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# Compartmentalization of lactic acid bacteria and yeasts in the fermentation of brined cucumbers*

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*Lactic acid bacteria were distributed between the brine and cucumbers in brine-fermented cucumbers. The percentage of bacterial cells located within the cucumbers, of the total cells produced, varied from* c. *8–51%, depending upon: (1) gas exchange treatment of the cucumbers before brining and (2) time of inoculation with* Lactobacillus plantarum *after brining. Oxygen exchange of the cucumbers before brining, and early inoculation of the brine, resulted in higher percentages of cells within the fermented cucumbers. Nitrogen exchange of the cucumbers before brining, and delay in inoculation of the brine for 2 days after brining resulted in lower percentages of cells within the cucumbers. No evidence was found to indicate that yeast cells were present within fermented cucumbers, even when the brines were inoculated with* Saccharomyces cerevisiae *and the cucumbers had been* $O_2$*-exchanged prior to brining. Yeasts, because of their greater size than bacteria, appear to be excluded from the cucumber interior.*

## Introduction

Maintenance of structural integrity of whole cucumbers during brine fermentation is highly important in regard to quality of the finished product for human consumption. Gaseous spoilage (bloater damage) can be a source of serious economic loss, and is related to $CO_2$ production by fermenting micro-organisms and the cucumbers during fermentation (Fleming et al. 1973b). The problem can be reduced by purging of $CO_2$ from the brine during fermentation (Costilow et al. 1977; Fleming et al. 1973a). A model for explaining the physical mechanism for bloater formation has been proposed (Fleming and Pharr 1980), and strengthened by further studies (Corey et al. 1983a, b). The model does not fully address the mechanism by which fermenting micro-organisms may be involved in bloater formation. Such an understanding may be necessary for obtaining more cost-effective alternatives to the current purging procedure employed by the pickle industry.

The microbial fermentation of brined cucumbers has been studied extensively in relation to types and numbers of micro-organisms that occur in the brine surrounding the cucumbers Yeasts (Etchells and Bell 1950), heterolactic

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acid bacteria (Etchells et al. 1968), homolactic acid bacteria (Fleming et al. 1973a; McFeeters et al. 1982), and *Enterobacteriaceae* (Etchells et al. 1945) occur during natural fermentation of cucumbers, and are sources of $CO_2$. Etchells et al. (1968) suggested that microbial fermentation occurs in the brine, with subsequent diffusion of fermentation gases into the cucumber, where it causes bloater formation. Samish et al. (1959), on the other hand, suggested that bloater damage was the result of microbial $CO_2$ production within the cucumber. They concluded that bacteria are present within fresh cucumbers in small numbers and multiply and produce $CO_2$ when the fruits are brined.

Daeschel and Fleming (1981) observed that lactic acid bacteria can enter and grow within cucumbers after they are brined. Exchange of the natural gases of cucumbers with oxygen just prior to brining significantly increased the entrance and subsequent numbers of the bacteria within the cucumbers. It was proposed that lactic acid bacteria entered brined cucumbers through stomata of the fruit epidermis and that movement into the fruit was due to a vacuum created within the fruit. Fleming et al. (1980) earlier postulated that the $O_2$ present in cucumbers is rapidly consumed, with accompanying production of $CO_2$, due to respiration when the fruit are immersed in brine. The $CO_2$, having a much greater solubility than the $O_2$ it replaced, is believed to dissolve in the tissue with a subsequent vacuum being formed. Corey et al. (1983a) confirmed that a partial vacuum occurs in oxygen-exchanged, brined cucumbers.

Objectives of the present study were to: (1) determine the effect of gas exchange and the time of inoculation of lactic acid bacteria on the final distribution of the bacteria between the brine and cucumber of completed fermentations; (2) determine the distribution of bacteria within various locations of the fermented fruit; and (3) test whether fermentative yeasts can enter and grow within brined cucumbers.

## Methods

### *Cucumbers*

Size No. 3 pickling cucumbers (3·8 to 5·1 cm in diameter) were obtained either from a commercial grower or from the North Carolina State University experimental farm. Only cucumbers free of disease and physical defects were used.

