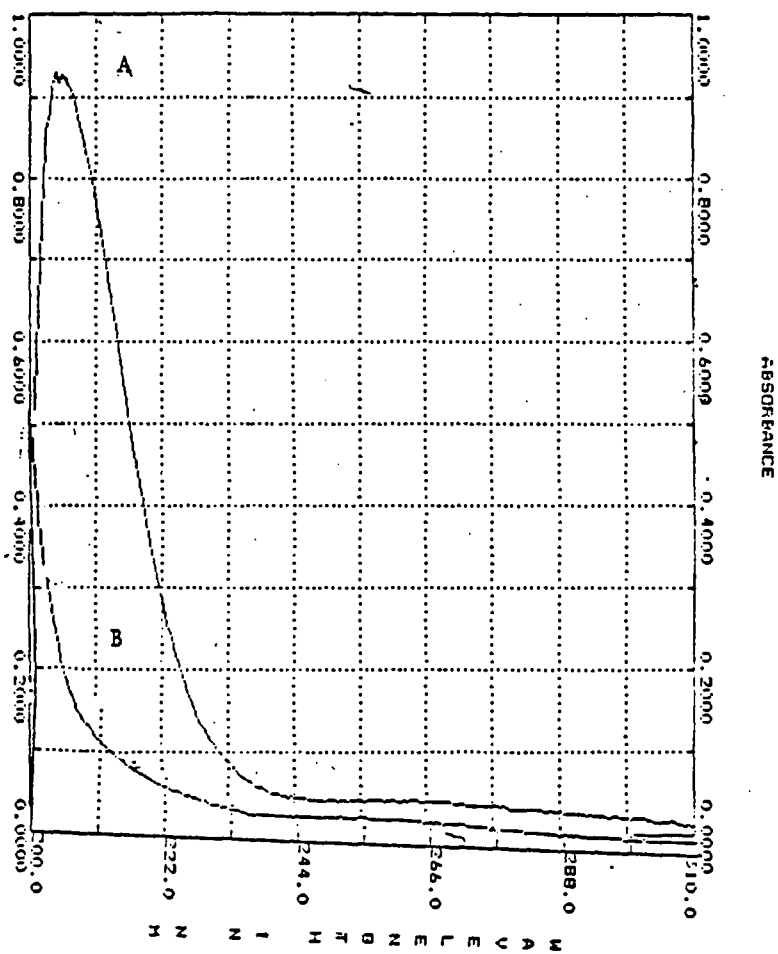


Fig. 2.10. Ultraviolet absorption spectrum of purified Microgard antimicrobial fraction before (A) and after storage at 2-5°C for 2 months (B).

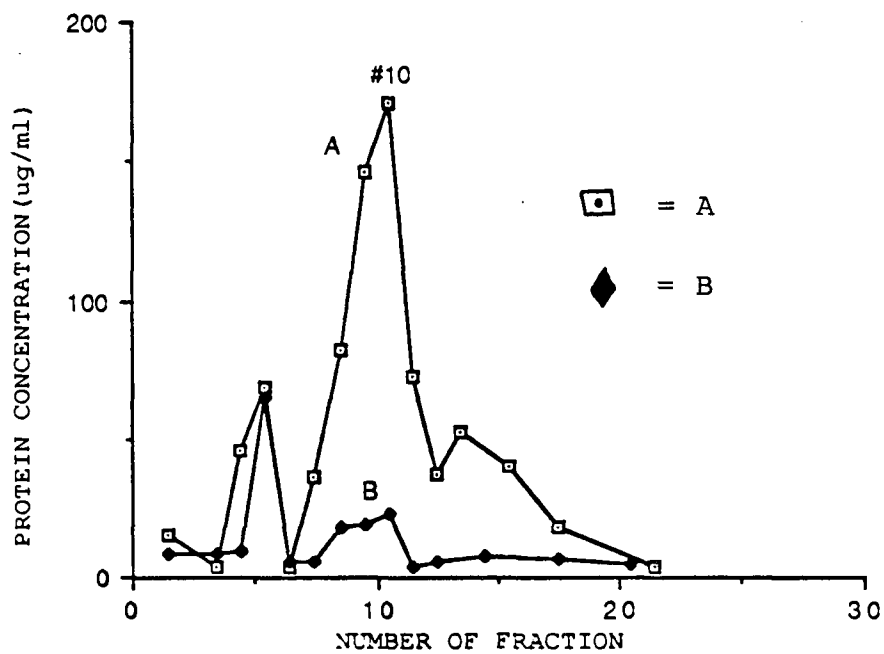


Fig. 2.11. Protein concentration in A; fractions of Microgard eluted from Sephadex G-10 and B; the more purified fraction (#10) obtained from A. The bioactive samples against *P. putida* (disc assay) in fraction A were 6-12 and in B was #6.

Table 2.1. Stability of Microgard towards boiling and autoclaving at different pH values as determined by the agar incorporation assay method against P. putida.

pH	<u>Inhibition when Microgard subjected to:</u>	
	*Boiling	*Autoclaving
1.5	n.d.	++
2.0	n.d.	++
3.0	++	n.d.
4.8	++	n.d.
5.30	++	++
5.9	++	n.d.
6.2	n.d.	++
6.7	++	n.d.
11.00	n.d.	++

* Boiling for 5 minutes; autoclaving at 121.1°C for 15 min.

++ no loss in the inhibition after heat treatments.

n.d. not determined.

Table 2.2 Amounts of volatile substances recovered from Microgard during GC Analysis.

Volatile Substance	Concentration ($\mu\text{g/g}$)
Acetaldehyde	9.66
Ethylacetate	18.18
Diacetyl	10.98
Isopentanal	2.50
Propanol	8.87
Ethylbutyrate	14.0
Acetoin	3.55
n-amylacohol	4.15

Table 2.3. Organic acid content of Microgard as revealed by HPLC Analysis.

Organic Acid	Concentration (ppm) ($\mu\text{g/g}$)
Citric	470.6
Lactic	8140.4
Acetic	4920.6
Propionic	1380.5

Table 2.4. Effect of treatment with various proteolytic enzymes on the inhibitory activity of Microgard. Enzymes were present at 0.1% at pH 6.9-7.1 (with pepsin at pH 3.0) and incubated for 15 hours at 37°C and then inactivated by boiling at pH 6.5 for 5 minutes, a procedure which does not affect Microgard activity.

Enzyme	Percent Inhibition of <i>P. putida</i> *
α -Chymotrypsin	0
Pepsin	0
Protease (papaya)	0
Trypsin	100

* Control plates without Microgard had 6×10^7 cfu/ml.

Table 2.5. Antagonistic effect of various substances on the inhibiting action of Microgard.

Antagonist	Percent Inhibition of <u>P. putida</u> *
Bovine Serum Albumin	0
Tween 80 + Lecithin	0
EDTA	100

* Control plate without Microgard had 6×10^7 cfu/ml.

REFERENCES

- Abdel-Bar, Nadia, N.D. Harris, and R.L. Rill. 1987. Purification and properties of an antimicrobial substance produced by Lactobacillus bulgaricus. J. Food Science 52(2): 411-415.
- Ahern, T.J., and A.M. Klivanov. 1985. The mechanism of irreversible enzyme inactivation at 100°C. Science 228 June: 1280-1283.
- Andersson, Rolf. 1986. Inhibition of Staphylococcus aureus and spheroplasts of gram-negative bacteria by an antagonistic compound produced by a strain of Lactobacillus plantarum. Int. J. Food Microbiology 3: 149-160.
- Andersson, R.E., M.A. Daeschel, and H.M. Hassan. 1987. Mode of action of Plantaricin SIK-83, a bacteriocin produced by Lactobacillus plantarum. FEMS Microbiology Reviews 46(3): p 69.
- Anderson, T.M., E.A. Bodie, N. Goodman, and R.D. Schwartz. 1986. Inhibitory effect of autoclaving whey-based medium on propionic acid production by Propionibacterium shermanii. Appl. Env. Microbiology 51(2): 427-428.
- Axelsson, L., T.C. Chung, W.J.D., and L.E. Lindgren. 1987. Discovery of a new antimicrobial substance produced by Lactobacillus reuteri. FEMS Microbiology Reviews 46(3): p 65.
- Basler, A.W., V. Der Hude, and M. Scheutwinkel. 1987. Screening of the food additive propionic acid for genotoxic properties. Fd. Chem. Toxic. 25(4): 287-290.
- Beattie, S.E., and G.S. Torrey. 1986. Toxicity of methanethiol produced by Brevibacterium linens toward Penicillium expansum. J. Agric. Food Chem. 34(1): 102-104.
- Bilinski, C.A., G. Innamator, and G.G. Stewart. 1985. Identification and characterization of antimicrobial activity in two yeast genera. Appl. Environmental Microbiology 50(5): 1330-1332.
- Blance, P., G.M. Faup, and G. Goma. 1987. Production of acetic and propionic acids from household refuse enzymatic hydrolysate by Propionibacterium acidipropionici. Biomass. 12: 49-55.

- Bodie, E.A., N. Goodman, and R.D. Schwartz. 1987a. Production of propionic acid by mixed cultures of Propionibacterium shermanii and Lactobacillus casei in autoclave-sterilized whey. J. Industrial Microbiology 1: 349-353.
- Bodie, E.A., T.M. Anderson, N. Goodman, and R.D. Schwartz. 1987b. Propionic acid fermentation of ultra-high temperature sterilized whey using mono- and mixed-cultures. Appl. Microbiol. Biotechnol. 25: 434-437.
- Branen, H.C.Go, and R.P. Genske. 1975. Purification and properties of antimicrobial substances produced by Streptococcus diacetylactis and Leuconostoc citrovorum. J. Food Science 40: 446-450.
- Brink, B. Ten., M. Minekus, J. Bol, and J.H.J. Huis. 1987. Production of antimicrobial compounds by lactobacilli. FEMS Microbiology Reviews 46(3): p 63.
- Buhnia, A.K., M.C. Johnson, and B. Ray. 1987. Direct detection of an antimicrobial peptide of Pediococcus acidilactici in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Industrial Microbiology 2:00-00.
- Bulgarelli, M.A., and L.A. Shelef. 1985. Effect of EDTA on growth for spores of Bacillus cereus. J. Food Science 50(3): 661-664.
- Cove, J.H., K.T. Holland, and W.J. Cunliffe. 1987. The effects of oxygen on cutaneous propionibacteria grown in continuous culture. FEMS Microbiology Letters 43: 61-65.
- Crow, V.L., and K.W. Turner. 1986. The effect of succinate production on other fermentation products in Swiss-type cheese. New Zealand J. Dairy Science and Tech. 21: 217-227.
- Crow, V.L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by Propionibacterium freudenreichii subsp. shermanii. Appl. Environmental Microbiology 53(10): 2600-2602.
- Deibel, R.H., and J.B. Evans. 1960. Modified benizidine test for the detection of cytochrome-containing respiratory systems in microorganisms. J. Bacteriology 79: 356-360.

