Inhibitory Effect of Lactobacillus plantarum on Salmonella infantis, Enterobacter aerogenes and Escherichia coli during Tempeh Fermentation

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ABSTRACT

Growth and inhibition of Salmonella infantis, Enterobacter aerogenes and Escherichia coli in fermenting soybeans during tempeh production were studied in presence and absence of Lactobacillus plantarum. In fermenting unacidified soybeans S. infantis grew by 7 log units in 40 h. E. coli and E. aerogenes grew by 6 and 7 log units respectively. A similar pattern of growth of the three test organisms in fermenting acidified beans was also noted. Further inoculation of unacidified cooked beans with L. plantarum resulted in a complete inhibition of the test organisms in the product. On acidified cooked beans a lower level of L. plantarum inoculum (10^2/g) was enough to show a complete inhibitory effect. The lowering of the pH in fermenting beans by L. plantarum might have played a role in the destruction of the test organisms.

Tempeh, which usually means a food product resulting from the fermentation of cooked soybeans by a Rhizopus species, has its origin in Indonesia (4). It has gained increasing popularity in the United States (18) and is available in the vegetarian market in The Netherlands as a meat-substitute (12). Due to its high protein content, there are suggestions that it could be introduced as a source of low-cost protein food in countries with a protein shortage (2).

Despite the large number of papers published on tempeh, data concerning its microbiology are scarce. Spore formers (16) and aerobic bacteria in general (9) were reported to be the microflora of tempeh and usar respectively. Recently, detailed studies were conducted on the microbiological safety of tempeh (18) and the microbiological quality of commercial tempeh (12).

Tempeh is a very nutritious medium for microorganisms and its production is susceptible to introduction of undesirable contaminants, pathogens and spoilage microorganisms. The lactic acid bacteria have been used by man in food and dairy fermentations to improve flavor and palatability, to preserve foods and to suppress many undesirable microorganisms. There are reports on inhibition of pathogens and undesirable microorganisms by lactic acid bacteria in milk (1,7), in skim milk (4,10,11) and in fermented meat products (5,13,17). This study was therefore undertaken to determine the inhibitory effect of Lactobacillus spp. on some Gram-negative rods during the fermentation of tempeh.

MATERIALS AND METHODS

 Cultures

The following bacterial cultures were used in this study. Salmonella infantis (S-9), Escherichia coli (WS 1323), Enterobacter aerogenes (WS 1292) and Lactobacillus plantarum (WS 1033), were obtained from the culture collection of Bakteriologisches Institut, S.V.F.A., Weihenstephan. Rhizopus oligosporus (CBS 338.62) was obtained from Centraalbureau voor Schimmelcultures, The Netherlands.

Methods of enumeration

 Cultures of S. infantis, E. coli and E. aerogenes were grown overnight at 37°C in nutrient broth and further diluted to give 3 x 10^9/ml. L. plantarum was also grown overnight at 37°C in MRS broth and diluted to give 3 x 10^9/ml. XLD agar plates were used to enumerate salmonella cultures. E. coli and E. aerogenes were enumerated on VRB agar plates and L. plantarum on MRS agar plates. Rhizopus oligosporus spores were harvested with 5 ml sterile water from potato dextrose agar slants after incubation at 30°C for 5 d. About 1.2 x 10^9 spores/ml of R. oligosporus were counted with a Thoma counting chamber. Viable R. oligosporus spores were not enumerated.

Preparation of tempeh

Tempeh was prepared following the methods of Steinkraus et al (14) with some modifications. For every experiment 200 g of hull-free, whole, dry soybeans were thoroughly washed with hot water and allowed to soak in 600 ml of water at 30°C for 24 h. an equal amount of soybeans was processed in a similar way except that 1.5 ml of glacial acetic acid was added to the soak water and soaking was done at room temperature for 24 h. The beans were then cooked in soak water at 100°C for 1 h., drained while still hot, spread on a layer of sterile cloth and covered with another layer of sterile cloth, thus giving them a fairly dry surface. The beans were allowed to cool to about 37°C and mixed with spores of R. oligosporus to get final inoculation level of ca 1.2 x 10^9/100 g cooked beans. Plastic petri dishes (100 x 15 mm, Greiner Labortech-
nik, W. Germany) were packed with about 60 g of inoculated beans and incubated at 30°C. It required about 30 h until the beans were tightly bound together by white mycelia.

