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A gastric acid condition enhances the microbial killing effect of ethanol

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ABSTRACT

Tolerance to extremely low pH (< pH 3) is a crucial strategy that enables microorganisms to survive in the human stomach cavity. We found that the killing effect of ethanol on dormant cysts of soil protist *Colpoda cucullus*, and pathogenic bacteria such as *Klebsiella pneumoniae* and enterohemorrhagic *Escherichia coli* O157 was enhanced under stomach acid's low pH condition. This result suggests that gastric-acid-tolerant microorganisms can be partly killed by drinking alcoholic beverages during meals.

Keywords: Acid-tolerant microorganisms, ethanol, bactericidal effect.

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INTRODUCTION

Some pathogenic bacteria such as *Klebsiella* and *Escherichia coli* O157, and protist's dormant cysts are tolerant to the acidic environment of the stomach, so they can move to the intestinal tract and proliferate to induce serious diseases (Miller and Kaspar, 1994; Benjamin and Datta, 1995; Aghi and Chhiber, 1999; Molina et al., 2003; Serrano-Luna et al., 2013). Dormant cysts of protists such as pathogenic *Entamoeba histolytica*, which causes the human parasitic infection known as amoebiasis, and those of nonpathogenic soil protist *Colpoda cucullus* are tolerant to extremely low pH (Serrano-Luna et al., 2013; Nakamura et al., 2020). The pathogenic bacterium *Klebsiella pneumoniae* causes infections such as pneumonia, urinary tract infections, bacteremias, and liver abscesses (Paczosa and Mecsas, 2016), and *Klebsiella* spp., which are colonized in the intestine, cause inflammatory bowel disease (Atarashi et al., 2017). The enterohemorrhagic *Escherichia coli* O157 produces a Shiga-like toxin that leads to serious gastrointestinal bleeding (Adamu et al., 2014).

The tolerance of such microorganisms to extreme acidic pH is closely related to their strategy of reducing the permeability of the cell membrane, which restricts the influx of H^+ into the cytoplasm (Mirete et al., 2017). In addition to this, acid tolerance mechanisms such as glutamate- and arginine-dependent acid-resistance systems which are based on the consumption of excessive cytoplasmic protons exist in some microorganisms, so that the cytoplasmic pH level was maintained at a constant level in acidic environments (Bearson et al., 2009; Guan and Liu, 2020).

Ethanol exerts several cytotoxic effects such as the increased membrane fluidity, the disruption of the physical structure of the plasma membrane, the protein denaturation (Goldstein,1986; Baker and Kramer,1999; Tóth et al., 2014). An increased membrane fluidity causes changes in membrane protein composition (Tóth et al., 2014). The denaturation of membrane proteins such as ion channels may disrupt the ion transport system.

It is believed that drinking low-alcohol drinks will not kill microorganisms in the stomach cavity, because the ethanol concentration necessary to disinfect hands is around 70%. However, even slight injury to the plasma membrane caused by a low concentration of ethanol under an extremely acidic pH condition such as stomach

cavity may be lethal for microorganisms, because H^+/Cl^- may flow down their concentration gradient.

MATERIALS AND METHODS

Culture and induction of dormant cysts of *Colpoda cucullus*

Vegetative cells of *Colpoda cucullus* Nag-1 were cultured at about 25°C in a 0.05% (w/v) infusion of dried wheat leaves (Sogame et al., 2013). To induce encystment, two-day cultured vegetative cells were collected by centrifugation (1500 x g, 2 min) and then suspended at a high cell density of >5,000 cells/ml in an encystment-inducing medium containing 0.1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2). The cell suspension (200 µl) was dispensed in watch glasses and kept for 1 to 2 weeks under humid conditions (Sogame et al., 2013).

Treatment of *Colpoda* with test solutions

Before treatment with test solutions, the medium in the cyst-adhered watch glass was discarded, and then the watch glass was washed with water 2 to 3 times and refilled with 300 µl of each test solution. After treatment for 1 h, the test solutions were again discarded, the glass was washed with running water for 10 min, and a fresh 0.05% infusion of wheat leaves was added to the glass to induce excystment (the process by which the vegetative form is reconstructed and escapes from the cyst). Cysts were randomly chosen, and the vacant cysts (excysted cysts) were counted at 24 h after excystment induction. The rate of excystment (survival rate) (Figure 1) was expressed as a percentage of the total number of observed cysts (100 cysts).