- Duff, J.T., G.G. Wright, and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. *J. Bacteriology* 72: 455-460.
- Dumont, Jean-Pierre, and J. Adda. 1978. Occurrence of sesquiterpenes in Mountain cheese volatiles. *J. Agric. Food Chem.* 26(2): 364-367.
- Fairclough, G.F., and J.S. Fruton. 1966. Peptide-protein interactions as studied by gel filtration. *Biochemistry* 5(2): 673-683.
- Fleming, H.P., J.L. Etchells, and R.N. Costilow. 1975. Microbial inhibition by an isolate of Pedicoccus from cucumber brines. *Appl. Microbiology* 30(6): 1040-1042.
- Flores-Galarza, R.A., B.A. Glatz, C.J. Bern, and L.D. Van Fossen. 1985. Preservation of high-moisture corn by microbial fermentation. *J. Food Protection* 48(5): 407-411.
- Gavrilova, N.N., and L.I. Zahharenko. 1987. Antagonistic activity of lactic acid bacteria and propionic acid bacteria from 'Kazakhsil' starters. *FEMS Microbiology Reviews* 46(3): p 67.
- Gonzalez, C.F., and B.S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in Pedicoccus acidilactici. *Appl. Environmental Micro.* 53(10): 2534-2538.
- Guem, J. Le., D. Thuault, and C.M. Bourgeois. 1987. Detection and activity of a bacteriocin produced by Lactobacillus plantarum. *FEMS Microbiology Reviews* 46(3): p 68.
- Haque, Z., and J.E. Kinsella. 1988. Emulsifying properties of food proteins: Bovine serum albumin. *J. Food Science* 53(2): 416-420.
- Henning, S., R. Metz, and W.P. Hammes. 1986. Studies on the mode of action of nisin. *International J. Food Microbiology* 3: 121-134.
- Herrero, A.A. 1983. End-product inhibition in anaerobic fermentations. *Trends in Biotechnology* 1(2): 49-53.
- Hettinga, D.H., and G.W. Reinbold. 1972. The propionic acid bacteria -- A review. III. Miscellaneous metabolic activities. *J. Milk and Food Technology* 35(7): 436-447.

- Hitchner, E.R. 1934. Some physiological characteristics of the propionic-acid bacteria. *J. Bacteriology* 28: 473-479.
- Hoover, D.G., P.M. Walsh, K.M. Kolaetis, and M.M. Daly. 1988. A bacteriocin produced by Pedococcus species associated with a 5.5-megadalton plasmid. *J. Food Protection* 51(1): 29-31.
- Hummel, J.P., and W.J. Dreyer. 1962. Measurement of protein-binding phenomena by gel filtration. *Biochemistry and Biophysics Acta*. 63: 530-532.
- Jay, J.M. 1982. Antimicrobial properties of diacetyl. *Appl. Environmental Microbiology* 44(3): 525-532.
- Jay, J.M. 1986. *Modern food microbiology*. Van Nostrand Reinhold Company, New York.
- Kabara, Jon J. 1978. Fatty acids and derivatives as antimicrobial agents -- A review. Ch. 1 In "The pharmacological effects of lipids," Jon J. Kabara, ed., American Oil Chem. Soc., Champaign, IL.
- Kato, Akio, A. Tanaka, N. Matsudemi, and K. Kobayashi. 1987. Deamidation of food proteins by protease in alkaline pH. *J. Agric. Food Chem.* 35(2): 224-227.
- Kim, D.S. 1987. Studies on antimicrobial agent produced by Lactobacillus acidophilus. In "Proceedings of the 3rd. AAAP Animal Science Congress. Volume 2. [In Food Science and Tech. Abstracts (1987) 19(3): 3B7.]
- King, N. 1948. A modification of the Voges-Proskauer test for rapid calorimetric determination of acetylmethylcarbinol plus diacetyl in butter cultures. *Dairy Industries* September: 860-878.
- Konishi, Y., Jun-ichi Kurisaki, S.K., and K. Yamanchi. 1985. Detection of antigenicity by radioimmune-assay and of trypsin inhibitory activity in heat or enzyme denatured ovomucoid. *J. Food Science* 50(5): 1422-1426.
- Lana, L., R. Grasselli, A. Lombardi, and M.P. Nuti. 1987. Isolation of bacteriocin(s)-producing streptococci active on lactate-fermenting clostridia. *FEMS Microbiology Reviews* 46(3): p 68.
- Langsrud, T., and G.W. Reinbold. 1973. Flavor development and microbiology of Swiss cheese -- A review. *J. Milk and Food Technology* 36(11): 531-542.

- Lee, S.Y., E.R. Vedamuthu, C.J. Washam, and G.W. Reinbold. 1970. Diacetyl production by Propionibacterium shermanii in milk cultures. *Canadian J. of Microbiology* 16: 1231-1242.
- Marwaha, S.S., R.P. Sethi, and J.F. Kennedy. 1983. Role of amino acids, betaine and choline in vitamin B₁₂ biosynthesis by strains of Propionibacterium. *Enzyme and Microbial Technology* 5(6): 454-465.
- Mehta, A.M., K.A. Patel, and P.J. Dave. 1983. Isolation and purification of an inhibitory protein from Lactobacillus acidophilus AC₁. *Microbios.* 37: 37-43.
- Nakagawa, M., A. Hirota, and H. Sakai. 1982. Terrecyclic acid A, A new antibiotic from Aspergillus terreus. *The Journal of Antibiotics* 35(7): 778-782.
- Oser, B.L. 1965. In "Hawk's physiological chemistry." 14th ed., p. 179. McGraw-Hill Book Co., New York.
- Oxford, A.E. 1944. Diplococcin, an anti-bacterial protein elaborated by certain milk streptococci. *Biochemistry* 38: 178-182.
- Perez-Mendoza, J.L., and F. Garcia-Hernandez. 1983. Fermentation of a waste-product from industrial processing of the lime (citrus aurantifolia Swingle) for vitamin B₁₂ production. *Biotechnology letters* 5(4): 259-264.
- Pritchard, G.G., J.W.T. Wimpenny, H.A. Morris, M.W.A.L., and D.E. Hughes. 1977. Effect of oxygen on Propionibacterium shermanii grown in continuous culture. *J. Gen. Microb.* 102(1): 223-33.
- Pulusani, S.R., D.R. Rao, and G.R. Sunki. 1979. Antimicrobial activity of lactic cultures: Partial purification and characterization of antimicrobial components produced by Streptococcus thermophilus. *J. Food Science* 44(2): 575-578.
- Raccach, Moshe. 1987. *Pediococci and biotechnology.* CRC Critical Reviews in Microbiology 14(4): 291-309.
- Ramanathan, S., G. Read, and W. Cutting. 1966. Purification of propionin, an antiviral agent from propionibacteria. *Proc. Soc. Exp. Biol. Med.* 123: 271-273.

- Reddy, N.S., and B. Ranganathan. 1983. Nutritional factors affecting growth and production of antimicrobial substances by Streptococcus lactis subsp. diacetylactis S1-67/C. J. Food Protection 46(6): 514-517.
- _____. 1983. Preliminary studies on antimicrobial activity of Streptococcus lactis Subsp. diacetylactis. J. Food Protection 46(3): 222-225.
- Reeves, Peter. 1972. The Bacteriocins. Pages 85-86. A. Kleinzeller, G.F. Springer and H.G. Wittman, eds. Springer-Verlag, NY.
- Ro, S.L., M. Woodburn, and W.E. Sandine. 1979. Vitamin B12 and ascorbic acid in Kimchi inoculated with Propionibacterium freudenreichii ss. shermanii. J. Food Science 44: 873-877.
- Rusul, G., F.E. El-Gazzar, and E.H. Marth. 1987. Growth and aflatoxin production by Aspergillus parasiticus NRRL 2999 in the presence of acetic or propionic acid at different initial pH values. J. Food Protection 50(11): 909-914.
- Sakaguchi, G., and S. Sakaguchi. 1966. Some observations on activation of Clostridium botulinum type E toxin by trypsin. In "Botulism," M. Ingram and T.A. Roberts ed., Chapman and Hall Limited, London.
- Salih, Mohammed Ali. 1985. Studies on growth metabolites produced by Propionibacterium shermanii. Ph.D. Thesis, Microbiology Dept., Oregon State University.
- Sandine, W.E. 1988. An introduction to lactococci and non-rodlike forms. Biochemie. In press.
- Schales, Otto. 1951. Effect of albumin on the antibacterial activity of diketones. Arch. Biochem. Biophys. 34: 56-63.
- Schillinger, U., and F.-K. Lucke. 1987. Antagonistic activities of lactic acid bacteria isolated from meats. FEMS Microbiology Reviews 46(3): p 65.
- Schutz, M., M. Sievers, and K. Klemm. 1987. Metabolically controlled continuous fermentation of lactose to propionate, acetate, and carbon dioxide. Milchwissenschaft 42(2): 71-74.

- Serot, T., X. Dousset, and N. Torcatis. 1987. Isolation and partial purification of antibacterial substances produced by Leuconostoc mesenteroides and Lactobacillus plantarum isolated from Kefir. FEMS Microbiology Reviews 46(3) p 66.
- Shaw, R.H., and J.M. Sherman. 1923. The production of volatile fatty acid bacteria with special reference to their action in cheese. J. Dairy Science 6: 303-309.
- Sherman, J.M., and R.H. Shaw. 1921. Associative bacterial action in the propionic acid fermentation. J. Gen. Physiology p. 657-658.
- Shibasaki, Isao, and N. Kato. 1978. Combined effect on antibacterial activity of fatty acids and their esters against gram negative bacteria. Ch. 2 In "The Pharmacological effects of lipids. J.J. Kabara ed., American Oil Chem. Soc., Champaign, IL.
- Shih, F.K. 1987. Deamidation of protein in a soy extract by ion exchange resin catalysis. J. Food Science 52(6): 1529-1531.
- Thomas, T.D. 1987. Acetate production from lactate and citrate by non-starter bacteria in cheddar cheese. New Zealand J. of Dairy Science and Tech. 22: 25-38.
- Tjaberg, T. Bjarne. 1974. Proteases of Clostridium botulinum. Acta. Vet. Scand. 15: 487-506.
- Vorob'eva, L.I., S. Al'-Sudani, and N.I. Kraeva. 1987. Peroxidase of propionic acid bacteria. Microbiology (Translated from Russian) 56(4): 590-593.
- Walker, D.W., and S.E. Gilliland. 1987. Buttermilk manufacturing using combination of direct acidification and citrate fermentation by Leuconostoc cremoris. J. Dairy Sci. 70(10): 2055-2062.
- Welch, Christi. 1987. Nutritional and therapeutic aspects of Lactobacillus acidophilus in dairy products. Cultured Dairy Products Journal 22(2): 23-26.
- Whitaker, J.R. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Analytical Chemistry 35(12): 1950-1953.
- Wood, H. Goff, and C.H. Werkman. 1936. Mechanism of glucose dissimilation by the propionic acid bacteria. Biochemistry Journal 30: 618-623.

CHAPTER 3

SCREENING MICROBIAL CULTURES FOR FERMENTATION
END PRODUCTS WHICH INHIBIT
OTHER MICROORGANISMS

ABSTRACT

Various screening tests for the presence of antimicrobial activity in fermentation end products were conducted on several cultures of lactic acid bacteria, propionic acid bacteria, sourdough starter bacteria, and a black-pigmented yeast. The psychotropic bacterium Pseudomonas putida was sensitive to fermentation end products produced by all cultures except the sourdough bacterium. Listeria monocytogenes, a food-borne pathogen, was inhibited by two species of lactic acid bacteria in the Pediococcus genus. Inhibition of the various indicator bacteria was offered at pH 5.3 but not at pH 5.8 or above. Propionibacterium shermanii expressed antagonistic activities when grown and maintained at pH 6.0 during fermentation. Adding lecithin and thioglycollate as well as maintaining the pH at 6.0 resulted in higher standard plate counts of P. shermanii prepared for storage by lyophilization.