**Inoculation of cooked beans with test organisms**

After inoculation with *R. oligosporus* spores, 400 g cooked beans were mixed with *S. infantis* suspensions to get a final inoculum level of ca. \(3 \times 10^2\)/g. The *Salmonella* inoculated beans were then divided into 200 g portions and one portion was further mixed with *L. plantarum* suspensions to obtain a final concentration of \(3 \times 10^2\)/g unacidified cooked beans or \(3 \times 10^6\)/g of acidified cooked beans. The other portion served as a *Lactobacillus*-free control. The same procedure was followed for *E. coli* and *E. aerogenes*. Inoculated beans were further sampled to check the inoculum level by plate counting as described below. The beans were incubated in petri dishes at 30°C for 2 d.

**Analysis of samples**

Ten grams of fermenting beans were sampled from petri dishes at intervals and blended for about 2 min in 90 ml of sterile water using a stomacher lab blender (Model 400, Seward UAC, London). A volume of 1 ml of the blended sample was poured plated with XLD, VRB and MRS agars. After further dilution in sterile water, volumes of 0.1 ml of appropriate dilutions were spread on predried surfaces of XLD, VRB and MRS agar plates. XLD and VRB plates were incubated at 37°C for 24 h and MRS plates were anaerobically incubated at 37°C for 48 h. Samples were plated in duplicates and results are average values.

**Determination of pH**

The pH of samples was measured by placing the electrode of the pH meter into a slurry containing 1/5 dilution of samples in sterile water.

**RESULTS**

Growth curves of *S. infantis* during fermentation of tempeh made from soybeans soaked in unacidified water with or without *L. plantarum* are shown in Fig. 1. *S. infantis* increased steadily in number by 7 log units in 40 h when allowed to grow in the absence of *L. plantarum*. Inoculation with *L. plantarum* resulted in a decrease of *S. infantis* count in the first 8 h. After 16 h, no *Salmonella* were detected in 1 g of material. In this time, *L. plantarum* grew by 1 log unit and the pH fell to 4.4. However, active mycelial growth in fermenting beans after 24 h resulted in rise of pH.

The growth of *E. coli* and *E. aerogenes* on unacidified soybeans is shown in Table 1. Both grew by 5 log units or more in 40 h. In associative inoculation with *L. plantarum* (Table 2), the growth of the test organisms was completely inhibited within 16 h. On the other hand, *L. plantarum* grew by about 1 log unit and the pH dropped to 4.4.

**TABLE 1. Growth of *E. aerogenes* and *E. coli* on fermenting unacidified soybeans.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th><em>E. aerogenes</em> cfu/g</th>
<th><em>E. coli</em> cfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.9</td>
<td>(2 \times 10^2)</td>
<td>(2 \times 10^2)</td>
</tr>
<tr>
<td>16</td>
<td>5.1</td>
<td>(2.5 \times 10^4)</td>
<td>(5.1 \times 10^2)</td>
</tr>
<tr>
<td>40</td>
<td>6.7</td>
<td>(2.7 \times 10^6)</td>
<td>(6.4 \times 10^6)</td>
</tr>
</tbody>
</table>

Table 3 shows the growth of the three test organisms on acidified soybeans. In 40 h fermentation time the number of each test organism increased by 7 log units or more. A constant rise in pH was observed during the fermentation. Inoculation of acidified beans with a small number of *L. plantarum* resulted in complete inhibition of the test organisms (Table 4). In 18 h the pH decreased by 1.2 units, *E. coli* was completely inhibited and the number of *S. infantis* and *E. aerogenes* decreased drastically. At the 40th h., no test organism was recovered.

