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**Original**

# Growth Inhibition of Putrefactive Lactic Acid Bacteria, *Leuconostoc* sp. by Histidine

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**In order to devise a useful method to detect the putrefactive lactic acid bacteria (LAB hereafter) *Leuconostoc* sp., ten amino acids of high solubility were tested for their effects to inhibit the growth of *Leuconostoc mesenteroides*. Against *L. mesenteroides* subsp. *mesenteroides* IFO3426 (hereafter referred to as MES) the most effective amino acid was histidine, followed by glycine, serine and valine in that order. Histidine, glycine and serine were similarly most effective against *L. mesenteroides* subsp. *dexranicum* IFO 3349 (hereafter referred to as DEX). Histidine was regarded as one of the most effective amino acids to inhibit the growth of both strains. It inhibited MES at a concentration of 7 % (w/v) and DEX at 5 % (w/v). It was inhibitory between 15 and 30℃ and between pH 7-9, and most inhibitory at pH 9. Of the 32 strains of *Leuconostoc* species other than *Leuconostoc oenos* examined in this study, 31 strains were inhibited with histidine with the exception being *Leuconostoc mesenteroides* subsp. *dextranicum* AHU 1680. Histidines did not inhibit the growth of putrefactive LAB other than *L. mesenteroides* and *Leuconostoc lactis*.**

*Key words* : Putrefactive lactic acid bacteria/*Leuconostoc* sp. /Growth inhibition/Histidine/Bacterial detection.

## INTRODUCTION

*Leuconostoc mesenteroides*, a hetero-type lactic acid bacteria, is well-known as a producer of ropy dextran slime. It is a putrefactive bacteria which causes acidic putrefaction and swelling, and produces ropy slime on fresh meat and meat products. However, putrefactive lactic acid bacteria (LAB hereafter) samples isolated from many foods and food products have often mistakenly been identified as *Lactobacillus* sp. (Morishita et al., 1985a, 1985b). There have been only a few papers on putrefactive *Leuconostoc* sp. (Makela et al., 1992; Morishita et al., 1985a, 1985b) although ropy slime production on fresh meat and meat products has often been observed. Therefore it is considered important to inhibit the growth of putrefactive *Leuconostoc* sp. on foods (Gardner, 1982). However, few such studies have been developed, because it is difficult to identify putrefactive *Leuconostoc* sp. and to distinguish them from *Lactobacillus* sp. according to their characteristics as given in Bergey's Manual of Systematic Bacteriology (Garvie, 1986).

On the other hand, there has been a paper from Komagata et al. (1968) which reported that lysozyme

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and some amino acids like glycine and alanine inhibited the growth of aerobic microbes; however, the effect of amino acids on putrefactive *Leuconostoc* sp. was not investigated. The present investigation was undertaken to study the effect of amino acids on LAB growth, and we found that the strains of *Leuconostoc mesenteroides* were inhibited specifically by histidine.

This paper reports the growth inhibition by histidine was widely seen among *L. mesenteroides* and *Leuconostoc lactis* strains. Furthermore, it discusses the conditions for inhibition of the growth of *L. mesenteroides* and reports that histidine did not have similar effects on other putrefactive LAB.

## MATERIALS AND METHODS

### Microorganisms

The strains used were obtained from Japanese Type Culture Collections of microorganisms and from the American Type Culture Collection. All cultures were maintained on Briggs agar (Briggs, 1953) and inoculated on a new batch of Briggs agar before the experiments.

### Medium

The following medium called GYP medium was used: Glucose 0.5 %(w/v), yeast extracts (Difco) 0.5 % (w/v), polypeptone (Nihon Pharmaceutical Co.) 0.5 % (w/v), $MgSO_4 \cdot 7H_2O$ 0.1 %(v/w). The pH of the medium was adjusted to 7.0, and followed by sterilization by Nalgen Type S Sterilization Filter Units (0.2 $\mu$m pore size, Curtin Matherson Scientific, Inc.).