INTRODUCTION

Traditionally, the lactic acid fermentation is used to prevent food spoilage by microorganisms, some of which may be pathogens. Several factors contribute to the antagonistic effect of lactic acid bacteria, including production of antibiotics (Andersson, 1986). Furthermore, it has been reported that yogurt possesses bacteriocidal and protozoicidal abilities (Pulsani et al., 1979). In this regard, Campylobacter jejuni was demonstrated to be killed in yogurt over a pH range of 4.2 - 5.3 (Cuk et al., 1987). Not only can lactic acid bacteria produce antibacterial substances, but they can also degrade blood serum cholesterol for which they are being considered as a therapeutic treatment (Welch, 1987).

Antibacterial compounds have been reported to be produced by various bacteria of the family Lactobacteriaceae. Examples of this family, which are associated with inhibitor production, are Lactobacillus acidophilus (Mehta et al., 1983; Kim, 1987; and Ferreira and Gilliland, 1988), Streptococcus lactis (Hurst, 1981; Henning et al., 1986; and Jay, 1986), and Lactobacillus plantarum (Andersson, 1986; Serot et al., 1987; Guern et al., 1987; and Andersson et al., 1987). Likewise, Lactobacillus bulgaricus and Lactobacillus reuteri have been found to produce antimicrobial substances effective against gram negative

bacteria (Abdel-Bar et al., 1987 and Axelsson et al., 1987). Furthermore, streptococcal species including S. thermophilus and S. diacetylactis were able to antagonize food spoilage and pathogenic bacteria (Pulusani et al., 1979; Reddy et al., 1983; and Branen et al., 1975). Also, metabolites of Leuconostoc citrovorum and L. mesenteroides were found to be inhibitory for gram positive and gram negative bacteria (Branen et al., 1975 and Serot et al., 1987).

Members of another genus of lactic acid bacteria, the Pediococcus, were shown to be able to produce bacteriocins, antimicrobial substances of a protein nature. These bacteriocins have been found to be produced by Pediococcus cerevisiae, P. acidilactici and a Pediococcus isolate from soy bean fermentation (Fleming et al., 1975; Buhnia et al., 1987; Gonzalez et al., 1987; Raccach, 1987; Hoover et al., 1988; and Nout et al., 1987).

During a previous study to investigate the production of antimicrobial substances by Propionibacterium shermanii, growth metabolites produced were found inhibitory for psychotrophs and several fungi (Salih, 1985). In an earlier study, an antiviral compound was isolated from Propionibacterium freudenreichii (Ramanathan et al., 1966). Furthermore, propionic acid, acetic acid and diacetyl, which are inhibitory to microorganisms (Jay, 1982 and Rusul et al., 1987), were found to be produced by

propionibacterial species (Langsrud et al., 1973; Hettinga et al.; 1972, Lee et al.; 1970, Shaw et al.; 1923, Blance et al., 1987; and Schutz et al., 1987).

Brevibacterium linens, important in the ripening of Brie cheese, was found by Beattie and Torrey (1986) to antagonize mold spore germination. Similarly, several workers were able to identify antibacterial substances produced by Aspergillus terreus (Nakagaw et al., 1982 and Hirota et al., 1982) and by yeasts such as Kluyveromyces thermotolerans (Sasaki et al., 1984 and Bilinski et al., 1985). In this regard, production of antimicrobial substances from yeast (killer yeasts) was associated with the immunity of the producer yeast against its own metabolites (Reeves, 1972).

Finally, Bifidobacterium organisms were found to offer protection against infections of the intestinal tract caused by Salmonella, Shigella and enteropathogenic E. coli (Collins and Hali, 1984) and therefore have been used in the manufacture of dry pharmaceutical preparations containing viable cells (Rasic and Kurmann, 1983).

It was the objective of this study to test for antibiotic production by various microorganisms and to determine the practicality of using these substances in food preservation.

MATERIALS AND METHODS

Cultures and Maintenance

Table 3.1 lists the source of each organism used in this study. Two groups of microorganisms were used. The first group included those used for production of inhibitory metabolites (Table 3.1). The second group represented the indicator organisms to be tested for inhibition by metabolites produced by organisms in the first group. Pseudomonas putida (Wesman Foods Inc., Beaverton, OR), Listeria monocytogenes ATCC 7644, Yersinia enterocolitica ATCC 23715, Lactobacillus plantarum C-11, Kluyveromyces marxianus var. marxianus ATCC 8554, Staphylococcus aureus, Salmonella paratyphi ATCC 9281, and Lactobacillus casei were included in the second group (indicators).

Lactic acid cultures used in the first group were maintained in deep agar tubes of MRS agar. Pathogenic bacteria, on the other hand, were kept on BHI agar slants and propionic acid bacteria were maintained in sodium lactate deep agar stabs. Yeast cultures were stored on Potato Dextrose Agar slants. All inert cultures were kept refrigerated.

Media and Fermentation Procedures

Following is the composition of the different media used with each bacterial strain tested for inhibitory metabolite production.

1) Propionic Acid Bacteria

Three different strains were used (see Table 3.1), and the growth of each strain was conducted with and without pH control.

A) Without pH control

Four different media were used to grow every strain of propionic acid bacteria. The four media were: (a) 11% nonfat dry milk (NDM), 0.2% yeast extract (YE) (Difco), 0.2% ammonium citrate dibasic (J.T. Baker), 0.5% sodium acetate trihydrate (J.T. Baker), 0.01% magnesium sulfate heptahydrate (Mallinckrodt), 0.005% manganese sulfate (Sigma) and 0.2% sodium phosphate dibasic (Mallinckrodt). The final pH after autoclaving was 6.5; (b) 7% sweet whey (Pollio Dairy Products, Campbell, N.Y.), 1% YE and 2% calcium carbonate (CaCO_3); (c) 11% NDM, 1% YE and 1% CaCO_3 ; and (d) 10% NDM, 1% YE, 1% sodium lactate (60% syrup, J.T. Baker) and 4% CaCO_3 .

Each medium was sterilized by autoclaving at 121.1°C (250°F) for 15 minutes followed by tempering to 25°C. Forty-eight hour old cultures of each respective bacterium were inoculated (4% by volume) into screw-capped bottles

containing 100 ml of the designated medium. The bottles were incubated at 30°C for 5 days followed by pasteurization at 63°C for 30 minutes in a water bath. The cell-free filtrate was obtained by centrifugation at 10,000 $\times g$ for 15 minutes. Then the clear supernatant was drawn carefully and adjusted to pH 5.2 - 5.3 with 1N sodium hydroxide (sterile) followed by filter sterilization (0.45 μm filters). Meanwhile, media that were not inoculated but subjected to the same conditions were used as controls. pH measurements were carried out daily on media which were sacrificed for such measurements.

B) pH control system

A multi-vessel, six-station fermentor was used to maintain the designated pH during fermentation. This fermentor contained six vessels (stainless steel flasks), each of which had a 2-liter capacity. The respective starter (*P. shermanii*) was activated in 10% NDM medium containing 0.5% YE for three successive times. Three different formulations (each one in duplicate) were used: (1) 9% NDM, (2) 18% NDM, and (3) 9% NDM + 9% Maltrin-M100 (Maltodextrin). Meanwhile, 0.5% YE and 0.6% sodium acetate (buffer) were added to each formula. Each formula was adjusted to pH 5.2 with lactic acid (85%, Kodak) and pasteurized in the same fermentor at 85°C for 45 minutes followed by cooling. Each flask containing the medium was inoculated aseptically with the active bacteria to give 5%

by volume of inoculum. Potassium hydroxide (1N) was used to neutralize the produced acids to maintain the pH at 5.2. The fermentation was at 30°C for 4 days (without stirring) and was followed by pasteurization (85°C for 45 minutes) to kill the starter cultures.

Antimicrobial assays

Cell-free filtrates were adjusted to pH 5.3, if needed, and bioassayed against P. putida using both the agar incorporation and agar well diffusion methods (Chapter I). The bioassays also were performed against L. monocytogenes ATCC 7644 (grown in BHI agar or tryptic soy agar, both at pH 5.3) and K. marxianus ATCC 8554 (Mycophile Agar) as the indicator organisms by applying the well assay (well diameter was 0.5 cm). Plates were incubated at 30°C for 48 hours.

2) Bifidobacterium longum ATCC 15707

Lyophilized culture was rehydrated in NDM (11% solids) containing 0.5% sodium acetate and 0.05% cysteine hydrochloride (Sigma) as a reducing agent (Collins and Hali, 1984). Sample and control media were incubated at 37°C for 5 days followed by centrifugation to obtain a clear supernatant. The pH was adjusted to 5.1 with 1N sodium hydroxide (sterile) and then filter sterilized (0.45 μ m filters).

Screening B. longum metabolites

The cell-free preparations (pH 5.1) obtained above were screened against P. putida and L. monocytogenes ATCC 7644 by using the well assay. The overnight culture of the respective indicator (1/100 dilution) was put in the agar medium followed by plating in petri plates. P. putida was inoculated into Plate Count Agar (pH 5.3) whereas L. monocytogenes ATCC 7644 was in TSA (pH 5.3). Any inhibition zones were measured after 2 days of incubation and compared to those obtained from uninoculated control media.

3) Lactobacillus sanfrancisco Strain T

An overnight culture was grown in a basal medium (3% inoculum by volume) containing 2% Maltose (Sigma), 0.3% Yeast Extract (Difco), 0.6% Trypticase (BBL), 0.03% Tween 80 (J.T. Baker), 0.001% magnesium sulfate (Sigma) and freshly-prepared yeast extract (Berg et al., 1981). However, this medium did not allow the bacterium to grow. Therefore a modified fresh yeast extract preparation was carried out by making a 20% (w/v) solution of Compressed Yeast (Fleischmann's) acidified to pH 3.6 with 3N hydrochloric acid followed by autoclaving at 121.1°C (250°F) for 30 minutes (hot extraction). The extract was added to the basal medium to a final concentration of 2% solids. The moisture in fresh yeast was reported to be 70% by Prescott and Dunn (1982). The inoculated medium along with the uninoculated control were maintained in an

incubator at 30°C for 5 days, after which the pH dropped from 6.5 to 4.6. Next, the fermentative product was adjusted to pH 5.30 followed by filter sterilization (0.45 μ m filters) to prepare cell-free filtrates.

Bioassays

P. putida (a 24-h culture) was added to Plate Count Agar (pH 5.3) to give 1/100 dilution. Then the seeded agar was poured in sterile petri plates and set aside to solidify. Wells were made with a sterile straw (0.5 cm. diameter) and each well was filled with the cell-free filtrate obtained above. The plates were incubated at 30°C for 2 days.

4) Black Yeast Isolate

This was grown in BHI broth with continuous stirring and incubation at 30°C for 6 days. Control broth (uninoculated) was subjected to the same conditions. After incubation, both sample and control media were adjusted to pH 5.3 with 10% sterile tartaric acid, and the adjusted products were filter sterilized to remove the cells and to prepare a cell-free extract which was used in later assays.