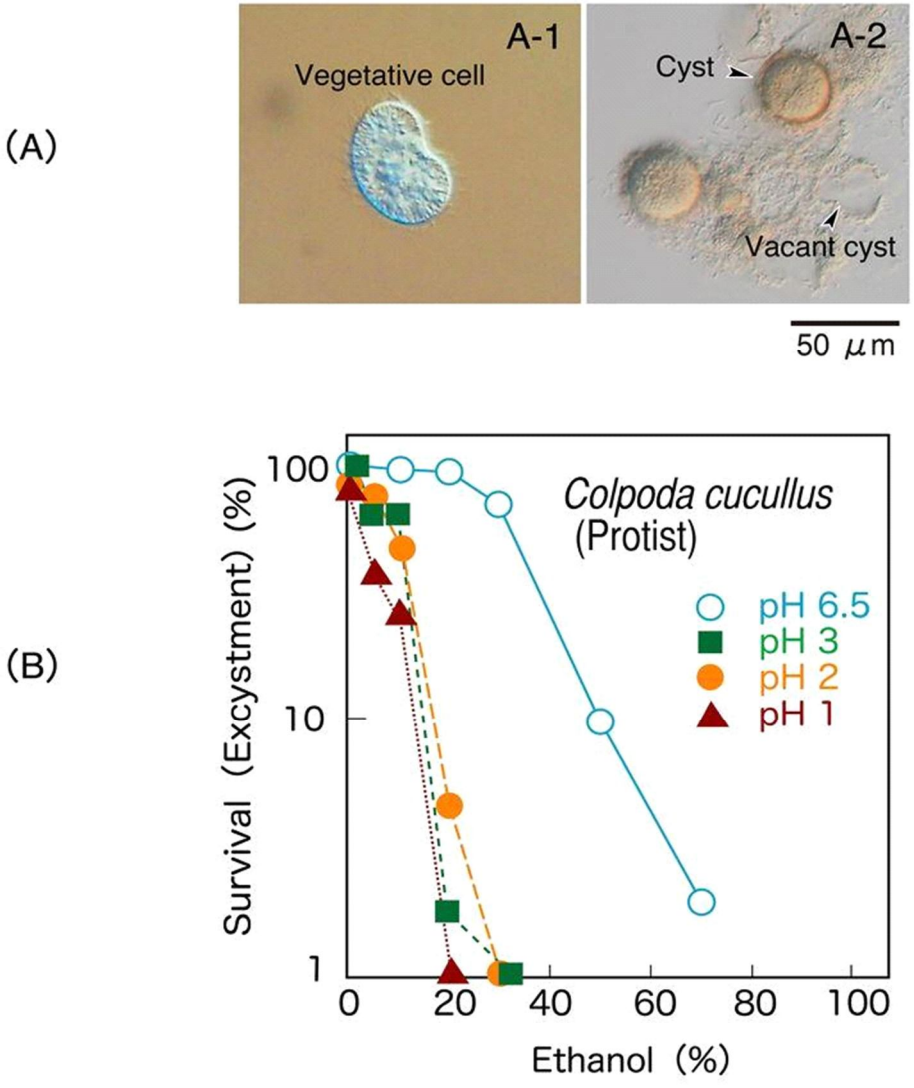

Figure 1. (A) Photomicrographs (Nomarski images) of a vegetative cell of *Colpoda cucullus* Nag-1 (A-1) and 1-week-old (a week after encystment induction) dormant cysts (A-2, arrowhead). A vacant cyst from which a vegetative cell had emerged is also indicated with an arrowhead. (B) Effect of ethanol on the *C. cucullus* excystment rate (survival rate) under acidic or nearly neutral pH conditions.

Culture of bacteria and treatment with test solutions

Klebsiella pneumoniae (strain 6081) was cultured at 37°C in 1.5% LB agar plates containing 1.5% agar (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), 1% polypeptone (Nihon Pharmaceutical Co. Ltd, Tokyo, Japan), 0.5% yeast extract (Oriental Yeast, Tokyo, Japan), and 1% NaCl (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). A sample of *E. coli* O157 isolated from a patient was cultured at 37°C in 1.5% agar plates (BBL Trypticase Soy Agar; Becton, Dickinson and Company, MD, USA). Cultured cells of *K. pneumoniae* and *E. coli* O157 were suspended in pure water so that the optical density at 650 nm (OD_{650}) became 0.23 in *K. pneumoniae*, and that at 600 nm (OD_{600}) became 0.2-0.25 in *E. coli* O157. Then, 1 µl of bacterial suspension was added to 1 ml of each test solution and kept for 1 h at room temperature. Thereafter, the bacteria were sedimented by centrifugation (8,000 × g for 10 min). The supernatant was discarded and then resuspended in 1 ml of pure water by gentle pipetting. Then, 100 ul of each bacterial suspension was spread over the surface of an agar plate and incubated for a day at 37°C. The survival rates were expressed as percentages of control colony-forming units (CFUs)/ml (derived from bacteria suspended in water without ethanol or HCl).

