

Inhibition of Lactic Acid Bacteria by Herbs 4775

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ABSTRACT

Increasing concentrations (0.5–8g/liter) of oregano, rosemary, sage, and thyme progressively delayed growth and acid production by *Lactobacillus plantarum* and *Pediococcus acidilactici* in a liquid medium. After the bacteriostatic activity was overcome, all four herbs strongly stimulated acid production. The relative inhibitory effect of the herbs toward both microorganisms was oregano >> rosemary = sage > thyme. *L. plantarum* was more resistant than *P. acidilactici* to the toxic effect of the herbs. Organisms from cultures exhibiting delayed fermentation in the presence of sublethal concentrations of an herb, when subcultured into fresh media containing identical herb concentrations, initiated fermentation without delay, indicating development of resistance to the herb's effect. Moreover, bacteria which had acquired a resistance to one herb were also resistant to the other three herbs.

INTRODUCTION

ANTIMICROBIAL PROPERTIES of spices such as cinnamon, clove, and mustard have been extensively studied. Less attention has been devoted to investigations of antimicrobial properties of the leafy spices or herbs such as oregano, rosemary, sage, and thyme. Beuchat (1976) reported that oregano and thyme were highly toxic to *Vibrio parahaemolyticus*: when present in growth media at a concentration of 0.5%. Shelef et al. (1980) found that rosemary and sage inhibited Gram-positive bacteria to a greater extent than Gram-negative bacteria. Julseth and Deibel (1974) reported that the growth of *Salmonella* in pre-enrichment cultures was inhibited in the presence of oregano. Llewellyn et al. (1981) found that thyme and oregano inhibited growth and aflatoxin production by three toxigenic *Aspergillus* strains, whereas rosemary supported good growth and aflatoxin production. Corran and Edgar (1933) reported that herbs, among them thyme and rosemary, did not inhibit yeast fermentation. Dold and Knapp (1948) considered sage and thyme to be ineffective against a number of pathogenic bacteria.

Past investigators have indicated that the antimicrobial factor of spices resides in the essential oil and/or oleoresin fraction. The essential oils of oregano, rosemary, sage, and thyme have been reported to have antibacterial (Collier and Nitta, 1930; Kellner and Kober, 1954; Maruzzella and Sicurella, 1960) and antifungal (Maruzzella and Liguori, 1958; Maruzzella, 1960) activity. Of the four herbs, the essential oils of oregano and thyme were generally found to be the most inhibitory and were considered among the most active of a large number of tested spice and herb essential oils.

Most of the reported studies on the antimicrobial activity of spices and herbs involved pathogenic microorganisms, and only a few reports involving lactic acid bacteria are available. These reports suggest that lactic acid bacteria are relatively resistant to the toxic effects of spices (Karaioannoglou et al., 1977; Salzer et al., 1977; Shelef et

al., 1980; Park et al., 1980) or their essential oils (Molina and Merzari, 1949; Anderson et al., 1953; Shcherbanovskii et al., 1973). Previous observations indicate that Lactacel MC starter culture (*Lactobacillus plantarum* and *Pediococcus cerevisiae*), used for preparation of fermented sausages, was relatively resistant to the antimicrobial action of 19 spices and herbs (Kissinger and Zaika, 1978; Zaika and Kissinger, 1979a, b). In most cases, the starter culture bacteria grew in a liquid medium containing up to 12g/liter spice; however, the microorganisms were severely inhibited by a few spices, notably oregano and clove. It was observed that growth in the presence of a variety of spices was accompanied by enhanced acid production relative to that in the unspiced control.

Previous investigations (Zaika and Kissinger, 1981) have indicated that while *L. plantarum* and *P. cerevisiae* can be completely inhibited by appropriate concentrations of oregano, the microorganisms can acquire resistance to the toxic effects by first being exposed to sublethal concentrations of the herb. One of the objectives of the present work was to compare viability and acid production by *L. plantarum* and *P. acidilactici* in the presence of the botanically-related herbs of the family *Labiatae* (oregano, rosemary, sage, and thyme), to determine if the starter bacteria could acquire resistance to the inhibitory effects of the other members of the *Labiatae*, and if resistance to one herb imparted resistance to the others.