Indicator bacteria

Both P. putida and L. monocytogenes ATCC 7644 were used to represent food spoilage and pathogenic bacteria, respectively. The cell-free fermentation preparations obtained as described above were biologically assayed

against both these bacteria using the disc method (1/2 inch disc diameter). Overnight cultures of each bacterium were inoculated into the agar medium to give a 1:100 final dilution. Plate Count Agar (PCA) and Tryptic Soy Agar (TSA), both at pH 5.3, were used as growth media for P. putida and L. monocytogenes, respectively. Each sterilized disc was dipped into the extract and placed onto the surface of the seeded agar (in petri plates). P. putida was incubated at 30°C whereas L. monocytogenes was at 30°C and 37°C. Both bacteria were incubated for 2-3 days. Control extract was similarly assayed against both bacteria.

5) Pediococci and L. plantarum

This group included Pediococcus cerevisiae A, Pediococcus acidilactici PAC 1.0, Pediococcus pentosaceus FBB-61 and Lactobacillus plantarum C-11. Each bacterium was activated in MRS broth at 30°C overnight before being inoculated (1% inoculum) in liquid medium (a) described above under propionic acid bacteria. However, 0.1% cysteine hydrochloride was added to this medium before autoclaving. Controls (uninoculated) and samples were incubated at 30°C for 11 days. The cell-free preparations (filtrate) were obtained by centrifugation at 10,000 xg for 20 minutes in a Beckman J2-21 model centrifuge at 5°C. The clear supernatants were adjusted to pH 5.2 with either 1N NaOH or 10% tartaric acid before filter sterilization

(0.45 μm filters.). Likewise, the supernatant obtained from Pediococcus cerevisiae A was adjusted to pH 5.8 and 7.0 in addition to pH 5.2, followed by filter sterilization and conduct of bioassays.

Pediococcus cerevisiae A was also grown in Elliker's Broth (Difco). This medium was inoculated with an overnight culture of bacteria at a 5% final concentration (v/v). Both the sample medium and the control were incubated at 30°C for 4 days, after which the pH dropped to 4.42. Then the fermented product was filter sterilized to obtain cell free preparations which was adjusted to pH 5.8 or 7.0 with 1N NaOH (sterile) and assayed.

Assay for Antimicrobial Activity

Cell-free extracts (pH 5.3) of each lactic acid bacterium were biologically assayed against P. putida (PCA), L. monocytogenes ATCC 7644 (TSA), Yersinia enterocolitica ATCC 23715 (Mackonky's Agar), K. marxianus ATCC 8554 (Mycophile Agar), and Lactobacillus plantarum C-11 (MRS agar). The disc assay was use for this experiment. Each medium was adjusted to pH 5.3 before autoclaving and the overnight culture of each bacterium was separately incorporated into the cool agar to provide a 1/100 dilution. Then the seeded agar was poured into petri plates and set aside to solidify. The sterilized disc (1/2 inch) was dipped in the appropriate cell-free preparation (growth metabolites) and placed onto the surface of the

seeded agar plates. These in turn were incubated aerobically at 30°C for 2 days except for L. plantarum C-11 which was incubated anaerobically in Gas Pak System (BBL). Furthermore, the cell-free preparations (pH 5.2) of Pediococcus cerevisiae A were further assayed by the agar well diffusion technique against P. putida (PCA), L. monocytogenes ATCC 7644 (TSA), Staphylococcus aureus (BHI agar), Salmonella paratyphi (BHI agar), and Lactobacillus casei (TSA, Gas Pak). Finally, the cell-free extracts adjusted previously to pH 5.8 and 7.0 (the pH of the agar medium was adjusted accordingly) were assayed against P. putida and L. monocytogenes ATCC 7644 by the well assay also. The plates were incubated at 30°C for 2 days.

Effect of pH on Production of Inhibitory Metabolites by Propionic Acid Bacteria

The fermentor mentioned previously in Materials and Methods was prepared for this experiment. An active culture of Propionibacterium shermanii 9616 (Wesman) was inoculated in the medium at 2% (v/v). The basal medium was 1% Glucose (Sigma), 0.5% Yeast Extract (Difco) and 0.5% Tryptone (Difco). Three different treatments (each in duplicate) were carried out. These were: the basal medium without pH maintenance at 6.0; the same as above with pH maintenance; and finally 10% Maltrin (maltodextrin) added to the basal medium with maintenance at pH 6.0. Each medium was sterilized by autoclaving at 121.1°C for 15

minutes followed by tempering to 30°C. Fermentation (static without stirring) temperature was kept at 30°C for four successive days during which 1N KOH was used as the neutralizing solution (automatic pH control) to maintain the pH at 6.0, if needed. Meanwhile, the pH dropped to 4.5 for those without pH maintenance. Samples taken from each treatment were adjusted to pH 5.3 and bioassayed against P. putida by using the agar well diffusion assay. Plate Count Agar adjusted to pH 5.3 as well was used as the growth medium for antibacterial screening. Diameters of the inhibition zones were measured after incubation at 30°C for 24 hours.

Production of Microgard Starters

This experiment was conducted with the fermentor using 2 approaches. Propionibacterium shermanii 9616 (Wesman) was activated and prepared by these two methods.

1) The First Method

The same experiment mentioned above under pH effect was used also for starter production. The standard plate counts (Standard Methods for Examination of Dairy Products, 1985) were conducted on the products (starter) of each treatment (3 treatments) by diluting each sample obtained in 0.1% peptone water (sterile) and the dilution steps carried out followed by plating on in sodium lactate agar

(Malik et al., 1968) and incubation at 30°C in Gas Pak System.

2) The Second Method

The basal medium contained 1% glucose, 0.5% YE, 0.5% Tryptone and 10% maltodextrin. Three different formulae (each in duplicate) were used beside the basal medium: 1% lecithin (Lecigram 5750 Vivon Chemical Company) and 0.1% sodium thioglycollate (Sigma); 1% lecithin only; and finally, 0.1% thioglycollate only. Each formula was sterilized (121°C/15 min.) and tempered to 30°C in the fermenter. Inoculum (2.5% by volume) of P. shermanii was added to each medium. The fermentation was carried out statically at 30°C for four days with automatic pH maintenance at 6.5 with 50% KOH. The standard plate counts were carried out as mentioned before.

RESULTS

Antimicrobial Spectrum of Propionic Acid Bacterial Metabolites

Table 3.2 shows that the indicator bacterium, P. putida, was severely inhibited (100% inhibition) by the metabolites of this group of bacteria as revealed by the agar incorporation assay. Meanwhile, inhibition zones of bacterial growth were noticed around the disc (disc assay) or the well (well assay). On the other hand, the growth metabolites of propionic acid bacteria in sweet whey medium (#b) were not inhibitory to P. putida during the agar incorporation method (Table 3.2). Furthermore, the metabolites under pH maintenance (fermentor experiment) gave basically the same inhibition zones of P. putida. L. monocytogenes ATCC 7644 was not inhibited and neither was K. marxianus ATCC 8554 (Tables 3.3 and 3.4). However, zones around the wells of L. monocytogenes were found but were not clear, while the Propionibacterium isolated from Swiss cheese gave relatively clearer zones in comparison with the other two propionibacteria.

Metabolites of B. longum ATCC 15707

These metabolites were found inhibitory for P. putida as revealed by the well technique, but small inhibition zones were obtained against P. putida. L. monocytogenes

ATCC 7644, on the other hand, was not inhibited by these metabolites. Table 3.4 shows the results.

Susceptibility of P. Putida to Sourdough Bacteria

L. sanfrancisco (sourdough) metabolites, were not able to suppress the growth of the indicator bacterium, P. putida, during the well assay at pH 5.30 (see Table 3.4).

Antibacterial Activity of the Black-Pigmented Yeast

During the disc assay, cell-free preparations of growth metabolites of the yeast isolate offered antagonistic activity against the psychotrophic bacterium, P. putida. However this inhibition was overcome after 48 hours at 30°C. In contrast, L. monocytogenes ATCC 7644 was not sensitive to the above mentioned metabolites. Table 3.4 summarizes the results.

Preliminary Screening of Pediococci and Lactobacillus Plantarum

Table 3.4 illustrates the results obtained from screening the possible antimicrobial activity of these organisms. Pediococcus cerevisiae A (cell-free filtrate) was the only bacterium that offered inhibitory activities against P. putida, L. monocytogenes and, to a less degree, against Y. enterocolitica as revealed during the well and disc assays at pH 5.3. However, P. acidilactici PAC 1.0 (cell-free filtrate) was only inhibitory for L. monocytogenes. Meanwhile, other indicator organisms were

not antagonized by cell-free filtrates (pH 5.3) obtained from P. cerevisiae A (Table 3.4). Furthermore, L. plantarum C-11 was not inhibited by its own metabolites under the susceptibility test.

When the screening test was conducted at pH 5.8 and 7.0 (pH of both cell-free extracts and the growth media for the indicator bacteria), both P. putida and L. monocytogenes (indicators) were not suppressed by the cell-free preparations of P. cerevisiae A during the well assay, as opposed to the inhibition offered at pH 5.3. Table 3.5 is a summary of these results.

Effect of pH on Production of Inhibitory Substances

As shown in Table 3.6, it was found that Propionibacterium shermanii was able to produce inhibitory metabolites for P. putida even if the producer bacterium was grown in medium maintained at pH 6.0 during fermentation.

Production of Starter Cultures

Maintenance of the pH at 6.0 resulted in 40% higher standard plate count results than those cultures under no pH maintenance. Table 3.7 summarizes the results.

In the second experiment, it was found that addition of both lecithin and thioglycollate to the basal medium led to approximately a 58% higher bacterial count than when lecithin was excluded. Meanwhile, the exclusion of

thioglycollate from the basal medium caused a 33% reduction in the bacterial count in comparison to those bacterial levels obtained when both lecithin and thioglycollate were incorporated. All the results obtained are shown in Table 3.8.

DISCUSSION

The data obtained in this study indicate that Propionibacterium shermanii metabolites were able to antagonize the growth of P. putida, a psychrotrophic bacterium, as determined by two bioassay methods. A previous study conducted by Salih (1985) reported the same finding, even though only the agar incorporation assay was used. In contrast, L. monocytogenes and K. marxianus were not inhibited by P. shermanii metabolites. Considering the possible effects of nutritional factors on the production of antimicrobial substances by P. shermanii, it was noteworthy that a combination of sweet whey and yeast extract in a buffer system did not support a minimal production of antimicrobial substances, despite bacterial growth. In this regard, among nine tested media, it has been observed that yeast extract dextrose broth offered good growth and maximum antimicrobial substance(s) production by Streptococcus lactis subsp. diacetylactis (Reddy et al., 1983). Also, while casein-sodium hydroxide hydrolysate supported the growth and biosynthesis of corrinoids (the central ring portions of vitamin B12) by P. shermanii, as opposed to casein hydrolysate and trypsin digested casein (Zodrow et al., 1963), methionine aided the production of methanethiol by B. linens which was inhibitory to mold spores (Beattie and Torrey, 1986). Furthermore, the expression of antibacterial activity by

yeasts was found only when methylene blue was added to the agar medium (Bilinski et al., 1985).

The present investigation indicated that P. shermanii was able to produce inhibitory metabolites when the pH was maintained at 6.0, whereas Salih (1985) showed that this bacterium produced the inhibitors at pH 5.3. However, the optimum pH for inhibitor production cannot be determined because both studies were carried out at two pH values. Several authors have reported the optimum pH values for inhibitory metabolite production by microorganisms (Bilinski et al., 1985; Lee et al., 1970; and Ferreira and Gilliland, 1988).

