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Acidic alcoholic beverages have a strong microbial killing effect

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Abstract
Low-concentration ethanol or weak acids alone have only a weak antimicrobial effect. However, when these are mixed to make acidic ethanol, the antimicrobial power increases synergistically. For example, when a kind of bacteria is suspended in a mixture of HCl (pH 3) and ethanol (10%), their survival rate drops to less than 0.01% compared to treatment with either HCl or ethanol alone. This occurs because ethanol molecules are incorporated into the lipid bilayer of the microbial cell membrane, increasing its permeability. As a result, H^+ which are abundant in the external medium flow into the microbial cells, lowering the internal pH. The drop in intracellular pH causes disorder in intracellular molecules and structures, ultimately leading to cell death. Therefore, in the early stage of a meal, alcoholic beverages mixed with acidic gastric juice will kill ingested microbes in the stomach. As the meal progresses, however, gastric acid becomes diluted, causing the microbe-killing effect to be lost. If so, sour alcoholic beverages may kill microbes regardless of the pH in the stomach. Actually, Japanese Ume liqueur (Umeshu), sour white liquor, and wine have a strong bactericidal effect, which may help reduce the risk of food poisoning.

Keywords: Sour alcoholic beverages, antimicrobial effect, inflow of H^+, ethanol, organic acids

Introduction
Because the stomach cavity is highly acidic (pH 1~2) in a fasting state, many of the microbes introduced orally are killed. The reason why microbes are killed by the stomach acid is that their membrane transport systems are disrupted by the denaturation of membrane proteins due to the extremely acidic pH of the external medium, and the intracellular molecules such as proteins are impaired by the drop in intracellular pH due to the inflow of H^+ [1]. However, pathogenic bacteria such as *Klebsiella* or *Escherichia coli* O157, and dormant cysts of protists such as *Entamoeba histolytica* are tolerant to the acidic environment of the stomach, so they can move to the intestinal tract and proliferate, inducing serious diseases [2-6].
In some microbes, tolerant to extremely low pH is closely related to their strategy of reducing the permeability of the cell membrane, thereby the inward flow of H^+ is restricted so that intracellular pH homeostasis is maintained [7, 8]. In addition to this, acid tolerance mechanisms based on the consumption of excessive cytoplasmic H^+ by amino acid decarboxylase systems (glutamate-, arginine-, lysine-, and ornithine-dependent acid-resistance systems) or H^+ pump-out systems exist in some microbes, thereby maintaining the cytoplasmic pH at a constant level even in highly acidic environments [1, 8, 9].

Microbial killing effect of acidic ethanol supplemented with HCl
Dormant cysts of the soil protist *Colpoda cucullus* are tolerant to extremely low pH [10], although it is not pathogenic. This acid tolerance may be due to the low permeability of their cell membranes, which restricts the inflow of H^+ even under highly acidic conditions [10]. In fact, measurements using BCECF-AM [(2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester)], a fluorescent probe for monitoring intracellular pH, showed that the intracellular pH of *Colpoda* cysts dropped to only 5.8 when they were suspended in 0.1 M HCl [10].
Almost all dormant cysts of *C. cucullus* were not killed when exposed to a 20% neutral ethanol solution (diluted with pure water) or to 0.1 M (pH 1), 0.01 M (pH 2), or 0.001 M (pH 3) HCl solutions without ethanol for 1 h. However, the cyst-killing effect was greatly enhanced in 20% acidic ethanol containing HCl (pH 1) [99% of dormant cysts were killed]

[11]. The pathogenic bacterium *Klebsiella pneumoniae* causes infections such as pneumonia, urinary tract infections, bacteremias, and liver abscesses [12], and *Klebsiella* spp. cause inflammatory bowel disease [13]. When cells of *K. pneumoniae* (strain 6081) were suspended for 1 h in various HCl solutions without ethanol, they were not killed in the pH range of 3-5. At neutral pH, almost all *K. pneumoniae* cells were not killed at an ethanol concentration of 10%. However, under acidic conditions (pH 3), the viability of *K. pneumoniae* cells was reduced to less than 0.01% of the initial population at an ethanol concentration of 10% [11].