MATERIALS & METHODS

Microorganisms

Frozen cultures of *Lactobacillus plantarum* (Lactacel 804, Microlife Technics, Sarasota, FL) and *Pediococcus acidilactici* (Lactacel, Microlife Technics) were used throughout the study. According to the supplier (personal communication), these are single-strain cultures.

Liquid medium

The fermentation medium was prepared by dissolving 3g beef extract (Difco), 5g tryptone (Difco), 20g sucrose, and 20g glucose in 1 liter of distilled water. The pH of the medium was adjusted to 6.5 with 6N H₂SO₄ (giving a post-sterilization pH of 5.7–6.3). Aliquots (250 ml) of the medium were dispensed into 500-ml Erlenmeyer flasks and sterilized for 15 min at 15 psi.

Herbs

All herbs used were commercially dried and ground. Commercially sterilized rosemary, sage, and thyme were obtained from Griffith Laboratories, Inc., Union, NJ. Oregano and oregano essential oil were obtained from Penn Herb Company, Philadelphia, PA. Oregano was sterilized with ethylene oxide in a Cryotherm Portable Sterilizer, series 8040 (American Sterilizer Company, Erie, PA). All herbs used in the experiment contained less than 100 organisms/g as determined by total aerobic plate counts.

Fermentation

Herbs were added aseptically to the flasks of sterile medium to provide concentrations of 0.5, 1, 2, 4, or 8g/liter. All flasks were inoculated with 2.5 ml of the thawed commercial starter culture diluted with 0.1% peptone water such that the initial bacterial population in the flasks ranged from 10² – 10⁴ cells/ml. The flasks were

incubated statically at 35°C for up to 7 days. Samples for bacterial counts and titratable acidity were taken at 24-hr intervals. Additional experiments were conducted using 3g/liter oregano and 8g/liter thyme, rosemary, and sage. For experiments with oregano essential oil, solutions (1 ml) of the oil in ethanol were added to the flasks containing medium to provide concentrations of 40 to 400 ppm. Ethanol (1 ml) was added to the control.

Titratable acidity

A 25 ml portion of each sample was centrifuged at 20200 X g for 15 min at 5°C. Ten ml of the supernatant, diluted with 50 ml of distilled water, were titrated with 0.1N NaOH to pH 7.0 with the aid of a Fisher Accumet Model 325 pH meter equipped with a Corning combination pH electrode. The titratable acidity was expressed in terms of ml of 0.1N NaOH/10 ml medium. The titratable acidities of uninoculated media were 0.33–0.65 ml.

Enumeration of bacteria

Bacterial counts were determined by conventional pour plate techniques using APT agar (Difco). Plates were incubated aerobically for 48 hr at 35°C.

Adaptation to herbs

A modification of a method developed previously (Zaika and Kissinger, 1981) was used to test for adaptation. Starter culture was inoculated into media containing either 3g/liter oregano, 8g/liter rosemary, 8g/liter sage, or 8g/liter thyme. When acid production began after a delay of several days, bacteria from the spice-containing samples were inoculated into medium containing the same spice or one of the other test spices.

RESULTS & DISCUSSION

Effect of herbs on starter cultures

The effect of oregano, rosemary, sage, and thyme on growth and acid production by *L. plantarum* is shown in Fig. 1A-D, respectively. For the sake of clarity of the graphs, not all of the herb concentrations tested (0.5–8g/liter) are shown in the figures. Addition of 0.5g/liter oregano to the medium did not affect growth of the microorganism, but did increase acid production by 2.5-fold. Increasing the oregano concentration to 2 and 4g/liter resulted in a delay in bacterial growth. Some bactericidal activity was evident during early stages of fermentation in cultures containing 2 and 4g/liter oregano and acid production was delayed until the inhibition of growth was overcome. After 7 days of fermentation the titratable acidity of the medium containing 4g/liter oregano was 2.5 times that of the control medium. Increasing the oregano concentration to 8g/liter was bactericidal to *L. plantarum*.