Bifidobacterium longum offered inhibition against P. putida but was not able to suppress the growth of L. monocytogenes. It has been reported in the literature that bifidobacteria antagonize the growth of enteric bacteria such as E. coli and enteric viruses (Rasic and Kurmann, 1983). The black-pigmented yeast antagonized P. putida during the disc assay and wide inhibition zones were obtained. However, L. monocytogenes was able to overcome the inhibitory metabolites of this yeast. Likewise, antibacterial activities were identified in some yeast such as Saccharomyces cerevisiae and Kluyveromyces thermotolerans (Sasaki et al., 1984 and Bilinski et al., 1985).

Among the lactic acid bacteria tested, only P. cerevisiae A inhibited both P. putida and L. monocytogenes at pH 5.3. However, P. acidilactici PAC 1.0 offered inhibition only against L. monocytogenes. Bacteriocins produced by pediococci were found inhibitory to some pediococcal species, lactobacilli and Staphylococcus aureus (Fleming et al., 1975; Gonzalez et al., 1987; and Buhnia et al., 1987). Pediococcus acidilactici (PAC 1.0) was found to produce less acids during growth compared to the two other pediococci strains when judged by pH. The pH dropped to 5.7 for P. acidilactici PAC 1.0, whereas in the instance of the other two pediococci, cultures reached pH 4.5 to 4.8 after 11 days of fermentation. In this regard, Raccach (1987) stated that the fermentation of lactose by pediococci is strain dependent and associated with a plasmid. Hence, the PAC 1.0 strain of pediococci may be unique for the lactose plasmid. Furthermore, addition of both lactose and galactose to the broth inoculated with either P. pentosaceus or P. acidilacti caused the pH to drop from 6.6 to 4.6 (Raccach, 1987).

Concerning the production of starter cultures of Propionibacterium shermanii through lyophilization, it was revealed during the present study that maintaining the pH during growth resulted in a higher standard plate count in comparison to growth without pH maintenance. In this regard, Blanc et al. (1987) stated that the growth of

Propionibacterium acidi-propionici was inhibited by propionic acid produced during fermentation. Therefore, lyophilization of propionibacteria for industrial purpose can be achieved by growing the culture in a pretested milk medium with pH maintenance before the lyophilization process (Kunz et al., 1983). Likewise, adding lecithin and thioglycollate to the basal medium with pH maintenance at 6.0 offered higher standard plate counts than either substance alone.

Finally, further characterization of these inhibitory substances should be pursued as well as searching for other microbial candidates inhibitory for food spoilage and pathogenic bacteria. Also, it will be of interest to investigate the contribution of genetic engineering of bacteria to antimicrobial substances production.

Table 3.1. Microorganisms tested for their ability to produce antimicrobial growth metabolites.

Strain	Source
<u>Propionibacterium shermanii</u>	ATCC 9616
<u>Propionibacterium shermanii</u> 9616	Wesman Foods Inc.
<u>Propionibacterium</u> isolate	Swiss cheese
<u>Bifidobacterium longum</u>	ATCC 15707
<u>Lactobacillus sanfrancisco</u> T	Sour dough
Black Yeast isolate	Wesman Foods Inc.
<u>Pediococcus cerevisiae</u> A	Microlife Technics
<u>Pediococcus pentosaceus</u> FBB-61	M.A. Daeschel
<u>Pediococcus acidilactici</u> PAC 1.0	M.A. Daeschel
<u>Lactobacillus plantarum</u> C-11	M.A. Daeschel

Table 3.2 The antagonistic effect of the growth metabolites of propionibacteria produced in several media when tested against P. putida.

Medium	*Sensitivity of the Indicator Bacteria
(a)	++
(b)	--
(c)	++
(d)	++
pH control system medium	++

* Determined by the agar incorporation method
(++ = inhibition; -- = no inhibition).

(a) 11% nonfat dry milk (NDM), 0.2% yeast extract (YE), 0.2% ammonium citrate, 0.5% sodium acetate, .01% magnesium sulfate, 0.005% manganese sulfate and 0.2% sodium phosphate.

(b) 7% sweet whey, 1% YE and 2% calcium carbonate (CaCO₃).

(c) 11% NDM, 1% YE and 1% CaCO₃.

(d) 10% NDM, 1% YE, 1% sodium lactate and 4% CaCO₃.

Table 3.3. The inhibitory ability of the antimicrobial substance(s) produced by propionibacteria against the indicator organisms.

Indicator Organism	Method of Testing	Sensitivity
<u>P. putida</u> (Wesman)	agar incorporation	++
	well assay	++
	disc technique	++
<u>L. monocytogenes</u> ATCC 7644	agar incorporation	--
	well assay	--
	disc technique	--
<u>K. marxianus</u> ATCC 8554	well assay	--
	disc technique	--

Table 3.4. Initial screening of the metabolites produced by several microorganisms.

Producer Organism	Test Organism Inhibited	Test Organism Not Inhibited
<u>Propionibacterium</u> <u>shermanii</u> 9616 (Wesman)	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644 <u>K. marxianus</u> ATCC 8554
<u>Propionibacterium</u> <u>shermanii</u> ATCC 9616	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644 <u>K. marxianus</u> ATCC 8554
<u>Propionibacterium</u> cheese isolate	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644 <u>K. marxianus</u> ATCC 8554
<u>B. longum</u> ATCC 15707	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644
<u>L. sanfrancisco</u> T	<u>P. putida</u>	-
Yeast isolate	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644
<u>Pediococcus</u> <u>cerevisiae</u> A	<u>P. putida</u> <u>L. monocytogenes</u> ATCC 7644 <u>Yersinia</u> <u>enterocolitica</u> ATCC 23715	<u>K. marxianus</u> ATCC 8554 <u>L. plantarum</u> C-11 <u>Salmonella paratyphi</u> <u>Staphylococcus aureus</u> <u>L. casei</u>

Table 3.4. Continued.

Producer Organism	Test Organism Inhibited	Test Organism Not Inhibited
<u>Pediococcus pentosaceus</u> FBB-61	None	<u>P. putida</u> <u>L. monocytogenes</u> ATCC 7644 <u>Y. enterocolitica</u> ATTC 23715 <u>K. marxianus</u> ATTC 8554 <u>L. plantarum</u> C-11
<u>Pediococcus acidilactici</u> PAC 1.0	<u>L. monocytogenes</u> ATCC 7644	<u>P. putida</u> <u>L. plantarum</u> C-11 <u>Y. enterocolitica</u> ATTC 23715 <u>K. marxianus</u> ATTC 8554
<u>Lactobacillus plantarum</u> C-11	None	<u>P. putida</u> <u>L. monocytogenes</u> ATCC 7644 <u>Y. enterocolitica</u> ATTC 23715 <u>K. marxianus</u> ATTC 8554 <u>L. plantarum</u> C-11

* Both disc and well assays were used at pH 5.30.

Table 3.5. Effect of pH of both the growth medium of the indicator bacteria and the cell-free preparation of Pediococcus cerevisiae A on antibacterial activity.*

pH	<u>Presence (+) or absence (-) of zones of inhibition against</u>	
	<u>P. putida</u>	<u>L. monocytogenes</u> ATCC 7644
5.30	+	+
5.8	-	-
7.00	-	-

* Determined by well assays.

Table 3.6. Production of antimicrobial substances by P. shermanii with or without pH maintenance.

Conditions	*Test bacteria sensitivity (<u>P. putida</u>)
pH maintained at 6.00	++
pH not maintained at 6.00	++

* During well assay.

Table 3.7. Standard plate counts of Propionibacterium shermanii under two pH treatments.

Treatment	Standard plate count as colony forming unit (Cfu/ml)
*Not maintaining the pH at 6.00	6.0 x 10 ⁹
*Maintaining the pH at 6.00	1.2 x 10 ¹⁰ (40%) ^a
*Maintaining the pH at 6.0 plus 10% Maltodextrin	1.0 x 10 ¹⁰ (20%) ^b

^aHigher percentage count than in 1.

^bLower percentage count than in 2.

*Medium composition was 1% glucose, 0.5% yeast extract and 0.5% tryptone.

Table 3.8. Effect of lecithin and/or sodium thioglycollate on the number of P. shermanii produced during preparation as starters.

*Basal medium used	Standard plate count (Cfu/ml)
Lecithin and thioglycollate	4.8 x 10 ⁹ (58%) ^a
Lecithin only	3.2 x 10 ⁹ (33%) ^b
Thioglycollate only	2.0 x 10 ⁹

^aHigher percentage than in 3.

^bLower percentage than in 1.

*1% glucose, 0.5% yeast extract and 0.5% tryptone.

REFERENCES

- Abdel-Bar, N., N.D. Harris and R.L. Rill. 1987. Purification and properties of an antimicrobial substance produced by Lactobacillus bulgaricus. J. Food Science 52(2):411-415.
- American Public Health Association. 1985. Standard methods for the examination of dairy products, 15th ed., American Public Health Association, Washington, D.C.
- Andersson, Rolf. 1986. Inhibition of Staphylococcus aureus and spheroplasts of gram-negative bacteria by an antagonistic compound produced by a strain of Lactobacillus plantarum. Int. J. Food Microbiology 3:149-160.
- Andersson, R.E., M.A. Daeshel and H.M. Hassan. 1987. Mode of action of Plantaricin SIK-83, a bacteriocin produced by Lactobacillus plantarum. FEMS Microbiology Reviews 46(3): p. 69.
- Axelsson, L., T.C. Chung, W.J. Dobrogosz and L.E. Lindgren. 1987. Discovery of a new antimicrobial substance produced by Lactobacillus reuteri. FEMS Microbiology Reviews 46(3): p. 65.
- Beattie, S.E. and G.S. Torrey. 1986. Toxicity of methanethiol produce by Brevibacterium linens toward Penicillium expansum. J. Agric. Food Chem. 34:102-104.
- Berg, R.W., W.E. Sandine and A.W. Anderson. 1981. Identification of a growth stimulant for Lactobacillus sanfrancisco. Appl. and Environmental Microbio. 42:786-788.
- Bilinski, C.A., G. Innamorato and G.G. Stewart. 1985. Identification and characterization of antimicrobial activity in two yeast genera. Appl. and Environmental Microbio. 50(5):1330-1332.
- Blanc, P., G.M. Faup and G. Goma. 1987. Production of acetic and propionic acids from household refuse enzymatic hydrolysate by Propionibacterium acidipropionici. Biomass. 12:49-55.

- Branen, A.L., H.C. Go and R.P. Genske. 1975. Purification and properties of antimicrobial substances produced by Streptococcus diacetilactis and Leuconostoc citrovorum. J. Food. Science 40:446-4450.
- Buhnia, A.K., M.C. Johnson and B. Ray. 1987. Direct detection of an antimicrobial peptide of Pedicoccus acidilactici in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Industrial Micro. 2:00-00.
- Collins, E.B. and B.J. Hali. 1984. Growth of Bifidobacteria in milk and preparation of Bifidobacterium infantis for a dietary adjunct. J. Dairy Science 67:1376-1380.
- Cuk, Z., A. Annan-Prah, M. Janc, and J. Zanc-Satler. 1987. Yoghurt: An unlikely source of Campylobacter jejuni/coli. J. Appl. Bacteriology 63:202-205.
- Ferreira, C.L. and S.E. Gilliland. 1988. Bacteriocin involved in premature death of Lactobacillus acidophilus NCFM during growth at pH 6. J. Dairy Science 71:306-315.
- Fleming, H.P., J.L. Etchells and R.N. Costilow. 1975. Microbial inhibition by an isolate of Pedicoccus from cucumber brines. Appl. Microbiology 30(6): 1040-1042.
- Gonzalez, C.F. and B.S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in Pedicoccus acidilactici. Appl. and Environmental Micro. 53(10):2534-2538.
- Guern, Le, T.D. and B.C.M. 1987. Detection and activity of a bacteriocin produced by Lactobacillus plantarum. FEMS Microbiology Reviews 46(3): p. 68.
- Hirota, A, M. Nakagawa and H. Sakai. 1982. Terrecyclic acid A, a new antibiotic from Aspergillus terreus. The Journal of Antibiotics 35(7):783-787.
- Henning, S., R. Metz and W.P. Hammes. 1986. Studies on the mode of action of nisin. International J. Food Micro. 3:121-134.
- Hettinga, D.H. and G.W. Reinbold. 1972. The propionic acid bacteria -- A review. J. Milk Food Tech. 35(7):436-447.