### *Gas exchange of cucumbers*

Cucumbers (1·9 kg) were washed, weighed and packed into 3·8 liter glass jars fitted with lids, gas inlets and brine reservoirs as described previously (Fleming et al. 1973a). Cucumbers were exposed to either $O_2$ or $N_2$ at a metered rate of 300 ml $min^{-1}$ for 1 h to exchange the internal atmosphere (Fleming et al. 1980). Nonexchanged (air) cucumbers served as controls.

### *Brining*

Brine composition and addition procedure were the same as previously described (Daeschel and Fleming 1981) except where indicated. Fermentation brines were purged with $N_2$ at a continuous flow rate of 5 ml $min^{-1}$ to prevent cucumber bloating (Fleming et al. 1975).

### *Micro-organisms, inoculation and enumeration*

Brines were inoculated with cells (log phase) of either *Lactobacillus plantarum* WSO or *Saccharomyces cerevisiae* Y-635 which had been grown in MRS broth (Difco Laboratories, Inc., Detroit, MI, USA) and YM broth (Difco Labs.), respectively. Cells were harvested by centrifugation (5900 × *g* for 10 min), washed twice with sterile, 0·85% saline and resuspended in saline. Plate counts of inocula, brines and cucumbers were with LBS agar (BBL Microbiology Systems, Cockeysville, MD, USA) for lactic acid bacteria and with acidified dextrose agar (5 ml of 10% w/v tartaric acid per 100 ml of dextrose agar, BBL) for yeasts. Counts were reported as CFU $g^{-1}$ or $ml^{-1}$. Fermented cucumbers were

rinsed thoroughly for 2 min under running tap water, aseptically transferred to sterile blender jars containing 200 ml of sterile water, and ground to a homogeneous slurry. Bacterial cells from the brines and cucumbers of completed fermentations were enumerated by direct microscopic count. Whole fermented cucumbers or designated areas thereof (Fig. 1) were blended to a homogeneous slurry and filtered through coarse filter paper (Reeve Angel 202, Whatman Laboratory Products Inc., Clifton, NJ) to remove the majority of gross particulate (>25 μm) matter. Brines were also filtered.

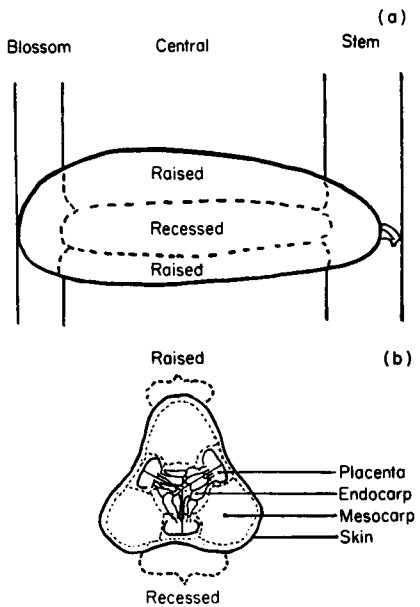


**Fig. 1.** Areas of the cucumber sampled for the enumeration of bacteria. Longitudinal (a) and cross-sectional (b) views of the cucumber are illustrated.

Samples were counted with a Petroff–Hauser counting chamber under a 40× phase objective, giving a total magnification of 600×. The counting procedure was standardized according to the protocol of Cassel (1965). A 95% confidence interval was used to determine the number of microscopic fields to be counted per sample. Reported values are means of duplicate counts of duplicate cucumbers. The effect of the filtration on retention of bacteria in cucumber slurry was determined by adding a known amount of cells (counted) to cucumber slurry and then counting the cells in the filtrate of that slurry. No significant differences in counts were observed. Control samples of whole fresh cucumbers and designated areas thereof were examined microscopically for bacteria. These samples also were enumerated by the total aerobic plate count using standard methods agar (BBL, Cockeysville, MD, USA). Bacterial pustules on the surface of brined cucumbers were enumerated visually under a stereomicroscope. Due to the limitations of accurately discerning pustules under the microscope, only pustules >0·3 mm in diameter were enumerated for comparative purposes. Pustules were measured with the aid of a comparator (Finescale Co., Orange, CA, USA).