Unfortunately, *E. coli* O157 cells were reduced by only 58% when they were suspended for 1 h in 10% ethanol (pH 3) supplemented with HCl [11]. This may be attributed to the strong acid tolerance of *E. coli* O157 mediated by acid-stress responses such as amino acid-dependent H^+ consumption systems [1, 8, 9, 14].

Bactericidal effect of sour alcoholic beverages

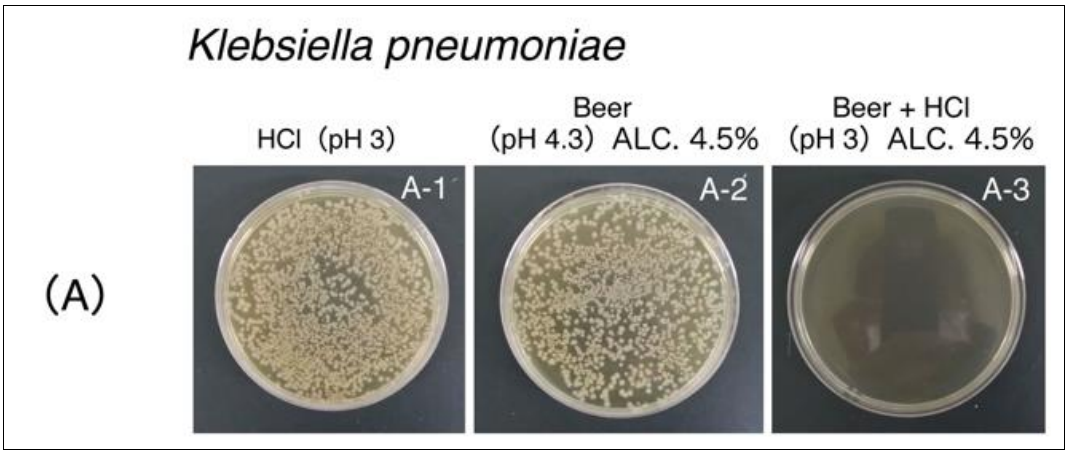
As shown in Fig. 1(A-1, A-2) [11], almost all *K. pneumoniae* cells remained viable when they were suspended for 1 h in 10^{-3} M HCl or beer (containing 4.5% ethanol). On the other hand, the viability of the cells was reduced to 0.1% of the initial population (equivalent to the number of cells suspended in water) when they were suspended in beer adjusted to pH 3 by the addition of HCl (Fig. 1, A-3). This result indicates that even acid-tolerant microbes will be killed by low-alcohol beverages in the stomach at the beginning of a meal. As the meal progresses, however, gastric acid becomes diluted, causing the microbial killing effect to be lost. If so, sour alcoholic beverages such as Japanese Ume (Japanese apricot) liqueur ('Umeshu' in Japanese), sour white liquor with lemon juice, wine, etc. may kill microbes regardless of the pH in the stomach.

Campylobacter jejuni causes a gastrointestinal tract infection (campylobacteriosis) characterized by severe diarrhea, fever, abdominal pain, nausea, and vomiting that usually lasts for several days [15]. Consumption of contaminated, undercooked or raw chicken meat or liver is often the source of campylobacteriosis, and recently this infection has accounted for about 70% of all bacterial food poisoning cases in Japan (https://www.orikane.co.jp/orikanelab/8211/; https://chubi.co.jp/template/wp-content/uploads/2021/04/b9099a00d36c8e23f80a95b621ee2301.pdf; https://www.yamaguti-coop.or.jp/wp/wp-content/uploads/2022/05/2022.5kensa.pdf). When *C. jejuni* cells were suspended for 10 min in commercially available Japanese Ume liqueur (pH 2.9, ALC. 10%) ('Sararitoshita Umeshu', Choya Umeshu Co., Ltd., Japan) (Fig. 1B-2) or sour white liquor supplemented with lemon juice (pH 3.3, ALC. 9%) ('-196 STRONG ZERO', Suntory Spirits Ltd., Japan) (Fig. 1B-3), the viability of the cells was reduced to less than 1% of the initial population (equivalent to the number of cells suspended in water; Fig. 1B-1). On the other hand, almost *C. jejuni* cells remained viable when they were suspended for 10 min in white liquor diluted with water (pH 6.8, ALC. 9%) (Fig. 1B-4).