Calcium chloride, tris(hydroxymethyl)aminomethane, ethanol and hydrochloric acid were purchased from FUJIFILM Wako Pure Chemical Co., Osaka, Japan.

RESULTS AND DISCUSSION

Figure 1A shows a *Colpoda* vegetative cell (A-1) and dormant cysts (A-2). When the dormant cysts were induced to excyst, vegetative cells were reconstructed within several hours, and escaped from the cysts. Because the vacant cysts (Figure 1A-2) are the exuviae from which the vegetative form has escaped, the excystment rate (%) corresponds to the survival rate (%). At pH 6.5 (pure water), few dormant cysts were killed upon exposure to an ethanol solution of 20% or less (open circles), or to 0.1 M (pH 1), 0.01 M (pH 2) or 0.001 M (pH 3) HCl solutions without ethanol (Figure 1B). However, the cell-killing effect of ethanol was greatly enhanced at 20% under an acidic condition (Figure 1B, closed triangles, closed circles and closed squares).

When cells of *K. pneumoniae* were suspended in various pH solutions without ethanol, they were not killed in the pH range 3-5 (HCl concentrations of 10^{-3}-10^{-5} M) (Figure 2A-1, 2C-1). Similarly, at pH 6.5, few *K. pneumoniae* cells were killed at ethanol concentrations below 10% (Figure 2A-2; 2C-2). Under an acidic condition (pH 3), on the other hand, 99.9% of *K. pneumoniae* cells were killed at ethanol concentrations below 10% (Figure 2A-3; 2C-2). Almost all *K. pneumoniae* cells remained viable when they were suspended in beer (containing 4.5% ethanol) (Figure 2A-4). On the other hand, 99.9% of cells were killed when they were suspended in beer adjusted to pH 3 by the addition of HCl (Figure 2A-5).

Almost all *E. coli* O157 cells remained viable after exposure to 0.001 M HCl (pH 3) without ethanol (Figure 2B-1, 2C-4), or to ethanol solutions (0-20%; pH 6.5) without the addition of HCl (Figure 2B-2, 2C-3). Under an acidic condition (pH 3), on the other hand, *E. coli* O157 cells were reduced by 58% at ethanol concentrations of 10% (Figure 2B-3; 2C-3). Almost all *E. coli* O157 cells remained viable when they were suspended in beer (containing 4.5% ethanol) (Figure 2B-4). On the other hand, the number of colonies was prominently reduced by 75% when the cells were suspended in beer adjusted to pH 3 by the addition of HCl (Figure 2B-5). It has been reported that the bactericidal effect of ethanol on *E. coli* O157 cells is enhanced under acidic conditions (pH 3) (Jordan et al., 1999). Our present results are consistent with this report.

The finding that acid-tolerant dormant cysts of non-pathogenic protist *C. cucullus* were killed by relatively low concentrations (20%) of ethanol under a low gastric pH condition (Figure 1B) suggested that acid-tolerant pathogenic protists such as *Entamoeba histolytica* (Serrano-Luna et al., 2013) may be killed by low concentrations of ethanol under a low gastric pH condition.

Although *K. pneumoniae* are indigenous to the oral cavity and intestines and nonpathogenic for healthy people, *Klebsiella* spp. which are colonized in the intestine cause inflammatory bowel disease even in healthy people (Atarashi et al., 2017). Our finding that a large number of acid-tolerant *Klebsiella* cells were killed by being suspended in low concentrations (2.5 to 10%) of ethanol or beer under a gastric acidic pH condition (pH 3) (Figure 2A-5) suggests that infection by *Klebsiella* spp. may be prevented by drinking light alcoholic beverages during a meal.

The present study showed that *E. coli* O157 cells were partly killed by lower concentrations of acidic ethanol (pH 3) and by beer supplemented with HCl (final pH of 3). An infective dose of *E. coli* O157 cells is estimated at < 100 cells (Weiss et al., 2019). Therefore, it is likely that drinking a moderate amount of alcoholic drinks possibly blocks *E. coli* O157 infection if *E. coli* O157 cells less than 100 cells are taken up orally.