Hull-free soybeans soaked in unacidified water at 30°C for 24 h had lower pH values (4.8-5.0) after cooking than those soaked in acidified water for 24 h (pH 5.8). A vigorous growth of microorganisms and a sharp drop in pH was observed in unacidified soak water in another experiment (unpublished data). The involvement of *L. plantarum* in the fermentation process did not markedly affect the odor of fresh tempeh or the taste of fat-fried tempeh.
**TABLE 2. Inhibition of E. aerogenes and E. coli by L. plantarum during fermentation of acidified soybeans.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
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<tr>
<td>0</td>
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<td>2x10^4</td>
<td>2x10^6</td>
<td>4.8</td>
<td>2x10^2</td>
<td>2x10^6</td>
</tr>
<tr>
<td>16</td>
<td>4.4</td>
<td>&lt;10</td>
<td>5.4x10^7</td>
<td>4.5</td>
<td>&lt;10</td>
<td>3x10^7</td>
</tr>
<tr>
<td>40</td>
<td>6.7</td>
<td>&lt;10</td>
<td>7x10^9</td>
<td>6.2</td>
<td>&lt;10</td>
<td>5.4x10^6</td>
</tr>
</tbody>
</table>

**TABLE 3. Growth of S. infantis, E. aerogenes and E. coli in fermenting acidified soybeans.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.8</td>
<td>2x10^2</td>
<td>2x10^6</td>
<td>3x10^2</td>
<td>5.8</td>
<td>3x10^2</td>
</tr>
<tr>
<td>16</td>
<td>6.1</td>
<td>5.6x10^8</td>
<td>6.1</td>
<td>7.4x10^4</td>
<td>6.1</td>
<td>3.3x10^3</td>
</tr>
<tr>
<td>40</td>
<td>6.7</td>
<td>1.5x10^9</td>
<td>6.7</td>
<td>1.1x10^9</td>
<td>6.7</td>
<td>5.1x10^8</td>
</tr>
</tbody>
</table>

**TABLE 4. Inhibition of S. infantis, E. aerogenes and E. coli by L. plantarum during fermentation of acidified beans.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
<th>pH</th>
<th>cfu/g</th>
<th>Lb. (cfu/g)</th>
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<td>7x10^6</td>
<td>5.8</td>
<td>3x10^2</td>
<td>7x10^6</td>
</tr>
<tr>
<td>16</td>
<td>4.6</td>
<td>2.2x10^9</td>
<td>4.6</td>
<td>2x10</td>
<td>2x10^9</td>
<td>4.6</td>
</tr>
<tr>
<td>40</td>
<td>5.8</td>
<td>&lt;10</td>
<td>1.3x10^10</td>
<td>&lt;10</td>
<td>6.5x10^6</td>
<td>6.1</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Although tempeh is a product with a high nutritive quality that can support the growth of most bacteria, there is little information on its microbiology. Steinkraus et al (14) indicated the presence of various amounts of bacterial contaminants and spoilage flora in tempeh from unacidified soybeans. Samson et al (12) reported high loads of Enterobacteriaceae, yeasts and lactic acid bacteria. The presence of S. aureus, B. cereus and E. coli was also shown. A high percentage of their samples had total aerobic count of more than 10^9/g. In evaluating the microflora of some commercial tempeh samples from München and its surroundings in our laboratory, high counts of total aerobic bacteria (ca 5 x 10^8/g), Enterobacteriaceae (ca 2 x 10^9/g), enterococci (ca 2 x 10^8/g) and S. aureus (ca 1.1 x 10^7/g) were observed.

The importance of a study of the danger to the consumer which a contaminated tempeh represents was clearly pointed out by Wood (20). Tanaka et al (18) demonstrated that Salmonella and other pathogens could grow on tempeh from unacidified soybeans by about 8 log units in 2 d. A similar trend was observed in this study with our test organisms. In contrast to the belief that acidification of soybeans during soaking inhibits growth of undesirable microorganisms, all our test organisms grew substantially on acidified beans. The pH of acidified beans was about 5.8 and this was not low enough to inhibit growth of the test organisms. Therefore, the contamination of acidified or unacidified soybeans by pathogens by tempeh makers can result in unsafe tempeh.