It has been reported that red and white wines have antimicrobial properties [16-21]. For example, the survival of *Salmonella enteritidis* [16, 17], *S. typhimurium* [19, 22], *Shigella sonnei* [16], *Escherichia coli* O157 [17], and vegetative cells of *Bacillus cereus* [21] were reduced to 0.001% or less of the initial population within 30 min when they were suspended in wine. The survival of cells of *C. jejuni* is also reduced to less than 0.001% of the initial population within 1 min, but the viability of a resistant strain of *C. jejuni* is reduced to only 0.3% when suspended in wine diluted at 1:2 [20]. On the other hand, it has been reported that wine has little effect on *E. coli* O157 survival in a model stomach system containing food and synthetic gastric fluid [22] and *B. cereus* spores [21].

Hypothetical mechanism of the synergistic microbial killing effect produced by acids and ethanol

It is suggested that the bactericidal effect of wine might be due to the combined effects [19, 20, 23] of ethanol, organic acids and low pH. This could apply to Japanese Ume liqueur and sour white liquor supplemented with lemon juice as well. Fig. 2 illustrates a possible mechanism that shows how these elements work together to produce a synergistic effect in killing microbes. Ethanol molecules may be integrated into the lipid bilayer, increasing the fluidity and permeability of the plasma membrane [24]. As a result, external dissociated and undissociated acids may more easily inflow. In this case, undissociated organic acids that enter microbial cells may dissociate to produce H^+, accelerating the decrease in cytoplasmic pH. This may cause a disorder in intracellular molecules and structures, ultimately leading to cell death.



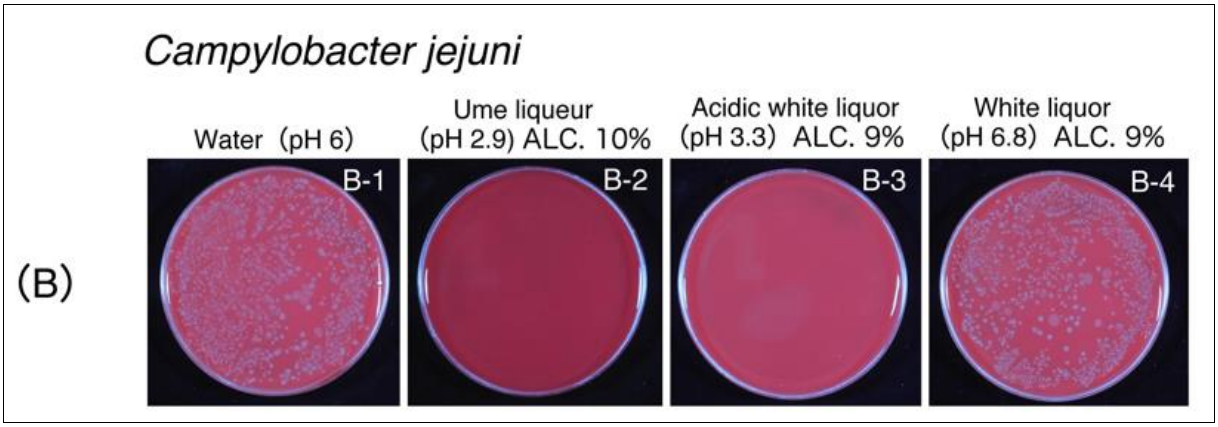


Fig 1: (A) Bactericidal effect of beer supplemented with HCl on *Klebsiella pneumoniae* (strain 6081). The cells of *K. pneumoniae* treated with each test solution for 1 h were rinsed in water prior to inoculation onto agar plates. Reproduced from [11] (Matsuoka *et al*., Microbiol Res Int. 9: 40-45, 2021). (B) Bactericidal effect of highly acidic alcoholic beverages, Japanese Ume liqueur (B-2) and sour white liquor supplemented with lemon juice (B-3) on *Campylobacter jejuni*. The pH and ethanol concentrations (ALC.) indicated in the photographs are the final values of the test solutions. The cells of *C. jejuni* treated with the test solutions for 10 min were inoculated onto agar plates without washing with water.