- Hoover, D.G., P.M. Walsh, K.M. Kolatis and M.M. Daly. 1988. A bacteriocin produced by Pedicoccus species associated with a 5.5-megadalton plasmid. J. Food Protection 51(1):29-31.
- Hurst, A. 1981. Nisin. Adv. Appl. Microbiology 27:85-123.
- Jay, J.M. 1982. Antimicrobial properties of diacetyl. Appl. and Environmental Micro. 44(3):525-532.
- _____. 1986. Food Preservation. Ch. 5. In "Modern Food Microbiology," 3rd edition. Van Nostrand Reinhold Company, New York.
- Kim, D.S. 1987. Studies on antimicrobial agent produced by Lactobacillus acidophilus. In "Proceedings of the 3rd. AAAP Animal Science Congress. Volume 2. [In Food Science and Tech. Abstracts (1987) 19(3): 3B7.]
- Kunz, B. and Chem-Ing, W. Blumel. 1983. Gewinnung lyophilisierter propionibacterium-kulturen fur den Einsatz in der Lebensmittel industrie. Lebensmittel-ustrie 30(4):156-158.
- Langsrud, T. and G.W. Reinbold. 1973. Flavor development and microbiology of Swiss cheese -- A review. J. Milk Food Tech. 36(11):531-542.
- Lee, S.Y., E.R. Vedamuthu, C.J. Washam and G.W. Reinbold. 1970. Diacetyl production by Propionibacterium shermanii in milk cultures. Canadian J. Microbiology 16:1231-1242.
- Malik, A.C., G.W. Reinbold and E.R. Vedamuthu. 1968. An evaluation of the taxonomy of Propionibacterium. Canadian J. Microbiology 14:1185-1191.
- Mehta, A.M., K.A. Patel and P.J. Dave. 1983. Isolation and purification of an inhibitory protein from Lactobacillus acidophilus AC₁. Microbios 37:37-43.
- Nakagawa, M., A. Hirota and H. Sakai. 1982. Terrecyclic acid A, a new antibiotic from Aspergillus terreus. The Journal of Antibiotics 35(7):778-782.
- Nout, M.J.R., T.M. Ronants-Van Laarhoven and R.H. Zwanikken. 1987. Inhibition of microbial growth by Pedicoccus spp. FEMS Microbiology Reviews 46(3): p. 63.

- Prescott and Dunn's. 1982. Production of Baker's yeast. Ch. 14. In Industrial Microbiology, G. Reed (ed.), p. 594. AVI Publishing Company, Westport, Connecticut.
- Pulusani, S.R., D.R. Rao and G.R. Sunki. 1979. Antimicrobial activity of lactic cultures: Partial purification and characterization of antimicrobial compound(s) produced by Streptococcus thermophilus. J. Food Science 44(2):575-578.
- Raccach, Moshe. 1987. Pedicocci and biotechnology. CRC Critical Reviews in Microbiology 14(4):291-309.
- Ramanathan, G.R. and W. Cutting. 1966. Purification of propionin, an antiviral agent from Propionibacteria. Proc. Soc. Exp. Biol. Med. 123:271-273.
- Rasic, J. Lj. and J.A. Kurmann. 1983. Pharmaceutical preparations. Ch. 8 In Bifidobacteria and their role, page 134. Birkhauser Verlag, Basel, Boston, Stuttgart.
- Reddy, N.S. and B. Ranganathan. 1983. Preliminary studies on antimicrobial activity of Streptococcus lactis subsp. diacetylactis. J. Food Protection 46(3):222-225.
- _____. 1983. Nutritional factors affecting growth and production of antimicrobial substances by Streptococcus lactis subsp. diacetylactis S₁-67/C. J. Food Protection 46(6):514-517.
- Reeves, Peter. 1972. The Bacteriocins. Pages 85-86. A. Kleinzeller, G.F. Springer and H.G. Wittman, eds. Springer-Verlag, NY.
- Rusul, Gulam, F.E. El Gazzar, and E.H. Marth. 1987. Growth and aflatoxin production by Aspergillus parasiticus NRRL 2999 in the presence of acetic or propionic acid at different initial pH values. J. Food. Protec. 50(11): 909-914.
- Salih, Mohammed Ali. 1985. Studies on growth metabolites produced by Propionibacterium shermanii. Ph.D. Thesis, Microbiology Dept., Oregon State University.
- Sasaki, T., J. Watari, M. Kohgo, N. Nishikaw and Y. Matsui. 1984. Breeding of a brewer's yeast processing anticontaminant properties. American Society of Brewing Chemists Journal 42(4):164-166.

- Schutz, M., M. Sievers and K. Klemm. 1987. Metabolically controlled continuous fermentation of lactose to propionate, acetate and carbon dioxide. *Milchwissenschaft* 42(2):71-74.
- Serot, T., X. Dousset and N. Torcatis. 1987. Isolation and partial purification of antibacterial substances produced by Leuconostoc mesenteriodes and Lactobacillus plantarum isolated from kefir. *FEMS Microbiology Reviews* 46(3): p. 63.
- Shaw, R.H. and J.M. Sherman. 1923. The production of volatile fatty acids and carbon dioxide by propionic acid bacteria with special reference to their action in cheese. *J. Dairy Science* 6:303-309.
- Welch, Christi. 1987. Nutritional and therapeutic aspects of Lactobacillus acidophilus in dairy products. *Cultured Dairy Products Journal* 22(2):23-26.
- Zodrow, K., J. Chelkowski, O. Stefaniak and D. Czarnecka. 1963. The effect of different casein hydrolysates on the growth and biosynthesis of carrinoids by Propionibacteria. *Acta Microbiologica Polonica* 12:259-262.

BIBLIOGRAPHY

- Abbar, F.M., and M.T. Mohamed. 1987. Occurrence of enteropathogenic E. coli serotypes in butter. *J. Food Protec.* 50(10): 829-831.
- Abdel-Bar, Nadia, N.D. Harris, and R.L. Rill. 1987. Purification and properties of an antimicrobial substance produced by Lactobacillus bulgaricus. *J. Food Science* 52(2): 411-415.
- Ahern, T.J., and A.M. Klibanov. 1985. The mechanism of irreversible enzyme inactivation at 100°C. *Science* 228 June: 1280-1283.
- Al-Hiti, M.M.A. and P. Gilbert. 1983. A note on inoculum reproducibility: A comparison between solid and liquid culture. *J. Appl. Bacteriology* 55: 173-175.
- American Public Health Association. 1985. Standard methods for the examination of dairy products. 15th ed. American Public Health Assoc., New York.
- Andersson, Rolf. 1986. Inhibition of Staphylococcus aureus and spheroplasts of gram-negative bacteria by an antagonistic compound produced by a strain of Lactobacillus plantarum. *Int. J. Food Microbiology* 3: 149-160.
- Andersson, R.E., M.A. Daeschel, and H.M. Hassan. 1987. Mode of action of Plantaricin SIK-83, a bacteriocin produced by Lactobacillus plantarum. *FEMS Microbiology Reviews* 46(3): p 69.
- Anderson, T.M., E.A. Bodie, N. Goodman, and R.D. Schwartz. 1986. Inhibitory effect of autoclaving whey-based medium on propionic acid production by Propionibacterium shermanii. *Appl. Env. Microbiology* 51(2): 427-428.
- Axelsson, L., T.C. Chung, W.J.D., and L.E. Lindgren. 1987. Discovery of a new antimicrobial substance produced by Lactobacillus reuteri. *FEMS Microbiology Reviews* 46(3): p 65.
- Ayres, J.C., J.G. Mundt, and W.E. Sandine. 1980. *Microbiology of foods*. W.H. Freeman and Company, San Francisco.

- Baer, A. 1987. Identification and differentiation of propionibacteria by electrophoresis of their proteins. *Milchwissenschaft* 42(7): 431-433.
- Basler, A.W., V. Der Hude, and M. Scheutwinkel. 1987. Screening of the food additive propionic acid for genotoxic properties. *Fd. Chem. Toxic.* 25(4): 287-290.
- Beattie, S.E., and G.S. Torrey. 1986. Toxicity of methanethiol produced by Brevibacterium linens toward Penicillium expansum. *J. Agric. Food Chem.* 34(1): 102-104.
- Benkerroum, Noredine. 1988. Personal communication. Microbiology Dept., Oregon State University, Corvallis, OR.
- Berg, R.W., W.E. Sandine and A.W. Anderson. 1981. Identification of a growth stimulant for Lactobacillus sanfrancisco. *Appl. and Environmental Microbio.* 42:786-788.
- Bilinski, C.A., G. Innamorator, and G.G. Stewartz. 1985. Identification and characterization of antimicrobial activity in two yeast genera. *Appl. Environmental Microbiology* 50(5): 1330-1332.
- Bishop, J.R., and C.H. White. 1986. Assessment of dairy product quality and potential shelf-life: A review. *J. of Food Protec.* 49(9): 739-753.
- Blanc, P., G.M. Faup, and G. Goma. 1987. Production of acetic and propionic acids from household refuse enzymatic hydrolysate by Propionibacterium acidipropionici. *Biomass.* 12: 49-55.
- Blanc, P., and G. Gowa. 1987. Kinetics of inhibition in propionic acid fermentation. *Bioprocess Engineering* 2: 175-179.
- Bodie, E.A., N. Goodman, and R.D. Schwartz. 1987a. Production of propionic acid by mixed cultures of Propionibacterium shermanii and Lactobacillus casei in autoclave-sterilized whey. *J. Industrial Microbiology* 1: 349-353.
- Bodie, E.A., T.M. Anderson, N. Goodman, and R.D. Schwartz. 1987b. Propionic acid fermentation of ultra-high temperature sterilized whey using mono- and mixed-cultures. *Appl. Microbiol. Biotechnol.* 25: 434-437.