### *Sugar determination*

Total reducing sugar in brine samples was quantitatively determined by the colorimetric method of Sumner and Somers (1944).

### *Statistical analysis*

Pustule enumeration and direct microscopic count data were analyzed with the Analysis of Variance and General Linear Model Programs of the Statistical Analysis of Variance and General Linear Model Programs of the Statistical Analysis System (SAS Institute 1979).

## Results and Discussion

Fresh cucumber samples contained less than $1 \times 10^6$ CFU $g^{-1}$ with a distribution gradient of cells getting progressively less numerous toward the middle of the cucumber fruit (Table 1). These values were obtained from the aerobic plate count. The relatively low numbers of bacteria in fresh cucumbers made enumeration by direct microscopic count impractical.

Lactic acid bacteria in completed fermentations were observed microscopically to be located in the brine, within the cucumbers, and upon the cucumber surface (as small colonies, i.e. pustules). The internal gas composition of the fresh cucumbers before brining had a significant effect on the distribution of bacterial cells between brine and cucumbers

**Table 1. Distribution of the naturally occurring microbial flora of the fresh cucumber fruit as determined by aerobic plate count.**

| Cucumber section | Sample[a] | Log CFU $g^{-1}$ |
|---|---|---|
| Whole | A | 5·70 |
| | B | 4·99 |
| Skin | A | 5·38 |
| | B | 5·75 |
| Mesocarp | A | 3·00 |
| | B | 3·36 |
| Endocarp | A | <1·00 |
| | B | <2·00 |

[a] Each sample consisted of a composite of four cucumbers.

as well as on the number of cells produced during fermentation (Table 2).

Cucumbers that were $O_2$-exchanged before brining and inoculated at the time of brining (Table 2, treatment No. 1) were observed to have a significantly higher proportion of cells within the cucumbers as compared with non-exchanged (treatment No. 2) or $N_2$-exchanged cucumbers (treatment No. 3). This confirms our previous observations (Daeschel and Fleming 1981) where it was shown that $O_2$-exchange greatly enhanced absorption of lactic acid bacteria into the brined cucumbers. Delay in inoculation of the brined cucumbers for 1 or 2 days resulted in a smaller proportion of cells being located within the fermented fruit as compared with inoculating immediately after brining (Table 2). This observation is consistent for $O_2$-exchanged (treatment Nos 1 vs 4), $N_2$-exchanged (treatment Nos 3 vs 6) and nonexchanged cucumbers (treatment Nos 2 vs 5 and 2 vs 7). Delayed inoculation permitted appreciable amounts of sugars to be present in the brine at the time of subsequent inoculation (Table 2). it seems reasonable that the presence of sugars in the brine at the time of inoculation favored the increased proportion of cells being located in the brine. In addtion, delaying inoculation would negate the $O_2$ exchange effect of drawing bacteria into the cucumber. Corey et al. (1983a) found that the partial vacuum of $O_2$-exchanged cucumbers is relieved within 4 h after brining.

The total number of cells produced per fermentation was significantly greater ($P < 0·05$) in the $O_2$-exchanged-0 time inoculation treatment as compared to all other treatments. Perhaps growth of bacterial cells within cucumbers requires less energy for physiological maintenance than cells in the more inhospitable brine, thus accounting for the higher cell numbers in this fermentation. The lower maintenance requirement could have resulted in the higher cell yield.

*Distribution of bacterial cells within brine-fermented cucumbers*

Fermented cucumbers from $O_2$-exchanged and nonexchanged treatments that were inoculated at 0 time were sectioned into specific areas (Fig. 1) and analyzed for distribution of bacterial cells by area. Significantly more cells were located in the skin area than in the endocarp area in both the $O_2^-$ and nonexchanged treatments (Table 3). Intermediate numbers of cells were observed in ther mesocarp areas from both treatments. Overall higher counts were observed in each of the areas of $O_2$-exchanged as compared with non-exchanged cucumbers.