The effect of rosemary, sage, and thyme on growth and acid production by *L. plantarum* was similar to that observed for oregano, except that those herbs were not as inhibitory. The bacteria survived, grew, and produced acid in media containing 8g/liter rosemary, sage, or thyme. The relative inhibitory activity of the herbs was oregano >> rosemary = sage > thyme. In all cases increasing concentrations of an herb caused a progressive delay in bacterial growth and acid production. However, after a growth lag,

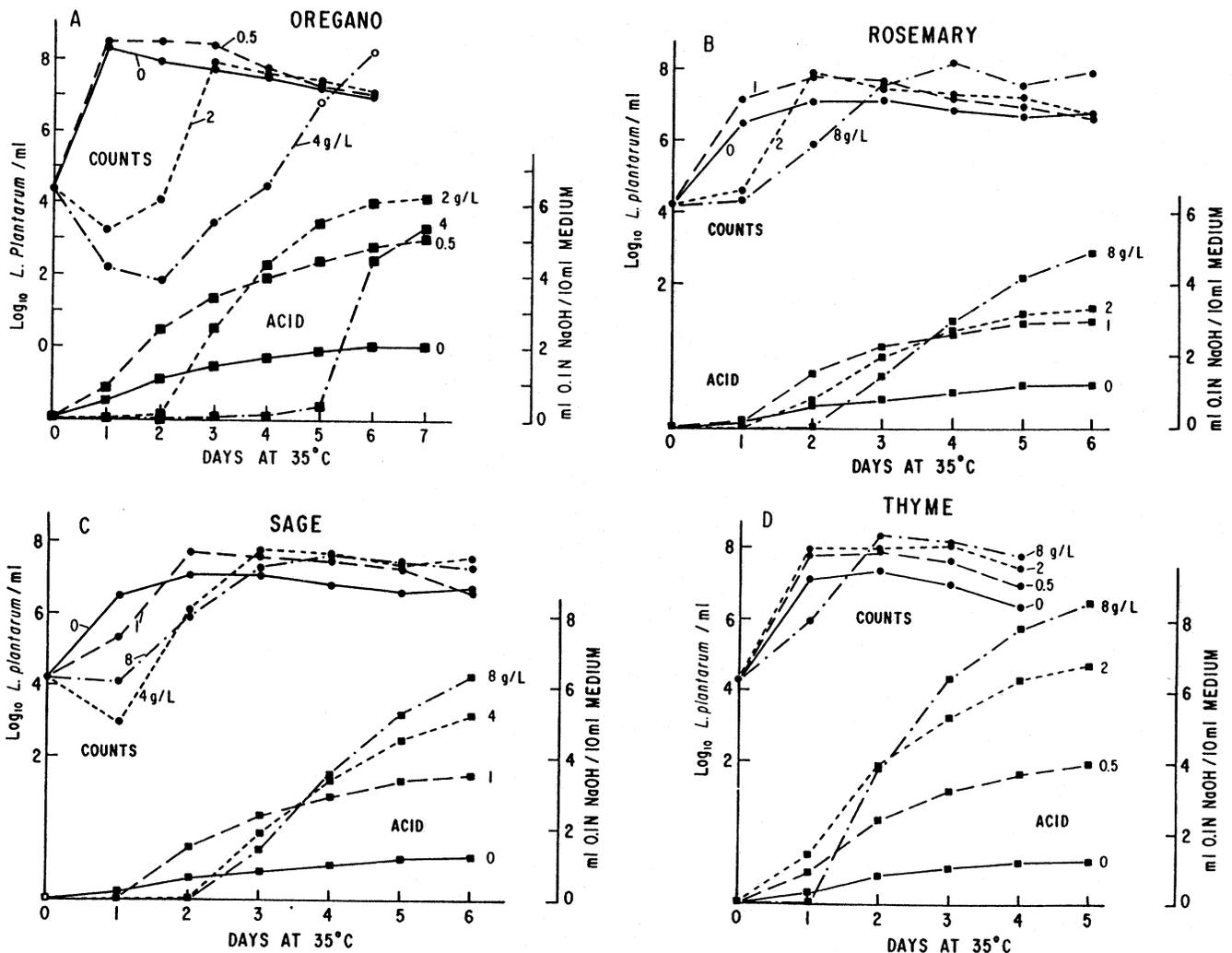


Fig. 1—Effect of herbs on growth and acid production by *L. plantarum*: oregano (A), rosemary (B), sage (C), thyme (D).

acid production increased with increase in herb concentration. Other experiments demonstrated that addition of 8g/liter thyme, sage, and rosemary resulted in titratable acidity values 7, 5, and 4 times greater, respectively, than those of control cultures after 6 days of fermentation, and acid production in the presence of 3g/liter oregano generally was three to four times higher than in the controls after 6 days.