### Cultivation

LAB at late log phase in 0.1 ml GYP medium was inoculated in another 10 ml of GYP medium containing 0 to 7 % (w/v) amino acids in the sterilized test tubes. The growth was measured by optical density at 660 nm ($OD_{660}$) on the spectrophotometer (Shimadzu Corporation). It attained a stationary phase at 30℃ after 30 h incubation.

### Measurement of the degree of growth inhibition

The late log phase cells of an 18-h culture were employed for experiments. The degree of growth inhibition was estimated from the ratio of decrease in the growth with the addition of 1 to 7 % (w/v) amino acids compared to the growth of the control without amino acids. The following amino acids with high solubility were used: L-Alanine, L-argininеHCl, glycine, L-histidineHCl, L-lysineHCl, L-proline, L-serine, sodium L-glutamate, L-threonine, and L-valine. Growth inhibition was defined to occur when there was 20 % less growth than in the control culture, or when the $OD_{660}$ did not reach 0.1, after the addition of amino acids and/or egg white lysozyme.

### Reagents

L-Alanine, L-argininеHCl, sodium L-glutamate, L-serine and L-threonine were presented to us from Ajinomoto Co., Inc. Lysozyme was obtained from Eisai Co., Ltd. Other reagents used were special grades.

## RESULTS AND DISCUSSION

### Inhibitory effects of amino acids on the growth of *L. mesenteroides*

Table 1 shows the effects of ten amino acids with high solubility on the inhibition of growth of two LAB, *L. mesenteroides* subsp. *mesenteroides* IFO3426 (MES hereafter) and *L. mesenteroides* subsp. *dexranicum* IFO 3349 (DEX hereafter). The amino acid with the greatest inhibitory effect on MES was histidine, and efficacy was shown in the order of histidine, glycine, serine and valine. On the other hand, histidine, glycine and serine showed the most inhibitory effects on DEX. Histidine was regarded as one of the most effective amino acids in inhibiting the growth of both strains.

**TABLE 1**. Effects of amino acids on the growth inhibition of *L. mesenteroides* subsp. *mesenteroides* IFO 3426 (MES) and *L. mesenteroides* subsp. *dextranicum* IFO3349 (DEX).

| Amino acid | Strain | |
|---|---|---|
| | MES | DEX |
| Alanine | ++ | ++ |
| Arginine | − | − |
| Glutamic acid | − | + |
| Glycine | +++ | +++ |
| Histidine | +++ | +++ |
| Lysine | ++ | ++ |
| Proline | + | − |
| Serine | +++ | +++ |
| Threonine | + | + |
| Valine | +++ | ++ |

The concentration of amino acids were 7 %(w/v). The degree of inhibition: 80 or more than 80 %, +++; 60-79 %, ++; 40-59 %, ++; 20-39 %, +; 0-19 %, −.

### Effect of cultural conditions on the inhibitory actions of histidine

The effect of the cultural conditions on the growth inhibition of MES and DEX by histidine was investigated. A single addition of 1 % (w/v) histidine

stimulated the growth of MES and DEX. However the growth of both strains was inhibited as the concentration of histidine increased above 1 % (w/v). MES was inhibited at 7 % (w/v) histidine and DEX at 5 % (w/v) (Fig. 1). The data show histidine was metabolized at low concentrations, but it acted inhibitorily against the microorganisms at higher concentrations.