Higher numbers of bacteria in the skin area, with regressively lower numbers toward the fruit center, indicate that entrance of bacteria is through the skin surface rather than through the stem of blossom ends. Bacterial pustule formation was observed to occur in areas of high stomatal frequency. We think that bacteria enter stomata and form colonies at those sites. The ability to grow to such

**Table 2. Effect of exchange gas and time of inoculation on the distribution of bacterial cells between brine and cucumbers in completed fermentations (21 days) as determined by direct microscopic count.**

| Treatment No. | Exchange gas | Time of inoculation after brining (days) | Reducing sugar (w/v) in brine at time of inoculation (%) | Log total number of cells[a] | Percentage distribution of cells | |
|---|---|---|---|---|---|---|
| | | | | | Cucumbers[b] | Brine[b] |
| 1 | $O_2$ | 0 | 0·02 | 12·54 | 51·1 | 48·9 |
| 2 | None | 0 | 0·02 | 12·40 | 31·5 | 68·5 |
| 3 | $N_2$ | 0 | 0·02 | 12·28 | 33·1 | 66·9 |
| 4 | $0_2$ | 1 | 0·17 | 12·18 | 22·8 | 77·2 |
| 5 | None | 1 | 0·17 | 12·18 | 14·5 | 85·5 |
| 6 | $N_2$ | 1 | 0·16 | 12·23 | 10·1 | 89·9 |
| 7 | None | 2 | 0·46 | 12·29 | 7·8 | 92·2 |

[a] Per fermentation jar (3784 ml). Cucumbers and brine occupied *c.* equal volumes within the jar. Brines were inoculated with *L. plantarum* WSO.
[b] Least significant difference at the 0·05 confidence limit ($LSD_{0·05}$) = 17·5.

**Table 3. Distribution of *L. plantarum* cells within $O_2$-exchanged and nonexchanged, brine-fermented cucumbers as determined by direct microscopic count.**

| Area | Log cells per g[a] | |
|---|---|---|
| | $O_2$-exchanged | Nonexchanged |
| Skin | 8·71 | 8·57 |
| Mesocarp | 8·31 | 7·50 |
| Endocarp | 8·15 | 7·33 |
| Placentae | 8·63 | 7·52 |
| Blossom | 8·50 | 7·99 |
| Stem | 8·52 | 8·37 |

[a] $LSD_{0·05}$ within and between columns = 0·34.

high densities as to form visual pustules indicates a good nutrient supply, perhaps to diffusion of nutrients from the cucumber into the brine via stomata.

*Distribution of bacterial pustules on the cucumber surface*

Bacterial pustules were enumerated in 'raised' and 'recessed' regions of the epidermis of fermented cucumbers. There are three such regions in a three-carpeled fruit (Fig. 1). From region samples of the approximate same size, significantly higher numbers of pustules were observed (Fig. 2) and enumerated in recessed than in raised regions. Recessed regions contained an average (six replicates) of 37·6 pustules per sample, whereas the raised regions contained an average of 5·7 pustules. A statistically significant difference ($P < 0·05$) was determined. Higher numbers of stomata are located in recessed as compared with raised regions of fermented cucumbers (Daeschel and Fleming 1983), which may account for higher numbers of pustules in recessed regions if bacteria enter the brined fruit through stomata.

*Yeast distribution in cucumber fermentations*

In a preliminary experiment with natural fermentation of brined cucumbers, it was observed that yeast populations were decidedly higher (>10-fold) in the brine than in the cucumbers. Lactic acid bacteria, however, were observed to be more numerous in the cucumbers (>5-fold) than in the brine. Believing that the yeasts may have been physically excluded from the fruit interior, experiments were designed to test for the

uptake of yeasts into brined cucumbers. Exchange of the internal gas of fresh cucumbers with $O_2$ did not increase the number of yeast cells enumerated from cucumbers that were brined and immediately inoculated with the yeast, *S. cerevisiae* (Table 4). On the other hand, $O_2$-exchange resulted in an increase of about 100-fold for lactic acid bacteria within the cucumber, which confirms our earlier study (Daeschel and Fleming 1981). Bacteria were observed microscopically within the cucumbers from $O_2$- and nonexchanged treatments. Yeasts were not observed microscopically within the cucumbers from either treatment. Apparently yeast cells, because of size, were excluded from the cucumbers. Smith et al. (1979) reported the mean stomatal pore diameter of open stomata on a size No. 3 fruit to be 8·7 μm. The diameter of *S. cerevisiae* ranges from (4·5–10·5) × (7·0 × 21·0) μm (Lodder 1970), excluding buds which would increase the diameter.