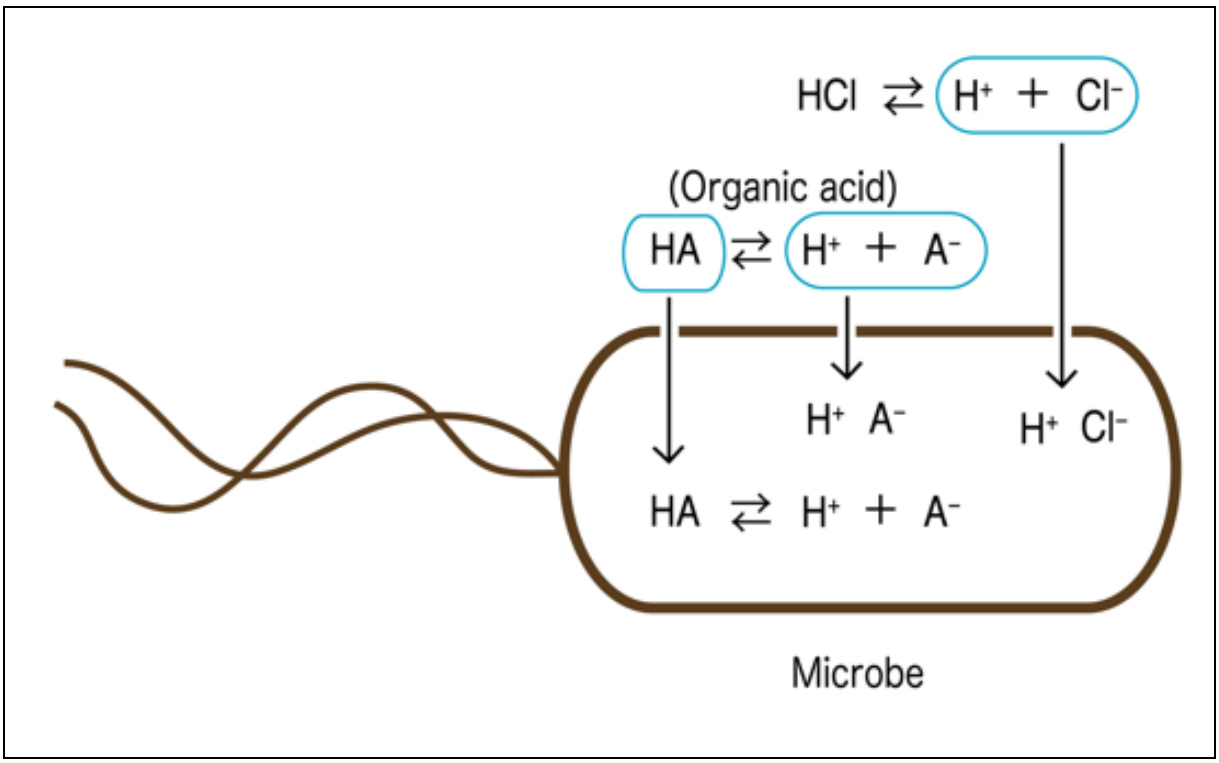


Fig 2: A schematic diagram showing how acids and ethanol work together to produce a synergistic effect in killing microbes.

Conclusion

It is generally believed that drinking low-alcohol beverages will not kill microbes such as food poisoning bacteria in the stomach cavity. However, in this paper, it was shown that even low-alcohol beverages have a strong microbial killing effect under gastric acidic pH conditions in the early part of the meal. In addition, strongly sour alcoholic beverages may kill microbes during a meal regardless of gastric pH.

It has been believed that wine and Japanese Ume liqueur are special alcoholic beverages with antimicrobial properties. However, the fact that even strongly acidic beer (pH 3.0) supplemented with HCl or sour white liquor supplemented with lemon juice (pH 3.3) has a strong bactericidal effect implies that not only wine and Japanese Ume liqueur but also other sour alcoholic beverages may have a strong microbial killing effect.

Conflict of Interest

The authors declare that they do not have a conflict of interest.

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