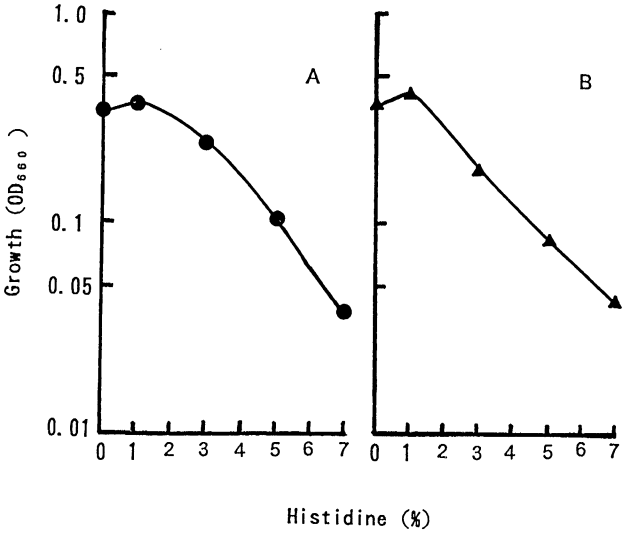


**FIG. 1**. Effect of histidine on the growth inhibition of *L. mesenteroides* subsp. *mesenteroides* IFO 3426 (MES) and *L. mesenteroides* subsp. *dextranicum* IFO3349 (DEX). Figs. A and B show the respective growth of MES and DEX.

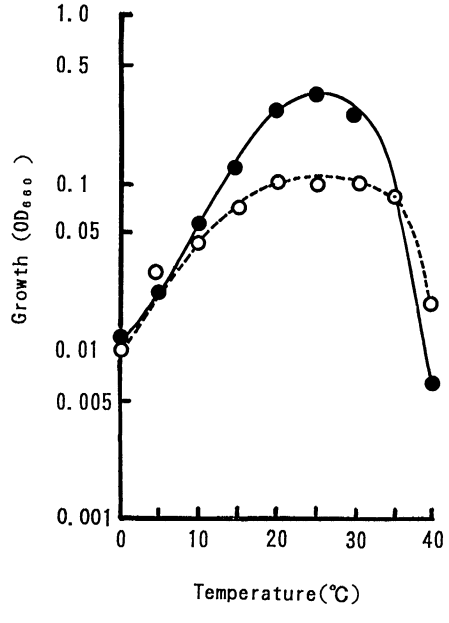


**FIG. 2**. Effect of temperature on the growth inhibition of *L. mesenteroides* subsp. *mesenteroides* IFO 3426 (MES). Symbols: ●, Growth without histidine; ○, Growth with 5 % (w/v) histidine.

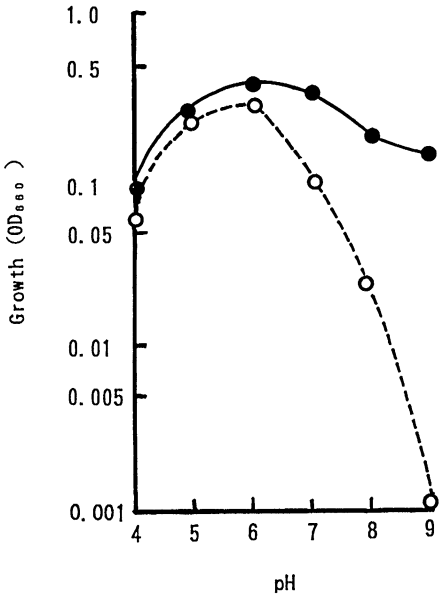


**FIG. 3**. Effect of pH on the growth inhibition of *L. mesenteroides* subsp. *mesenteroides* IFO3426 (MES). Symbols: ●, Growth without histidine; ○, Growth with 5 % (w/v) histidine.

As shown in Fig. 2, histidine was most effective between 15 and 30℃ against MES. No effect was observed beyond the above temperature range.

The growth of MES was not influenced between pHs 4 and 6, but as pH was raised from 7 to 9, the inhibitory effect increased and the growth at pH 9 with the addition of histidine was only 6.6 % that of the control growth (Fig. 3).

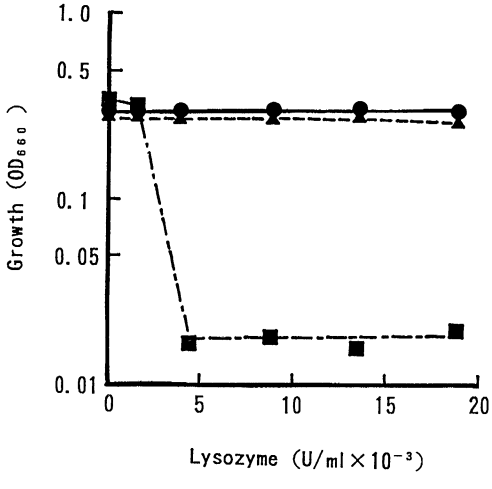


**FIG. 4**. Effect of lysozyme on the growth inhibition of *L. mesenteroides* subsp. *mesenteroides* IFO3426 (MES), *L. mesenteroides* subsp. *dextranicum* IFO3349 (DEX) and *Lactobacillus plantarum* ATCC 8014 (PLA). Symbols: ●, MES; ▲, DEX; ■, PLA.

