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Prevalence of alcohol-tolerant and antibiotic-resistant bacterial pathogens on public hand sanitizer dispensers

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SUMMARY

Background: Since the advent of the COVID-19 pandemic, alcohol-based hand sanitizer dispensers (HSDs) have been installed in most public and clinical settings for hygiene purposes and convenient application.

Aim: To determine whether sanitizer-tolerant bacterial pathogens can colonize HSDs, spreading diseases and antibiotic resistance.

Methods: Sampling was conducted from operational automatic HSDs, specifically the dispensing nozzle in direct contact with sanitizer. Culture-dependent cultivation of bacteria and MALDI-TOF were employed to assess microbiological contamination. Bacterial isolates were selected for rapid killing and biofilm eradication assays with alcohol treatment. Antibiotic minimum inhibitory concentration assays were performed according to the Clinical and Laboratory Standards Institute guidelines. Virulence potential of bacterial isolates was evaluated in the *Caenorhabditis elegans* infection model.

Findings: Nearly 50% of HSDs from 52 locations, including clinical settings, food industry, and public spaces, contain microbial contamination at 10^3 – 10^6 bacteria/mL. Bacterial identification revealed *Bacillus cereus* as the most frequent pathogen (29%), while *Enterobacter cloacae* was the only Gram-negative bacterial pathogen (2%). Selecting *B. cereus* and *E. cloacae* isolates for further evaluation, these isolates and associated biofilms were found to be tolerant to alcohol with survival up to 70%. They possessed resistance to various antibiotic classes, with higher virulence than laboratory strains in the *C. elegans* infection model.

Conclusion: HSDs serve as potential breeding grounds for dissemination of pathogens and antibiotic resistance across unaware users. Proper HSD maintenance will ensure protection of public health and sustainable use of sanitizing alcohols, to prevent emergence of alcohol-resistant pathogens.

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Introduction

Alcohols are widely used to control microbial infection in clinical settings globally. Due to the COVID-19 pandemic, alcohol-based hand sanitizer dispensers (HSDs) have also been installed in various locations, such as home, food and beverage settings, and public spaces. This has enabled convenient application by public users for rapid hygiene maintenance. Although manual HSDs remain in use, their hand-operated levers harbour most pathogens whereas dispensing nozzles remain sterile [1]. This drives the increasing use of automatic and contact-free HSDs with sensors that detect the hands placed under the nozzle spout, and dispensers that pump the alcohol directly on to the outstretched palms, thereby reducing the spread of potential pathogens.

Hand sanitizers of various brands can kill nearly all pathogens, but recent studies have shown that hospital-acquired clinical isolates may gain tolerance to alcohols [2,3]. Microbial contamination was also found in alcohol-manufacturing plants [4]. Mutations in carbohydrate metabolism enable bacteria to survive at higher alcohol concentrations [3]. Formation of multicellular biofilms with their sticky exopolymeric matrix acting as a physical barrier can protect bacteria from alcohol killing [5,6].

This raises the question of whether widespread use of HSDs and similar devices enables the colonization of alcohol-tolerant bacteria, especially in the dispensing nozzle spout in direct contact with alcohol, with a potential to cause the spread of microbial diseases. Our study aimed to determine whether bacteria were present in direct contact with hand sanitizer, with factors accounting for antibiotic resistance, biofilm formation, and virulence potential.

Methods

Microbiological sampling from hand sanitizer dispensers

Ethical approval was granted by the Research Safety Subcommittee, Hong Kong Polytechnic University (ARSA-21134-DEPT-ABCT). Standard microbiological sampling, detection and enumeration of bacteria from swabs were performed in accordance with Public Health England standard methods [7]. Sampling was achieved by swabbing the entire area of mouth opening of the nozzle from the working hand sanitizer dispenser by using the sterile 3M™ Quick Swab which contained the Lethen neutralizing buffer used to neutralize the disinfectant effect. Samples were collected from 52 local sites in October 2021 for examination on the day of collection or within 12 h of collection.