The effect of the four herbs on growth and acid production by *P. acidilactici* (Fig. 2A-2D) was similar to that noted for *L. plantarum* (Fig. 1A-1D). *P. acidilactici* was more sensitive than *L. plantarum* to the toxic effect of high concentrations of the herbs. While acid production by *P. acidilactici* was stimulated in the presence of the herbs, the degree of stimulation was not as great as that observed for *L. plantarum*. The relative inhibitory activity of the herbs toward *P. acidilactici* was oregano \gg rosemary = sage > thyme. In cultures containing 0.5g/liter oregano bacterial growth was not affected, while titratable acidity was doubled; however, a concentration of 4g/liter (data not shown) was bactericidal. *P. acidilactici* initially declined in numbers in media containing 4 and 8g/liter rosemary and for similar concentrations of sage, but later grew and produced acid. Significant inhibition of *P. acidilactici* by

thyme was observed only at the 8g/liter level. Rosemary, sage, and thyme appeared to stimulate acid production by *P. acidilactici* to a similar extent. Titratable acidity values after 5 days in media containing 8g/liter of the herbs were approximately three times higher than in control cultures.

Essential oils of the botanically related members of the *Labiatae* family have a number of constituents in common (Rhyu, 1979). The oils of oregano and thyme both contain carvacrol and thymol as major constituents. Antimicrobial activity for both compounds has been reported (Katayama and Nagai, 1960; Kellner and Kober, 1955). Other constituents of the essential oils of the four herbs studied (such as p-cymene, 1,8-cineole, d-linalool, thujone, and α -terpineol) have been reported to exhibit antimicrobial activity (Kellner and Kober, 1955).

Although the composition of oregano and thyme oils may be similar, the inhibitory activity of oregano observed in the present work was much greater than that of thyme. This difference may be due to the essential oil content of the two herbs. According to Shankaracharya and Natarajan (1971), good commercial samples of the herbs should have the following essential oil content: oregano 4% (minimum), rosemary 0.3–2.0%, sage 1.5–3.0%, and thyme 1.5–2.5%.