### Effect of histidine and egg white lysozyme on growth of MES and DEX

Lysozyme is a well-known enzyme which lyses bacterial cell walls, especially in case of Gram-positive bacteria (Salton and Pavlik, 1960). Lysozyme-sensitive *Lactobacillus plantarum* ATCC8014 (hereafter referred to as PLA) was used as an experimental control, and the growth inhibition of MES and DEX was investigated. Figure 4 shows that 0.004 U/ml lysozyme was enough to inhibit the growth of PLA, but the enzyme was less effective on MES and DEX than PLA, even when the concentration employed was as high as 0.18 U/ml. The results suggested that the growth inhibition with lysozyme might widely differ for various species of LAB, and PLA was especially sensitive to the enzyme.

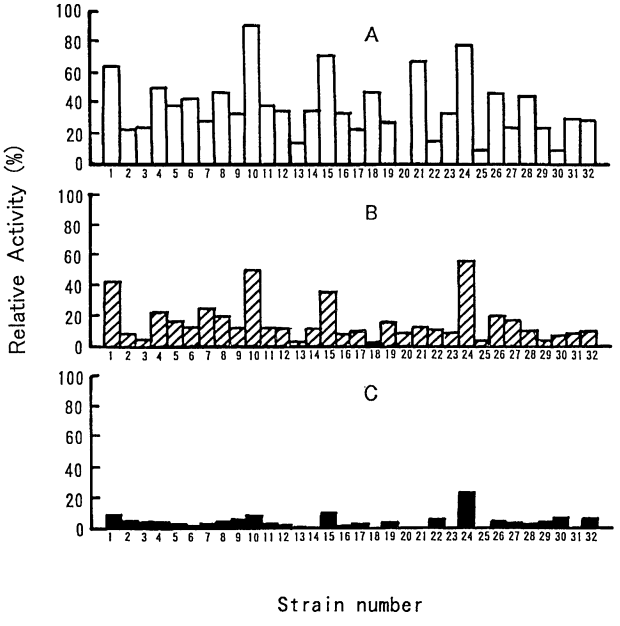


**FIG. 5**. Effect of histidine on the growth inhibition of various strains of *Leuconostoc* sp. A, B and C show the growth of *Leuconostoc* sp. with 3, 5, 7 % (w/v) histidine, respectively. The numbers of the abscissae show the strains of *Leuconostoc* sp. as follows: Numbers 1 to 17 are *L. mesenteroides* subsp. *mesenteroides*; 1, AHU1068; 2, AHU1656; 3, ATCC10830a; 4, ATCC10877; 5, ATCC10878; 6, ATCC 10879; 7, ATCC 10880; 8, ATCC 10883; 9, ATCC11449; 10, ATCC12291; 11, ATCC14430; 12, ATCC14935; 13, ATCC23386; 14, ATCC27258; 15, IFO3426(MES); 16, IFO3832; 17, IFO1060. Numbers 18 and 19 are *L. mesenteroides* subsp. *cremoris*. 18, IAM 1087; 19, NRIC1538. Numbers 20 to 22 are *Leuconostoc lactis*. 20, ATCC15520; 21, ATCC 19256; 22, IFO 12455. Numbers 23 to 30 are *L. mesenteroides* subsp. *dextranicum*. 23, AHU1076; 24, AHU1680; 25, ATCC17071; 26, ATCC17072; 27, IAM1122; 28, IFO3349 (DEX); 29, IAM10076; 30, NRIC1539. Numbers 31 and 32 are *Leuconostoc mesenteroides* var. *sake*. 31, IAM10060; 32, IAM10069.