Bacterial isolation

Samples were vortexed briefly to aid the release of microbes into the diluent, followed by transfer and spreading on standard Petri dishes each containing 20 mL lysogeny broth agar (LBA) for growth of microbes. The Petri dishes were incubated in room temperature, where bacterial colony growth was observed every day for three days. Colonies with unique phenotype (morphology, shape, and colour) were selected for further experiments and stored with 50% (v/v) glycerol at -80°C .

Matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

Per manufacturer's instructions in the MALDI Biotyper® Protocol Guide (Bruker Daltonics, Hilden, Germany), a bacterial colony was directly smeared as a thin film on to a sample position on a MALDI target plate, then overlaid with 1 μL formic acid (70%) and 1 μL of α -cyano-4-hydroxycinnamic acid (HCCA) solution + 10 mg/mL of HCCA in standard solution within 30 min and dried at room temperature. Standard solution was prepared with 50 vol% acetonitrile, 47.5 vol% Milli-water (Millipak® 40 Gamma Gold; Sigma–Aldrich, Burlington, MA, USA) and 2.5 vol% trifluoroacetic acid. As previously described, the sample plate was performed with MALDI-TOF MS, under control by FlexControl ultraflex TOF/TOF software [8]. Each spectrum had a summation of 200 laser shots with a mass range of 2000–20,000 Da. The spectrum from each microbe was matched against each main spectrum in the microbe library. The range from 2.000 to 3.000 indicates the high confidence identification and 1.700 to 1.999 indicates low confidence identification.

Bacterial colony-forming units (cfu) assay

As previously described, bacterial cultures were serially diluted, grown on LBA and incubated at 37°C for 16 h [9]. Colonies were enumerated and the cfu/mL was tabulated by (no. of colonies \times dilution factor)/volume of culture plate.

Alcohol killing assay

Bacterial cells from overnight cultures were washed with 0.9% NaCl (w/v) saline and their OD 600 nm was adjusted to 0.3 in LBA containing various concentrations (0%, 4.38%, 8.75%, 17.50%, 25%, 35%, and 70%) of ethanol. For rapid killing with ethanol, the bacterial cells were incubated at 37°C for 10 min. The bacterial populations were enumerated with cfu assay as described above.

Biofilm eradication assay

As previously described, the minimum biofilm eradication concentration (MBEC) assay (previously known as Calgary Biofilm device) was employed by using Nunc Immuno TSP Lids (Thermo Fisher Scientific, Waltham, MA, USA) [10]. The bacterial isolates were cultivated in 200 μL LB media to enable biofilm formation on the peg surfaces at 37°C for 24 h. After washing the biofilms three times with 0.9% NaCl (w/v) saline, mature biofilms on the peg lids were fitted into 96-well microtitre plates containing six different concentrations of ethanol (70%, 35%, 17.5%, 8.75%, 4.375%, and 0%). After 24 h at 37°C , biofilm cells were disrupted into saline by sonication in ice-water bath for 10 min, followed by 15 s rigorous vortexing for three times. For quantification of bacterial numbers, cfu assay was employed as described above.

Endospore staining assay

A 10 μL aliquot of bacterial sample was added to the centre of the glass slide, where the sample was air-dried for 5 min and heat-fixed. A few drops of 1% Malachite Green stain were added