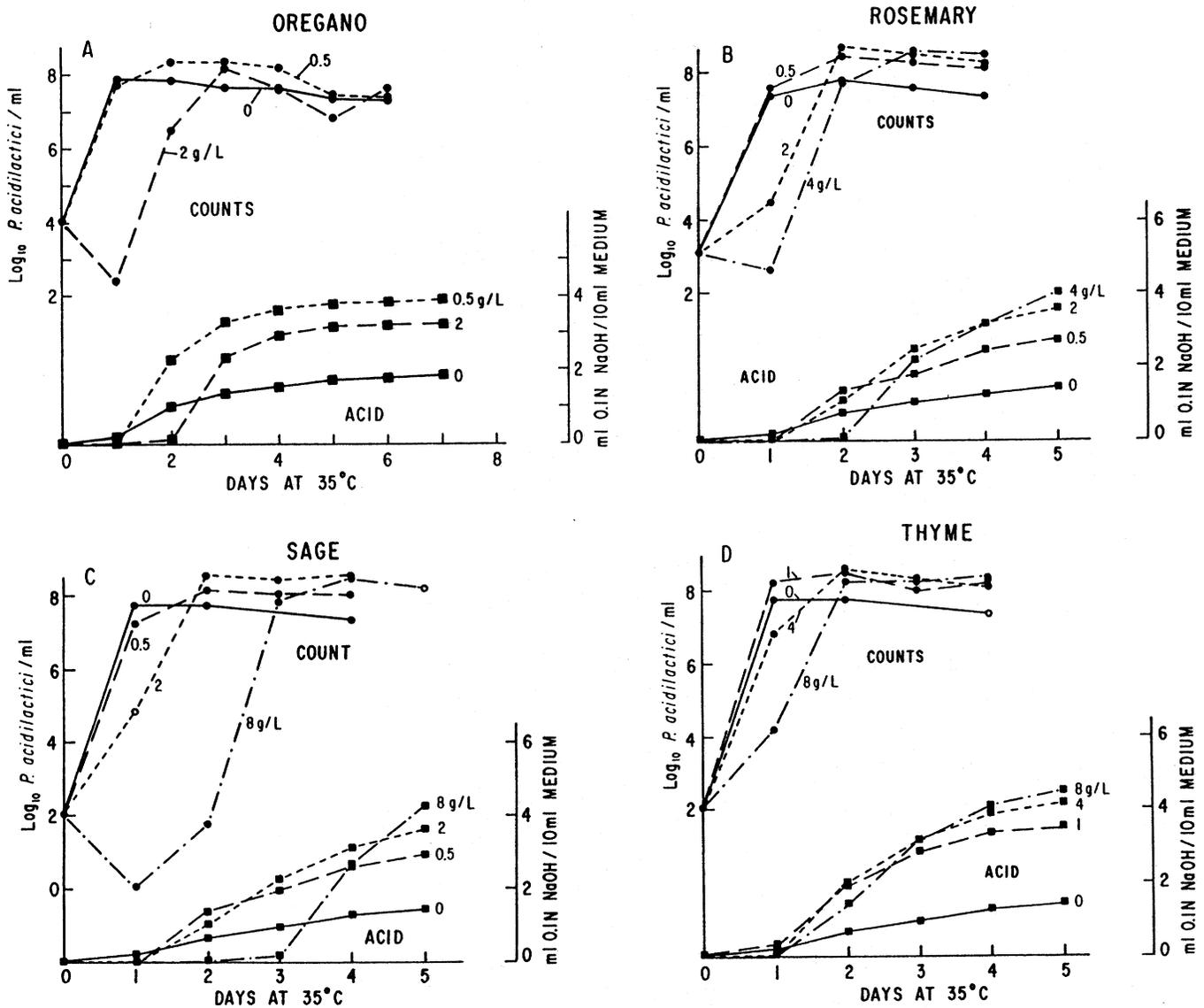


Fig. 2—Effect of herbs on growth and acid production by *P. acidilactici*: oregano (A), rosemary (B), sage (C), thyme (D).

Adaptation to herbs

A sequential subculturing scheme (Fig. 3) was used to test for adaptation of the starter bacteria to the toxic effects of the herbs. When *L. plantarum* was inoculated into media containing sublethal concentrations of oregano (3g/liter) or thyme (8g/liter), growth and acid production

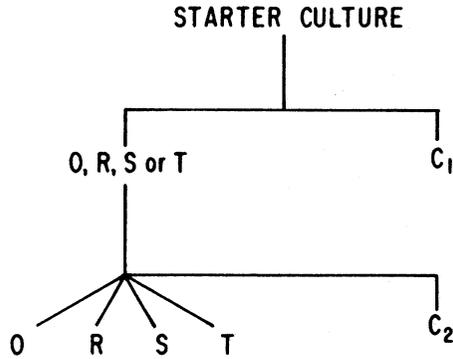


Fig. 3—Sequential subculturing of starter culture bacteria exposed to herbs. C_1 , C_2 = control medium; O, R, S, T = medium containing oregano, rosemary, sage, and thyme, respectively.