### Growth inhibition of *L. mesenteroides* by histidine

Thirtytwo strains of *Leuconostoc* species other than *Leuconostoc oenos* were collected from the Japanese and American type culture collections to investigate the sensitivity of histidine. According to Fig. 5 twentyfive out of the 32 strains were inhibited with the addition of 5 % histidine, while thirtyone strains were inhibited with the addition of 7 % (w/v) histidine. There was an exception of *L. mesenteroides* subsp. *dextranicum* AHU1680 (no. 24 in Fig. 5), in which case more than 80 % the growth was not inhibited with the addition of 5 % to 7 % (w/v) histidine. The results showed that the inhibition by histidine was a common characteristic among *L. mesenteroides* and *Leuconostoc lactis*.

### Effect of histidine and lysozyme on various LABs

Effects of histidine and lysozyme on the inhibition of the growth of putrefactive LAB were investigated. According to Fig. 6, a single addition of egg white lysozyme inhibited the growth of five strains other than PLA, such as *Lactobacillus sake* IAM10077, *Pediococcus botuliacidofaciens* AHU 1430,

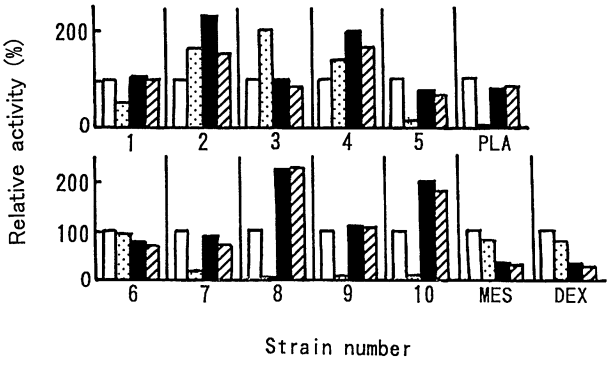


**FIG. 6**. Effects of lysozyme and histidine on the inhibition of growth of various putrifactive LAB. Symbols show: open bar, growth in GYP medium alone; dotted bar, growth with 0.23U /ml lysozyme; closed bar, 5 % histidine; shaded bar, 0.23U /ml lysozyme and 5 % histidine. The numbers of the abscissae show the strains as follows: Number 1, *Lactobacillus acidophilus* AHU1042; No. 2, *Lactobacillus brevis* ATCC4006; No. 3, *Lactobacillus casei* AHU1896; No. 4, *Lactobacillus delbrueckii* subsp. *lactis* IAM1173; No. 5, *Lactobacillus sake* IAM10077; No. 6, *Pediococcus acidilactici* AHU1670, No.7, *Pediococcus botuliacidofaciens* AHU1430; No. 8, *Streptococcus faecalis* AHU1112, No. 9, *Streptococcus lactis* AHU1089; No. 10, *Streptococcus thermophilus* AHU1661; PLA, *Lactobacillus plantarum* ATCC8014; MES, *Leuconostoc mesenteroides* subsp. *mesenteroides* IFO3426; DEX, *Leuconostoc mesenteroides* subsp. *mesenteroides* IFO3349.

*Enterococcus faecalis* AHU1112, *Lactococcus lactis* AHU1089, *Streptococcus thermophilus* AHU1661. However, no LAB strains tested were inhibited by the single addition of histidine except MES and DEX. The combination of egg white lysozyme with histidine showed an additive effect on the inhibition of those five strains.

The effect of histidine on the growth inhibition of *Leuconostoc* sp. was observed to be so specific and characteristic that a histidine inhibition test could be recommended as an useful screening method for *L. mesenteroides* subsp. *mesenteroides, L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris, L. mesenteroides* var. *sake* and *Leuconostoc lactis*, with the exception of *L. mesenteroides* subsp. *dextranicum* AHU1680. According to fluorophotometric examination (Endo, 1983), both strains of MES and DEX did not produce histamine (unpublished data). Therefore histidine inhibition of *Leuconostoc* sp. growth may occur because of some physical or chemical effect. It seems to act like a cationic surfactant, since histidine inhibited the growth of both strains at alkaline pH.

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