In general, the reduced permeability of the cell membrane to H^+ is responsible for microorganisms' tolerance to extreme acidic pH (Mirete et al., 2017). On the other hand, it is known that ethanol physically injures the plasma membrane (Tóth et al., 2014). If ethanol injures the plasma membrane of a microorganism that is placed in an extreme acidic medium, H^+/Cl^- may immediately inflow to disrupt the intracellular pH environments, thereby resulting in a disorder of intracellular molecules and structures and leading to cell death (Figure 3).

It has been reported that a strong acid tolerance of *E. coli* O157 may be mediated by several acid-stress responses, such as the glutamate and arginine-dependent buffering systems (Foster, 2004; Bearson et al., 2009; Guan and Liu, 2020). The result (Figure 2C-3) that *E. coli* O157 cells are tolerant to ethanol even under

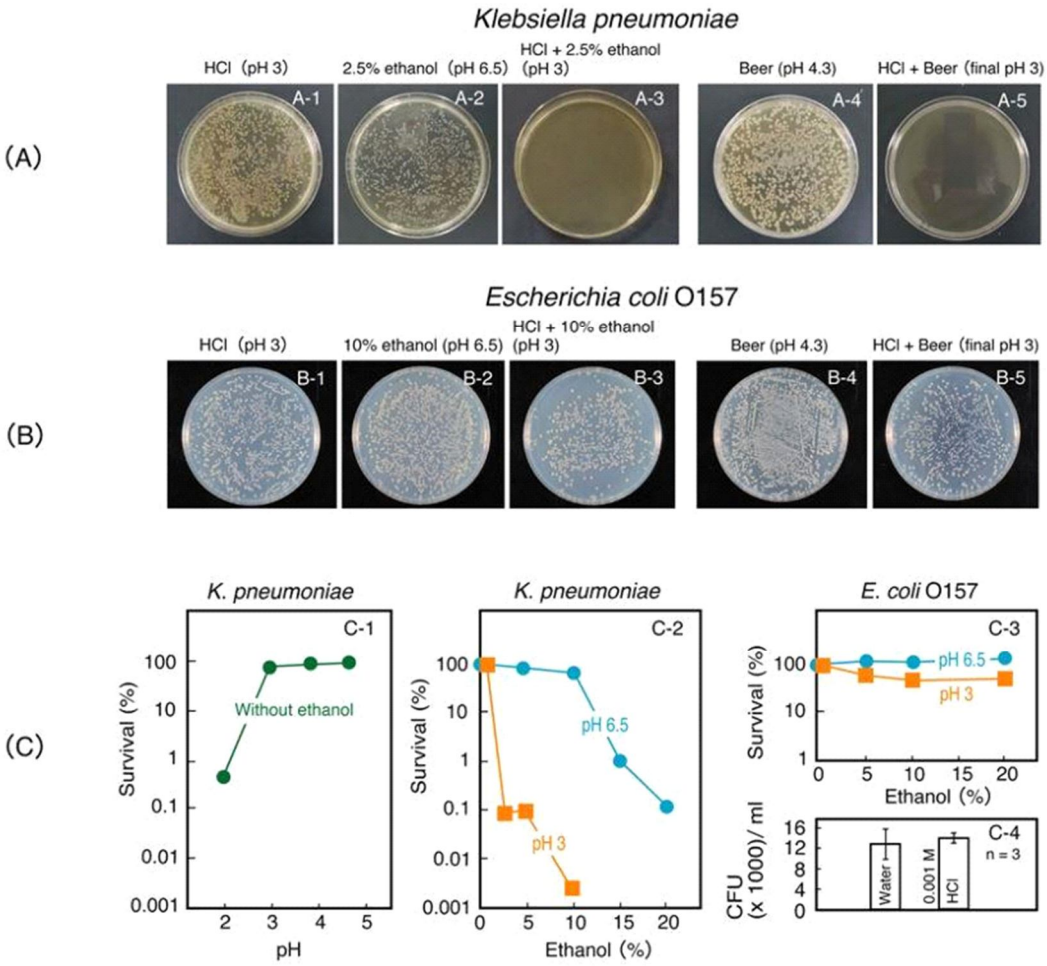

Figure 2. Bactericidal effect of ethanol under a gastric acid condition.
(A) Colonies of *Klebsiella pneumoniae*. (A-1) 0.001 M HCl (pH 3); (A-2) 2.5% ethanol; (A-3) 2.5% ethanol containing 0.001 M HCl (pH 3); (A-4) Beer; (A-5) Beer (pH 3) supplemented with HCl. The cells of *K. pneumoniae* in A-1, A-2 and A-3 were obtained from one of the batch cell preparations, and the cells in A-4 and A-5 were obtained from another batch sample.
(B) Colonies of *Escherichia coli* O157. (B-1) 0.001 M HCl (pH 3); (B-2), 10% ethanol; (B-3) 10% ethanol containing 0.001 M HCl (pH 3); (B-4) Beer; (B-5) Beer (pH 3) supplemented with HCl. The cells of *E. coli* O157 used in B1, B2, and B3 were obtained from one of the batch cell preparations, and the cells in B-4 and B-5 were obtained from another batch sample.
(C) Effects of pH and ethanol concentrations on the survival rate (%) of *K. pneumoniae* (C-1, C-2) and *E. coli* O157 (C-3; C-4). (C-1) Survival rate (%) of *K. pneumoniae* cells treated with various pH solutions (10^{-2} -10^{-5} M HCl) without ethanol. (C-2) Effect of ethanol concentration on the survival rate (%) of *K. pneumoniae* under an acidic (pH 3) (closed squares), or a nearly neutral (pH 6.5) (without HCl) pH condition (closed circles). (C-3) Effect of ethanol concentration on the survival rate (%) of *E. coli* O157 under an acidic (pH 3) (closed squares) or a nearly neutral (pH 6.5) (without HCl) pH condition (closed circles).