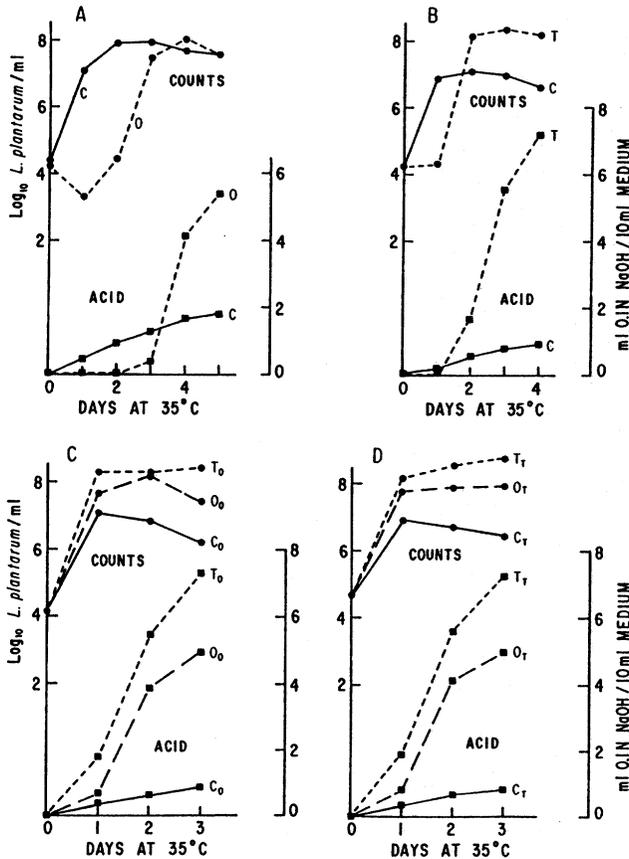


Fig. 4—Growth and acid production by *L. plantarum*: (A) unadapted cells cultured in control medium, C, and in medium containing 3g/liter oregano, O. (B) unadapted cells cultured in control medium, C, and in medium containing 8g/liter thyme, T. (C) adapted cells subcultured from O after 3 days; C_0 = control medium; O_0 = medium containing 3g/liter oregano; T_0 = medium containing 8g/liter thyme. (D) adapted cells subcultured from T after 2 days; C_T = control medium; O_T = medium containing 3g/liter oregano; T_T = medium containing 8g/liter thyme.

(Fig. 4A, B) were delayed in a manner as previously noted. After the bacterial population in the medium containing oregano or thyme increased to 10^8 cells/ml, cells were transferred to fresh media containing the same concentrations of the herbs (Fig. 4C, D). In this case, no inhibition of growth due to the presence of the herbs was evident. In fact, the herbs appeared to stimulate growth, possibly due to additional nutrients or cofactors supplied by the herbs. Cells grown in the presence of one herb acquired resistance to the other. Similar experiments with *P. acidilactici* indicate that this organism can also acquire resistance to the toxic effects of oregano, rosemary, sage, and thyme when exposed to sublethal concentrations of these herbs. For example, in the presence of 8g/liter rosemary or 8g/liter sage, bacterial counts decreased considerably before growth began (Fig. 5A). After 3 days of incubation, when the bacterial population reached 10^8 cells/ml, bacteria from the rosemary-containing medium were subcultured into fresh media containing the same concentrations of rosemary and sage. As shown in Fig. 5B, *P. acidilactici* acquired resistance to inhibition not only to rosemary, but to sage also.

Additional experiments using adaptation and challenge combinations of oregano, rosemary, sage, and thyme indicated that both starter cultures can acquire multiple resistance to the inhibitory effects of various herbs. In all cases, acid production by the adapted cultures was strongly stimulated by the presence of an herb. Adaptation of the bacteria also allowed them to tolerate herb levels normally bactericidal. For example, *L. plantarum*, previously grown in the presence of 3g/liter oregano, was able to survive, grow, and produce acid when subcultured into a medium containing 8g/liter oregano, a concentration normally bactericidal (Fig. 6).

When the starter cultures were incubated in media containing various concentrations of oregano essential oil, little if any inhibition of growth and acid production was noted in the presence of 40 ppm oregano oil, while levels >200 ppm were bactericidal to both organisms. As in the case of the herb, bacteria exposed to sublethal concentrations of oregano oil were able to overcome the inhibition and to develop resistance to the toxic effect of oregano oil or oregano. It should be noted that the essential oil did not enhance acid production by the bacteria.