- Brady, M.S., and S.E. Katz. 1987. Simplified plate diffusion system for microbial assays of antibiotics. *J. Assoc. of Anal. Chem.* 70(4): 641-646.
- Branen, H.C. Go, and R.P. Genske. 1975. Purification and properties of antimicrobial substances produced by Streptococcus diacetylactis and Leuconostoc citrovorum. *J. Food Science* 40: 446-450.
- Brink, B. Ten., M. Minekus, J. Bol, and J.H.J. Huis. 1987. Production of antimicrobial compounds by lactobacilli. *FEMS Microbiology Reviews* 46(3): p 63.
- Britz, T.J., and P.L. Steyn. 1980. Comparative studies on propionic acid bacteria. *Phytophylactica* 12: 89-103.
- Brocklehurst, T.F. and B.M. Lund. 1988. The effect of pH on the initiation growth of cottage cheese spoilage bacteria. *Int. J. Food Microbiology* 6(1):43-49.
- Buhnia, A.K., M.C. Johnson, and B. Ray. 1987. Direct detection of an antimicrobial peptide of Pedicoccus acidilactici in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Industrial Microbiology* 2:00-00.
- Bulgarelli, M.A., and L.A. Shelef. 1985. Effect of EDTA on growth for spores of Bacillus cereus. *J. Food Science* 50(3): 661-664.
- Bullerman, L.B. and J.H. Rupnow. 1984. A laboratory manual for food microbiology. Institute of Agriculture and National Resources, Lincoln, Nebraska.
- Christen, G.L., and M. Senica. 1987. Casein, fluorescein isothiocyanate as a substrate for measuring protease production by psychotrophic bacteria. *J. Food Protec.* 50(9): 744-749.
- Collins, E.B. and B.J. Hali. 1984. Growth of Bifidobacteria in milk and preparation of Bifidobacterium infantis for a dietary adjunct. *J. Dairy Science* 67:1376-1380.
- Cousin, M.A. 1982. Presence and activity of psychotrophic microorganisms in milk and dairy products: A review. *J. Food Protec.* 45(2): 172-207.
- Cove, J.H., K.T. Holland, and W.J. Cunliffe. 1987. The effects of oxygen on cutaneous propionibacteria grown in continuous culture. *FEMS Microbiology Letters* 43: 61-65.

- Crow, V.L., and K.W. Turner. 1986. The effect of succinate production on other fermentation products in Swiss-type cheese. *New Zealand J. Dairy Science and Tech.* 21: 217-227.
- Crow, V.L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by Propionibacterium freudenreichii subsp. shermanii. *Appl. Environmental Microbiology* 53(10): 2600-2602.
- Cuk, Z., A. Annan-Prah, M. Janc, and J. Zajc-Satler. 1987. Yoghurt: An unlikely source of Campylobacter jejuni/coli. *J. Appl. Bacteriology* 63: 202-205.
- Debevere, J.M. 1987. The use of buffered acidulant systems to improve the microbiological stability of acid foods. *Food Microbiology* 4: 105-114.
- Deibel, R.H., and J.B. Evans. 1960. Modified benizidine test for the detection of cytochrome-containing respiratory systems in microorganisms. *J. Bacteriology* 79: 356-360.
- DeMan, J.C., M. Rogosa, and M.E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriology* 231: 130-135.
- Doyle, M.P. and J.L. Schoeni. 1987. Isolation of E. coli O157:H7 from retail fresh meat and poultry. *Appl. and Envir. Microbiology* 50(10): 2394-2396.
- Duff, J.T., G.G. Wright, and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. *J. Bacteriology* 72: 455-460.
- Dumont, Jean-Pierre, and J. Adda. 1978. Occurrence of sesquiterpenes in Mountain cheese volatiles. *J. Agric. Food Chem.* 26(2): 364-367.
- Eklund, T. 1985. Inhibition of microbial growth at different pH levels by benzoic and propionic acids and esters of p-hydroxybenzoic acid. *Int. J. Food Micro.* 2: 159-167.
- Elliker, P.R., W.E. Sandine, B.A. Hauser, and W.K. Moseley. 1964. Influence of culturing cottage cheese dressing with different organisms on flavor and keeping quality. *J. Dairy Science* 47: 680.
- Fairclough, G.F., and J.S. Fruton. 1966. Peptide-protein interactions as studied by gel filtration. *Biochemistry* 5(2): 673-683.

- Ferreira, C.L. and S.E. Gilliland. 1988. Bacteriocin involved in premature death of Lactobacillus acidophilus NCFM during growth at pH 6. *J. Dairy Science* 71:306-315.
- Fleet, G.H., and M.A. Mian. 1987. The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiology* 4: 145-155.
- Fleming, H.P., J.L. Etchells, and R.N. Costilow. 1975. Microbial inhibition by an isolate of Pedicoccus from cucumber brines. *Appl. Microbiology* 30(6): 1040-1042.
- Flores-Galaza, R.A., B.A. Glatz, C.J. Bern, and L.D. Van Fossen. 1985. Preservation of high-moisture corn by microbial fermentation. *J. Food Protection* 48(5): 407-411.
- Food and Drug Administration. 1984. Bacteriological analytical manual (BAM). 6th ed. U.S. Food and Drug Administration, Washington, D.C.
- Franco, Bernadette D.G.M., B.E.C. Guth, and L.R. Trabuls. 1987. Enterotoxigenic E. coli isolated from foods in Sao Paulo, Brazil. *J. Fd. Pr.* 50(10): 832-834.
- Freese, E., C.W. Sheu, and E. Galliers. 1973. Function of lipophilic acids as antimicrobial food additives. *Nature* 241: 321-325.
- Gavrilova, N.N., and L.I. Zahharenko. 1987. Antagonistic activity of lactic acid bacteria and propionic acid bacteria from 'Kazakhsil' starters. *FEMS Microbiology Reviews* 46(3): p 67.
- Gonzalez, C.F., and B.S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in Pedicoccus acidilactici. *Appl. Environmental Micro.* 53(10): 2534-2538.
- Guem, J. Le., D. Thuault, and C.M. Bourgeois. 1987. Detection and activity of a bacteriocin produced by Lactobacillus plantarum. *FEMS Microbiology Reviews* 46(3): p 68.
- Haque, Z., and J.E. Kinsella. 1988. Emulsifying properties of food proteins: Bovine serum albumin. *J. Food Science* 53(2): 416-420.
- Henning, S., R. Metz, and W.P. Hammes. 1986. Studies on the mode of action of nisin. *International J. Food Microbiology* 3: 121-134.

- Herrero, A.A. 1983. End-product inhibition in anaerobic fermentations. *Trends in Biotechnology* 1(2): 49-53.
- Hettinga, D.H., and G.W. Reinbold. 1972. The propionic acid bacteria -- A review. III. Miscellaneous metabolic activities. *J. Milk and Food Technology* 35(7): 436-447.
- Hirooka, E.Y. Sonia, P.C. Desalzburg, and M.S. Bergdall. 1987. Production of staphylococcal enterotoxin A and thermonuclease in cream pies. *J. Food Protec.* 50(11): 952-955.
- Hirota, A., M. Nakagawa and H. Sakai. 1982. Terrecyclic acid A, a new antibiotic from Aspergillus terreus. *The Journal of Antibiotics* 35(7):783-787.
- Hitchner, E.R. 1934. Some physiological characteristics of the propionic-acid bacteria. *J. Bacteriology* 28: 473-479.
- Hogg, G.M., M.F. Patterson, and J.G. Barr. 1987. Correlation between antibiotic sensitivity testing by conventional and conductivity measurements. *J. Appl. Bact.* 62: 189-195.
- Holt, J.G., ed. 1977. *The shorter Bergey's manual of determinative bacteriology*. 8th ed. Williams and Wilkins, Baltimore.
- Hoover, D.G., P.M. Walsh, K.M. Kolaetis, and M.M. Daly. 1988. A bacteriocin produced by Pedicoccus species associated with a 5.5-megadalton plasmid. *J. Food Protection* 51(1): 29-31.
- Hummel, J.P., and W.J. Dreyer. 1962. Measurement of protein-binding phenomena by gel filtration. *Biochemistry and Biophysics Acta.* 63: 530-532.
- Hurst, A. 1981. Nisin. *Adv. Appl. Microbiology* 27:85-123.
- Ingram, E., K.T. Holland, G. Gowland, and W.J. Cunliffe. 1983. Studies of the extracellular proteolytic activity produced by Propionibacterium acnes. *J. Appl. Bact.* 54: 263-271.
- Ismaeel, N., J.R. Furr and A.D. Russell. 1986. Reversal of the surface effects of chlorhexidine diacetate on cells of Providencia stuartii. *J. Appl. Bact.* 61: 373-381.

- Jay, J.M. 1982. Antimicrobial properties of diacetyl. *Appl. Environmental Microbiology* 44(3): 525-532.
- Jay, J.M. 1986. *Modern food microbiology*. Van Nostrand Reinhold Company, New York.
- Juven, B.J., and J. Kanner. 1986. Effect of ascorbic, isoascorbic and dehydroascorbic acids on the growth and survival of Campylobacter jejuni. *J. of Appl. Bact.* 61: 339-345.
- Kabara, Jon J. 1978. Fatty acids and derivatives as antimicrobial agents -- A review. Ch. 1 In "The pharmacological effects of lipids," Jon J. Kabara, ed., American Oil Chem. Soc., Champaign, IL.
- Kato, Akio, A. Tanaka, N. Matsudemi, and K. Kobayashi. 1987. Deamidation of food proteins by protease in alkaline pH. *J. Agric. Food Chem.* 35(2): 224-227.
- Kim, D.S. 1987. Studies on antimicrobial agent produced by Lactobacillus acidophilus. In "Proceedings of the 3rd. AAAP Animal Science Congress. Volume 2. [In Food Science and Tech. Abstracts (1987) 19(3): 3B7.]
- King, N. 1948. A modification of the Voges-Proskauer test for rapid calorimetric determination of acetylmethylcarbinol plus diacetyl in butter cultures. *Dairy Industries* September: 860-878.
- Konishi, Y., Jun-ichi Kurisaki, S.K., and K. Yamanchi. 1985. Detection of antigenicity by radioimmune-assay and of trypsin inhibitory activity in heat or enzyme denatured ovomucoid. *J. Food Science* 50(5): 1422-1426.
- Kunz, B. and Chem-Ing, W. Blumel. 1983. Gewinnung lyophilisierter p;ropionibacterium-kulturen fur den Einsatz in der Lebensmittel industrie. *Lebensmittelin-ustrie* 30(4):156-158.
- Kvenberg, J.E., and D.L. Archer. 1987. Economic impact of colonization control of food-born disease. *Food Technology* July: 77-81.
- Lana, L., R. Grasselli, A. Lombardi, and M.P. Nuti. 1987. Isolation of bacteriocin(s)-producing streptococci active on lactate-fermenting clostridia. *FEMS Microbiology Reviews* 46(3): p 68.

- Langsrud, T., and G.W. Reinbold. 1973. Flavor development and microbiology of Swiss cheese -- A review. *J. Milk and Food Technology* 36(11): 531-542.
- Lee, S.Y., E.R. Vedamuthu, C.J. Washam, and G.W. Reinbold. 1970. Diacetyl production by Propionibacterium shermanii in milk cultures. *Canadian J. of Microbiology* 16: 1231-1242.
- Magan, N., and J. Lacey. 1986. Water relations and metabolism of propionate in two yeasts from hay. *J. Appl. Bact.* 60: 169-173.
- Malik, A.C., G.W. Reinbold, E.R. Vedamuthu. 1968. An evaluation of the taxonomy of propionibacterium. *Can. J. Micro.* 14: 1185-1191.
- Marwaha, S.S., R.P. Sethi, and J.F. Kennedy. 1983. Role of amino acids, betaine and choline in vitamin B₁₂ biosynthesis by strains of Propionibacterium. *Enzyme and Microbial Technology* 5(6): 454-465.
- Mehta, A.M., K.A. Patel, and P.J. Dave. 1983. Isolation and purification of an inhibitory protein from Lactobacillus acidophilus AC₁. *Microbios.* 37: 37-43.
- Miyakoshi, S., H.U. Chiyami, and coworkers. 1987. Distribution of the methylcitric acid cycle and B-oxidation pathway for propionate catabolism in fungi. *Agric. Biol. Chem.* 51(9): 2381-2387.
- Moon, N.J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *J. Appl. Bact.* 55: 453-460.
- Moran, A.P., and M.E. Upton. 1987a. Factors affecting production of coccoid forms by Campylobacter jejuni in solid media during incubation. *J. of Appl. Bact.* 62: 527-537.
- _____. 1987b. Effect of medium supplements, illumination and superoxide dismutase on the production of coccoid forms of Campylobacter jejuni ATCC 29428. *J. Appl. Bacteriology* 62(1): 43-51.
- Morris, C.E. 1985. Derived from whey, it's a milk replacer. *Food Engineering* March: 63.
- Moustafa, H.H., and E.B. Collins. 1969. Effect of selected food additives on growth of Psuedomonas fragi. *J. Dairy Science* 52(3): 335-340.