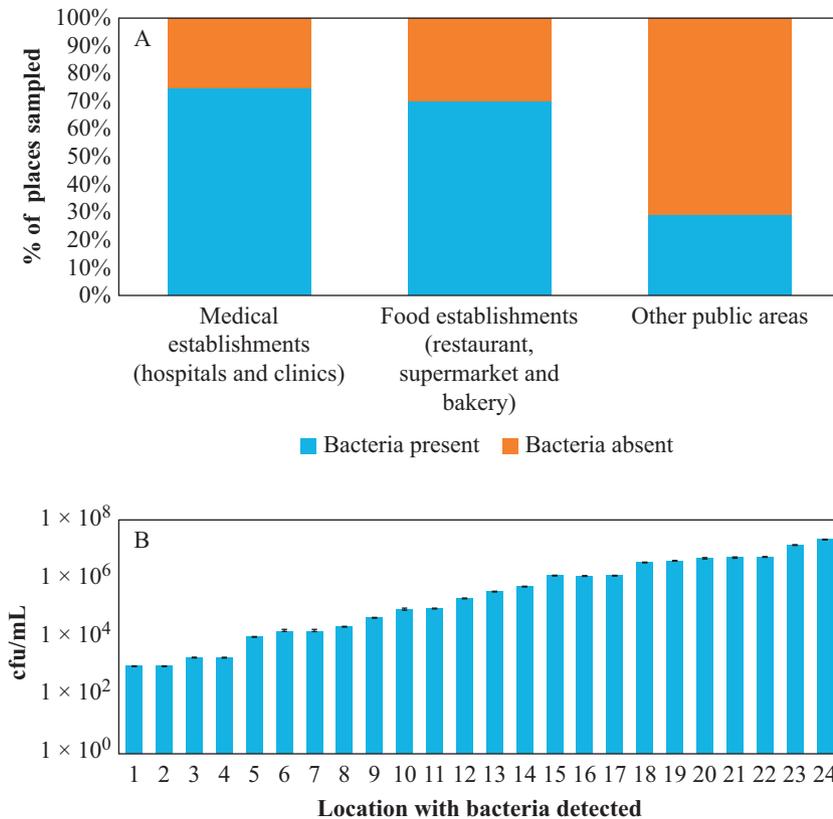


Figure 1. Characteristics of the microbes found on dispensing nozzle of hand sanitizer dispensers. (A) Study profile of samples collected from different locations for microbiological analysis. (B) Bacterial cfu from each dispenser contaminated with bacteria (presented in ascending order). Means and SD from triplicate experiments are shown.

to the fixed sample and steamed for 5 min. Distilled water was used to wash away the stain, followed by addition of a few drops of safranin to stain bacterial samples for 30 s. Distilled water was also used to wash away the remaining stain. Representative brightfield images of the bacterial cells and endospores were captured by a brightfield microscope (Zeiss, Oberkochen, Germany) under $\times 100$ objective.

Antibiotics susceptibility assay

The minimum inhibitory concentration (MIC) testing of antibiotics on bacterial isolates was determined according to the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA, USA) guidelines [11–13]. Bacteria were cultivated in 200 μ L Mueller–Hinton (MH) media with various antibiotic concentrations in 96-well plates (Nunc; Thermo Fisher Scientific). The OD_{600nm} values of each well were quantified at 0 h, 8 h and 24 h with a microplate reader (Tecan Infinite 2000; Tecan Austria GmbH, Grödig, Austria), where the MIC was determined at the antibiotic concentration with no bacterial growth.

C. elegans infection assay

As previously described, the Bristol N2 wild-type *C. elegans* provided by the Caenorhabditis Genetics Center, the University of Minnesota was maintained [14,15]. For nematode killing assay, the bacterial isolates were first cultivated as bacterial lawns on peptone–glucose–sorbitol agar (PGS; 1% Bacto-

Peptone/1% NaCl/1% glucose/0.15 M sorbitol/1.7% Bacto-Agar) at 37 °C for 24 h [16]. Thirty stage L3 nematodes were transferred from the maintenance Petri dish with *Escherichia coli* OP50 to triplicate bacterial testing Petri dishes with a titanium wire picker. The co-cultures were incubated at room temperature for five days and observed for live/dead nematodes under a stereomicroscope (Zeiss).

Statistical analysis

The results were expressed as means \pm standard deviation (SD). Data groups were compared using one-way analysis of variance and Student's *t*-test to evaluate associations between independent variables, and the *P*-values were obtained. Three independent trials were conducted in triplicate for each experiment.

Results

Characteristics of the microbes found on dispensing nozzle of hand sanitizer dispensers

The nozzle spout exits of 50 operational HSDs located in different places were sampled, ranging from clinical settings (hospitals and clinics) to commercial settings (restaurants and supermarkets) (Figure 1A). The HSDs originate from common brands used locally, where the hand sanitizers contain ethanol concentrations ranging from 60% to 75% (w/w). Nearly half of