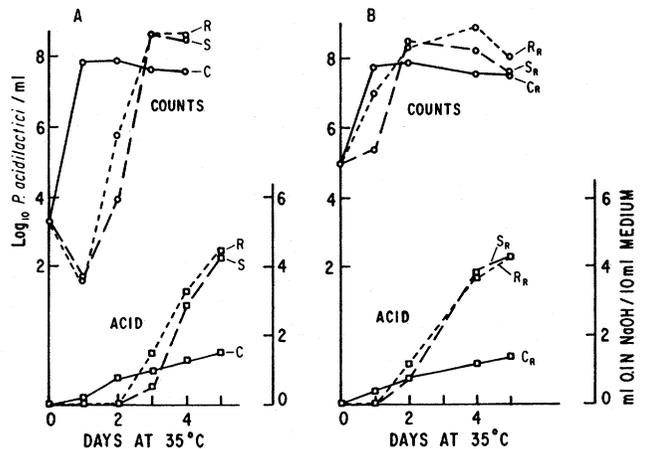


Fig. 5—Growth and acid production by *P. acidilactici*: (A) unadapted cells cultured in control medium, C, in medium containing 8g/liter rosemary, R, and in medium containing 8g/liter sage, S. (B) adapted cells subcultured from R after 3 days; C_R = control medium; R_R = medium containing 8g/liter rosemary; S_R = medium containing 8g/liter sage.

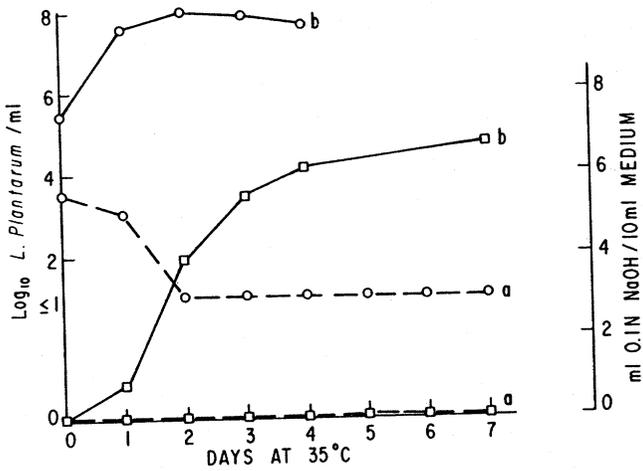


Fig. 6—Growth and acid production by *L. plantarum* in media containing 8g/liter oregano: (a) before adaptation, (b) after adaptation, subcultured from medium containing 3g/liter oregano after 3 days. ○ = bacterial count; □ = titratable acidity.

The possibility was considered that adaptation may be the result of recovery of the starter organisms from injury due to frozen storage. A comparison was made of inocula prepared from the thawed commercial starter with and without a preliminary culturing in the liquid medium. The results for *P. acidilactici* inoculated into control media and media containing 3g/liter oregano, shown in Fig. 7, were similar for both types of inoculum. In both cases, growth and acid production were delayed to a similar extent in the presence of oregano. This and other experiments indicated that injury of the starter bacteria due to freezing was not an important factor in their adaptation to herbs.

The mechanism by which the starter cultures acquire resistance awaits future research, as does the mechanism of their inhibition by spices and their components. The possibility that other food-borne microorganisms could respond to the inhibitory effect of spices in a similar way should be examined.

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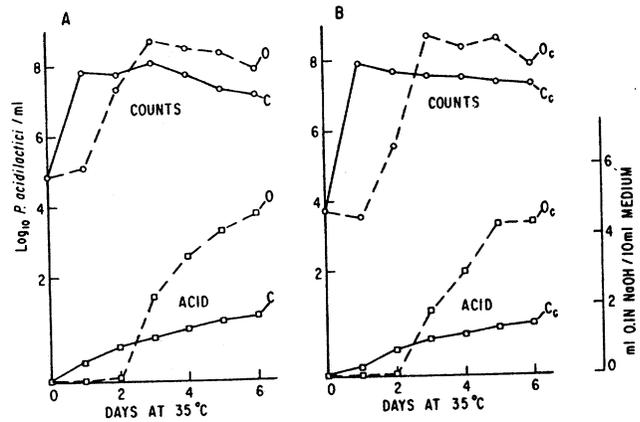


Fig. 7—Growth and acid production by *P. acidilactici*: (A) inoculum from thawed commercial culture. C = control medium; O = medium containing 3g/liter oregano. (B) inoculum from broth medium; C, after 3 days at 35°C. C_C = control medium; O_C = medium containing 3g/liter oregano.