(C-4) Colony-forming unit (CFU)/ml of *E. coli* O157 cells incubated in water (pH 6.5) or 0.001 M HCl. Columns and attached bars correspond to the means and SE (n=3).

an extremely low pH condition may be attributable to such acid-stress responses.

The pathogenic bacteria such as *Klebsiella* spp. and *E. coli* O157 are colonized in the intestine and cause inflammatory bowel disease and serious gastrointestinal bleeding (Adamu et al., 2014; Atarashi et al., 2017). These pathogenic bacteria are tolerant to the acidic environment of the stomach, so they can move to the intestinal tract and proliferate (Miller and Kaspar, 1994; Benjamin and Datta, 1995; Aghi and Chhiber, 1999; Molina et al., 2003). It is often thought that drinking low alcoholic beverages will not kill microorganisms in the stomach cavity, since the ethanol concentration required to disinfect hands is around 70%. Contrary to this, the

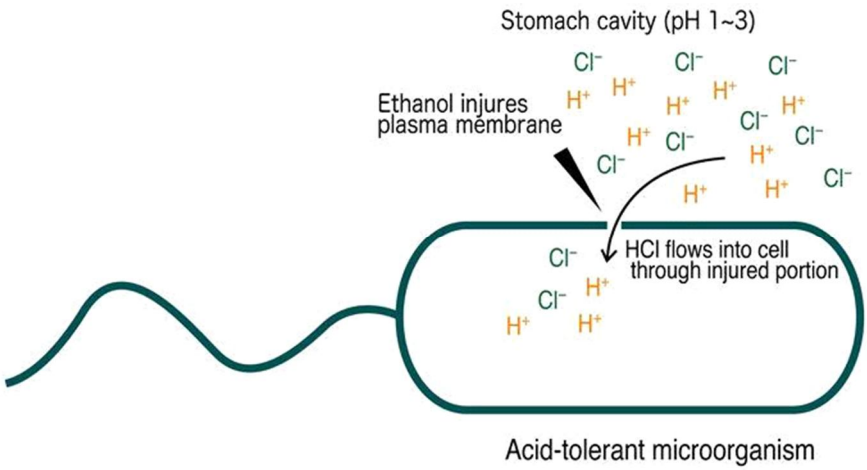

Figure 3. Schematic diagram showing how gastric-acid-tolerant microorganisms are killed by ethanol under a gastric acidic condition.

present study suggested that low alcoholic beverages may partly kill gastric-acid-tolerant microorganisms in the stomach cavity.

CONCLUSION

It is generally believed that drinking low-alcohol drinks will not kill microorganisms in the stomach cavity. Contrary to this, we showed that even low concentrations of ethanol have a microbial killing effect under a gastric pH condition (pH 1 to 3), suggesting that low alcoholic beverages could partly kill gastric-acid-tolerant microorganisms in the stomach cavity. Further examination of other acid-tolerant pathogenic microorganisms should be required.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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