- Nakagawa, M., A. Hirota, and H. Sakai. 1982. Terrecyclic acid A, A new antibiotic from Aspergillus terreus. The Journal of Antibiotics 35(7): 778-782.
- Nout, M.J.R., T.M. Ronants-Van Laarhoven and R.H. Zwanikken. 1987. Inhibition of microbial growth by Pedicoccus spp. FEMS Microbiology Reviews 46(3): 63.
- Oser, B.L. 1965. In "Hawk's physiological chemistry." 14th ed., p. 179. McGraw-Hill Book Co., New York.
- Oxford, A.E. 1944. Diplococcin, an anti-bacterial protein elaborated by certain milk streptococci. Biochemistry 38: 178-182.
- Palumbo, Samuel A. 1986. Is refrigeration enough to restrain foodborne pathogens? J. Food Protec. 49(12): 1003-1009.
- Perez-Mendoza, J.L., and F. Garcia-Hernandez. 1983. Fermentation of a waste-product from industrial processing of the lime (citrus aurantifolia Swingle) for vitamin B₁₂ production. Biotechnology letters 5(4): 259-264.
- Phillips, J.D., and M.W. Griffiths. 1987. The relations between temperature and growth of bacteria in dairy products. Food Microbiology 4(2): 173-185.
- Prescott and Dunn's. 1982. Production of Baker's yeast. Ch. 14. In Industrial Microbiology, G. Reed (ed.), p. 594. AVI Publishing Company, Westport, Connecticut.
- Pritchard, G.G., J.W.T. Wimpenny, H.A. Morris, M.W.A.L., and D.E. Hughes. 1977. Effect of oxygen on Propionibacterium shermanii grown in continuous culture. J. Gen. Microb. 102(1): 223-33.
- Pulusani, S.R., D.R. Rao, and G.R. Sunki. 1979. Antimicrobial activity of lactic cultures: Partial purification and characterization of antimicrobial components produced by Streptococcus thermophilus. J. Food Science 44(2): 575-578.
- Raccach, Moshe. 1987. Pediococci and biotechnology. CRC Critical Reviews in Microbiology 14(4): 291-309.
- Ramanathan, S., G. Read, and W. Cutting. 1966. Purification of propionin, an antiviral agent from propionibacteria. Proc. Soc. Exp. Biol. Med. 123: 271-273.

- Rasic, J. Lj. and J.A. Kurmann. 1983. Pharmaceutical preparations. Ch. 8 In *Bifidobacteria and Their Role*, p. 134. Birkhauser Verlag, Basel, Boston, Stuttgart.
- Ray, Bibek, and C. Johnson. 1984. Sensitivity of cold-stressed Campylobacter jejuni to solid and liquid selective environments. *Food Microbiology* 1: 173-176.
- Reddy, N.S., and B. Ranganathan. 1983. Nutritional factors affecting growth and production of antimicrobial substances by Streptococcus lactis subsp. diacetylactis S1-67/C. *J. Food Protection* 46(6): 514-517.
- _____. 1983. Preliminary studies on antimicrobial activity of Streptococcus lactis Subsp. diacetylactis. *J. Food Protection* 46(3): 222-225.
- Reeves, Peter. 1972. The Bacteriocins. Pages 85-86. A. Kleinzeller, G.F. Springer and H.G. Wittman, eds. Springer-Verlag, NY.
- Ro, S.L., M. Woodburn, and W.E. Sandine. 1979. Vitamin B₁₂ and ascorbic acid in Kimchi inoculated with Propionibacterium freudenreichii ss. shermanii. *J. Food Science* 44: 873-877.
- Rose, Sally A. 1985. A note on yeast growth in media used for the cultivation of Lactobacilli. *J. Appl. Bact.* 59: 153-156.
- Rosenow, E.M., and E.H. Marth. 1987. *Listeria*, listeriosis and dairy foods: A review. *Cultured Dairy Prod. Journal* 22(4): 13-17.
- Rusul, G., F.E. El-Gazzar, and E.H. Marth. 1987. Growth and aflatoxin production by Aspergillus parasiticus NRRL 2999 in the presence of acetic or propionic acid at different initial pH values. *J. Food Protection* 50(11): 909-914.
- Saito, Isao, H. Oshima, N. Kawamura, K. Uno, and M. Yamada. 1987. Determination of sorbic, dehydroacetic, and propionic acids in cheese by liquid chromatography and gas chromatography. *J. Assoc. of Anal. Chem.* 70(3): 507-509.
- Sakaguchi, G., and S. Sakaguchi. 1966. Some observations on activation of Clostridium botulinum type E toxin by trypsin. In "Botulism," M. Ingram and T.A. Roberts ed., Chapman and Hall Limited, London.

- Salih, Mohammed Ali. 1985. Studies on growth metabolites produced by Propionibacterium shermanii. Ph.D. Thesis, Microbiology Dept., Oregon State University.
- Sandine, W.E. 1988. An introduction to lactococci and non-rodlike forms. Biochemie. In press.
- Sasaki, T., J. Watari, M. Kohgo, N. Nishikaw and Y. Matsui. 1984. Breeding of a brewer's yeast processing anticontaminant properties. American Society of Brewing Chemists Journal 42(4): 164-166.
- Schales, Otto. 1951. Effect of albumin on the antibacterial activity of diketones. Arch. Biochem. Biophys. 34: 56-63.
- Schillinger, U., and F.-K. Lucke. 1987. Antagonistic activities of lactic acid bacteria isolated from meats. FEMS Microbiology Reviews 46(3): p 65.
- Schutz, M., M. Sievers, and K. Klemm. 1987. Metabolically controlled continuous fermentation of lactose to propionate, acetate, and carbon dioxide. Milchwissenschaft 42(2): 71-74.
- Schwartz, G.W., H.N.S. Kaufman, and J.E. Snoke. 1948. The specific esterase activity of trypsin. J. Biol. Chem. 172: 221-239.
- Serot, T., X. Dousset, and N. Torcatis. 1987. Isolation and partial purification of antibacterial substances produced by Leuconostoc mesenteroides and Lactobacillus plantarum isolated from Kefir. FEMS Microbiology Reviews 46(3) p 66.
- Shaw, R.H., and J.M. Sherman. 1923. The production of volatile fatty acid bacteria with special reference to their action in cheese. J. Dairy Science 6: 303-309.
- Sherman, J.M., and R.H. Shaw. 1921. Associative bacterial action in the propionic acid fermentation. J. Gen. Physiology p. 657-658.
- Shibasaki, Isao, and N. Kato. 1978. Combined effect on antibacterial activity of fatty acids and their esters against gram negative bacteria. Ch. 2 In "The Pharmacological effects of lipids. J.J. Kabara ed., American Oil Chem. Soc., Champaign, IL.
- Shih, F.K. 1987. Deamidation of protein in a soy extract by ion exchange resin catalysis. J. Food Science 52(6): 1529-1531.

- Shotwell, O.L., G.A. Bennett, W.F. Kwolek, and C.W. Hesseltine. 1984. Effect of propionic acid on aflatoxin levels of stored Georgia 1981 Dent corn samples. *J.A.O.C.S.* 61(3): 526-528.
- Smith, D.A., E.M. Mikolajcik, and J.B. Lindamood. 1987. Causative organisms and chemical nature of the Swiss cheese rind rot defect. *Cultured Dairy Prod. Journal* 22(4): 9-12.
- Somers, E.B., and S.L. Taylor. 1987. Antibotulinal effectiveness of nisin in pasteurized process cheese spreads. *J. Food Protec.* 50(10): 842-848.
- Speck, M. L., ed. 1984. Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D.C.
- Stanfield, J.T., C.R. Wilson, W.H. Andrews, and G.J. Jackson. 1987. Potential role of refrigerated milk packaging in the transmission of listeriosis and salmonellosis. *J. Food Protec.* 50(9): 730-732.
- Suihko, M.L., and V. Makinen. 1984. Tolerance of acetate, propionate and sorbate by Saccharomyces cerevisiae and Torulopsis holmii. *Food Microbiology* 1: 105-110.
- Tham, Wilhelm, and L.J. Hajdu. 1987. Comparison of 6 media for the isolation of Staphylococcus aureus from foods. *Food Microbiology* 4(2): 133-146.
- Thomas, T.D. 1987. Acetate production from lactate and citrate by non-starter bacteria in cheddar cheese. *New Zealand J. of Dairy Science and Tech.* 22: 25-38.
- Tjaberg, T. Bjarne. 1974. Proteases of Clostridium botulinum. *Acta. Vet. Scand.* 15: 487-506.
- Vallejo-Cordoba, B., S.N., W.D. Powrie, and T. Beveridge. 1987. Extended shelf life of frankfurters and fish frankfurter-analogs with added soy protein hydrolysates. *J. Food Science* 52(5): 1133-1136.
- Vedamuthu, E.R., and G.W. Reinbold. 1967. The use of candle oats jar incubation for the enumeration, characterization and taxonomic study of propionibacteria. *Milchwissenschaft* 22(7): 428-431.
- Vorob'eva, L.I., S. Al'-Sudani, and N.I. Kraeva. 1987. Peroxidase of propionic acid bacteria. *Microbiology* (Translated from Russian) 56(4): 590-593.

- Walker, D.W., and S.E. Gilliland. 1987. Buttermilk manufacturing using combination of direct acidification and citrate fermentation by Leuconostoc cremoris. J. Dairy Sci. 70(10): 2055-2062.
- Washington, J.A., ed. 1985. Laboratory procedures in clinical microbiology. Springer-Verlag, New York.
- Weber, G.H., and W.A. Broich. 1986. Shelf life extension of cultured dairy foods. Cultured Dairy Products Journal November: 19-23.
- Welch, Christi. 1987. Nutritional and therapeutic aspects of Lactobacillus acidophilus in dairy products. Cultured dairy products journal 22(2): 23-26.
- Wheeler, K.A., A.D. Hocking, J.I. Pitt, and A.M. Anggawatl. 1986. Fungi associated with Indonesian dried fish. Food Microbiology 3(4): 351-357.
- Whitaker, J. R. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Analytical Chemistry 35(12): 1950-1953.
- Wood, H. Goff and C. H. Werkman. 1936. Mechanism of glucose dissimilation by the propionic acid bacteria. Biochemistry Journal 30: 618-623.
- Zodrow, K., J. Chelkowski, O. Stefaniak and D. Czarnecka. 1963. The effect of different casein hydrolysates on the growth and biosynthesis of carrinoids by Propionibacteria. Acta Microbiologica Polonica 12: 259-262.