*Practical implications*

Our observations that bacteria can enter and grow within brined cucumbers, but yeasts are excluded, sheds new light on the mechanism of microbially caused bloater damage. The $CO_2$ produced by *L. plantarum* WSO has been shown to

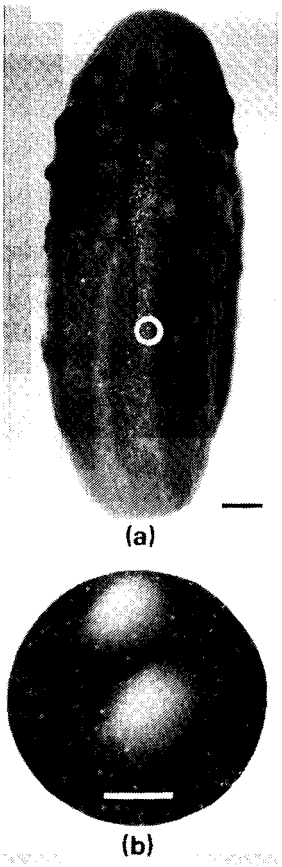


**Fig. 2.** (a): Bacterial pustules predominantly located on a recessed surface region of a fermented cucumber, bar = 1 cm. (b): Higher magnification of inset showing individual pustules, bar = 0·5 mm.

**Table 4. Effect of $O_2$-exchange on entrance of yeasts and lactic acid bacteria into brined cucumbers.**[a]

| Exchange gas | Brine inoculum: Species | Brine inoculum: Log CFU ml$^{-1}$ brine | Log CFU ml$^{-1}$ or g after 24 h: Brine | Log CFU ml$^{-1}$ or g after 24 h: Cucumber |
|---|---|---|---|---|
| $O_2$ | *L. plantarum* | 6·00 | 6·90 | 6·00 |
| None | *L. plantarum* | 6·00 | 7·08 | 3·89 |
| $O_2$ | *S. cerevisiae* | 5·83 | 5·14 | <2·00 |
| None | *S. cerevisiae* | 5·83 | 5·36 | <2·10 |

[a] Cover brine consisted of 5% w/v NaCl and 0·16% w/v acetic acid. Reported plate counts for cucumbers are mean values of duplicate treatments. A composite of two blended cucumbers was analyzed from each duplicate.

originate mainly from decarboxylation of malic acid, the primary acid in pickling cucumbers (McFeeters et al. 1982). If malic acid of the cucumber is decarboxylated during bacterial growth within the fruit, bloater damage would likely be more severe. If inoculation is delayed to allow diffusion of malic acid from the fruit, bloater damage would likely be less severe. Similarly, delay in inoculation to allow sugar diffusion into the brine would favor more bacteria in the brine and, therefore, less severe bloater damage. Furthermore, delay in inoculation of $O_2$-exchange cucumbers would cause fewer bacteria to be drawn into the fruit. Thus, manipulation of the time of inoculation and the prebrining exchange gas may be useful methods of reducing the incidence of bloater damage by lactic acid bacteria. Recent success in obtaining mutants of lactic acid bacteria that do not decarboxylate malic acid ($MDC^-$) (Daeschel et al. 1984) could alter the importance of bacterial entrance into fermenting cucumbers. Efforts are underway to develop $MDC^-$ cultures that perform well in fermentations, and are inconsequentional if drawn into the cucumbers.

Yeasts, because of their exclusion from cucumbers, presumably induce bloater formation from $CO_2$ produced in the brine. Thus, time of inoculation and $O_2$ exchange would not be expected to have the same influence with yeast as compared with lactic fermentation.

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