Based on these possible dangers, it was attempted, in this study, to make use of lactic acid bacteria to inhibit the growth of the test organisms. L. plantarum was found to be effective in inhibiting the growth and survival of the test organisms in tempeh from acidified or unacidified soybeans.

In associative culture of test organisms with *L. plantarum*, the pH decreased steadily in the first half of the fermentation. However, as the mycelia started to grow actively, the pH increased despite a further steady growth of *L. plantarum*. Active growth of mycelia was also accompanied by a sharp rise in temperature in the fermenting beans (ca 10°C above the incubation temperature). Dutta et al (3) reported that acid production by lactic acid bacteria in skim milk was nearly optimum at 32°C.

The growth patterns of *S. infantis* on acidified and unacidified beans were essentially alike. However, inoculation of acidified beans with *L. plantarum* at a level higher than 10^2/g resulted in very low pH and complete inhibition of mold growth. In such cases tempeh could not be produced. Acidification of soawater had no effect either on growth of *E. coli* and *E. aerogenes* or their inhibition by *L. plantarum*.

The inhibition of the test organisms by *L. plantarum* in this study seems to correlate the drop in pH. In other solid state fermentation studies on meat products (5,13,17), the acid conditions in the fermenting products produced by lactic acid bacteria were believed to contribute greatly to the destruction of pathogens during fermentation. Inhibition of salmonellae (10,19), Enterobacter spp. (11) and *E. coli* (4) in milk and milk products was also attributed to low pH and acid produced by lactic acid bacteria. However, other modes of inhibition are also possible. Hydrogen peroxide was reported to be partially responsible for inhibition of foodborne pathogens (8), and many strains of lactic acid bacteria produce a variety of antibiotics (6).

Various recommendations are forwarded to avoid the presence of food-borne pathogens and spoilage microorganisms from bioprocessed foods (6,7). Although it may be difficult to suggest that traditional procedures should be changed in areas where tempeh is traditionally consumed, the application of *L. plantarum* may be considered in commercial scale production of tempeh.
REFERENCES

starter cultures and food-borne pathogens. *Streptococcus diacetylactis*

Econ. Microbiol. 4:115-140.

of incubation temperature on acid and flavor production in milk by lactic

4. Frank, J. F., and E. H. Marth. 1977. Inhibition of Enteropathogenic *E. coli*
by homofermentative lactic acid bacteria in skim milk. J. Food Prot.
40:749-753.

the manufacture and storage of a fermented sausage product. J. Milk
Food Technol. 33:185-191.


cultures and food-borne pathogens: Lactic streptococci versus staphylo­

cillus acidophilus* towards intestinal and food borne pathogens in asso­


num* in skim milk during fermentation by lactic acid bacteria. J.
Milk Food Technol., 35:482-488.

in skim milk during fermentation by lactic acid bacteria. J. Food
Prot. 43:720-728.

quality of commercial tempeh in The Netherlands. J. Food Prot. 50:92-
94.

Survival of *Salmonella typhimurium* in Lebanon bologna. J. Milk Food

A pilot plant process for the production of dehydrated tempeh. Food
Technol., 19:63-98.

15. Steinkraus, K. H., Y. B. Han, J. B. Van Buren, M. I. Providenti and D.

during the fermentation and frying of tempeh. Ed. Chem. 3:165-170.

1980. Inhibition of botulinum toxin in bacon by acid development. J.
Food Prot. 43:450-457.

1985. Evaluation of the microbiological safety of tempeh made from
unacidified soybeans. J. Food Prot. 48:438-441.

murium* and *S. aureus* in cottage cheese whey. J. Milk Food Technol.
36:19-22.