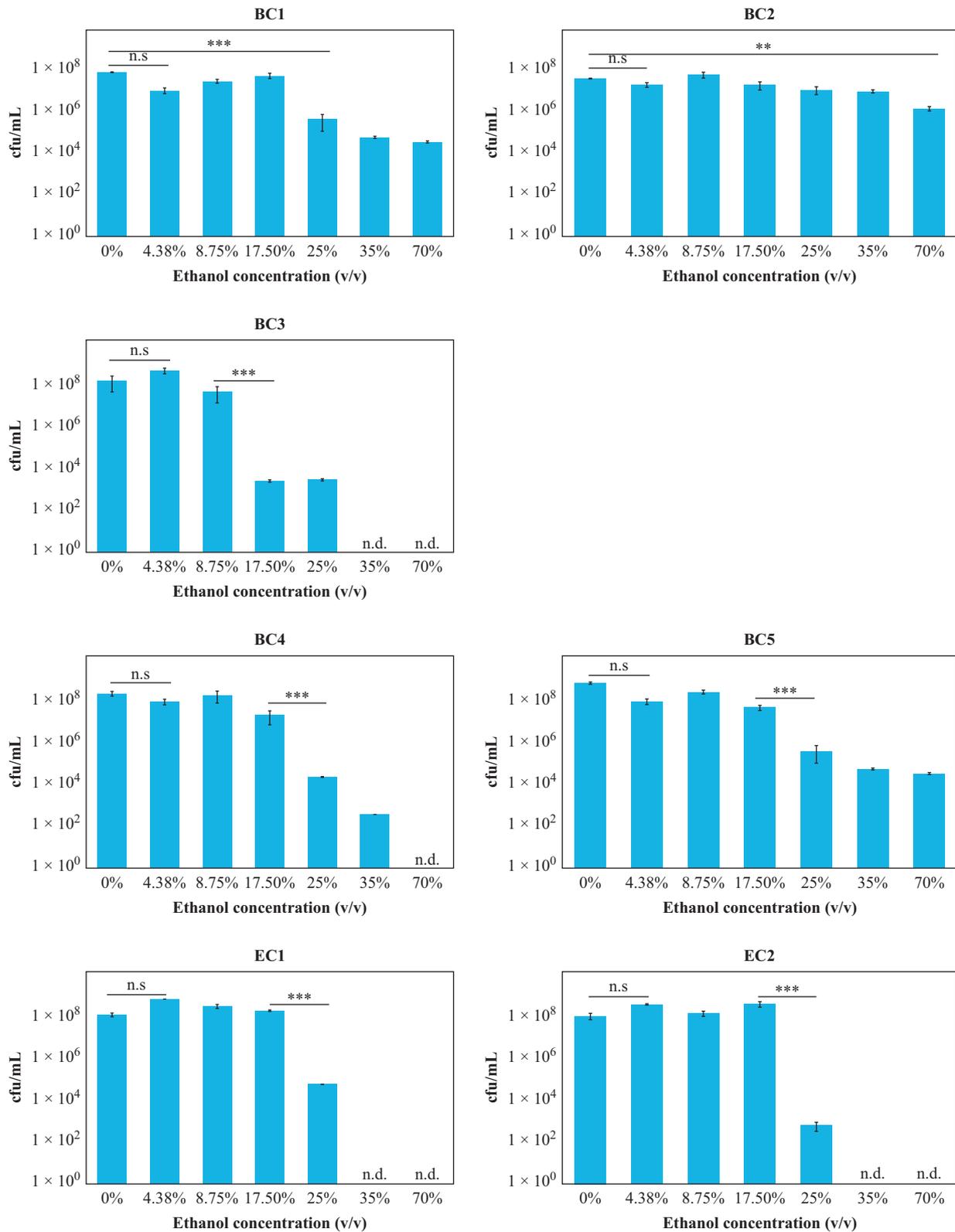


Figure 2. Microbes are tolerant to killing by low levels of alcohol after 10 min of treatment. Means and SD from triplicate experiments are shown. BC, *Bacillus cereus*; EC, *Enterobacter cloacae*. *** $P < 0.001$; n.s., not significant; n.d., not detectable.

the HSDs contained microbial contaminants, where bacteria grew on LBA, with a large range of bacterial numbers, from 10^3 to 10^6 cfu/mL (Figure 1B). The unique colonies were selected

and collected for initial evaluation using MALDI-TOF. Most bacterial isolates were identified as Gram-positive bacteria, where nearly 30% bacterial isolates were identified as

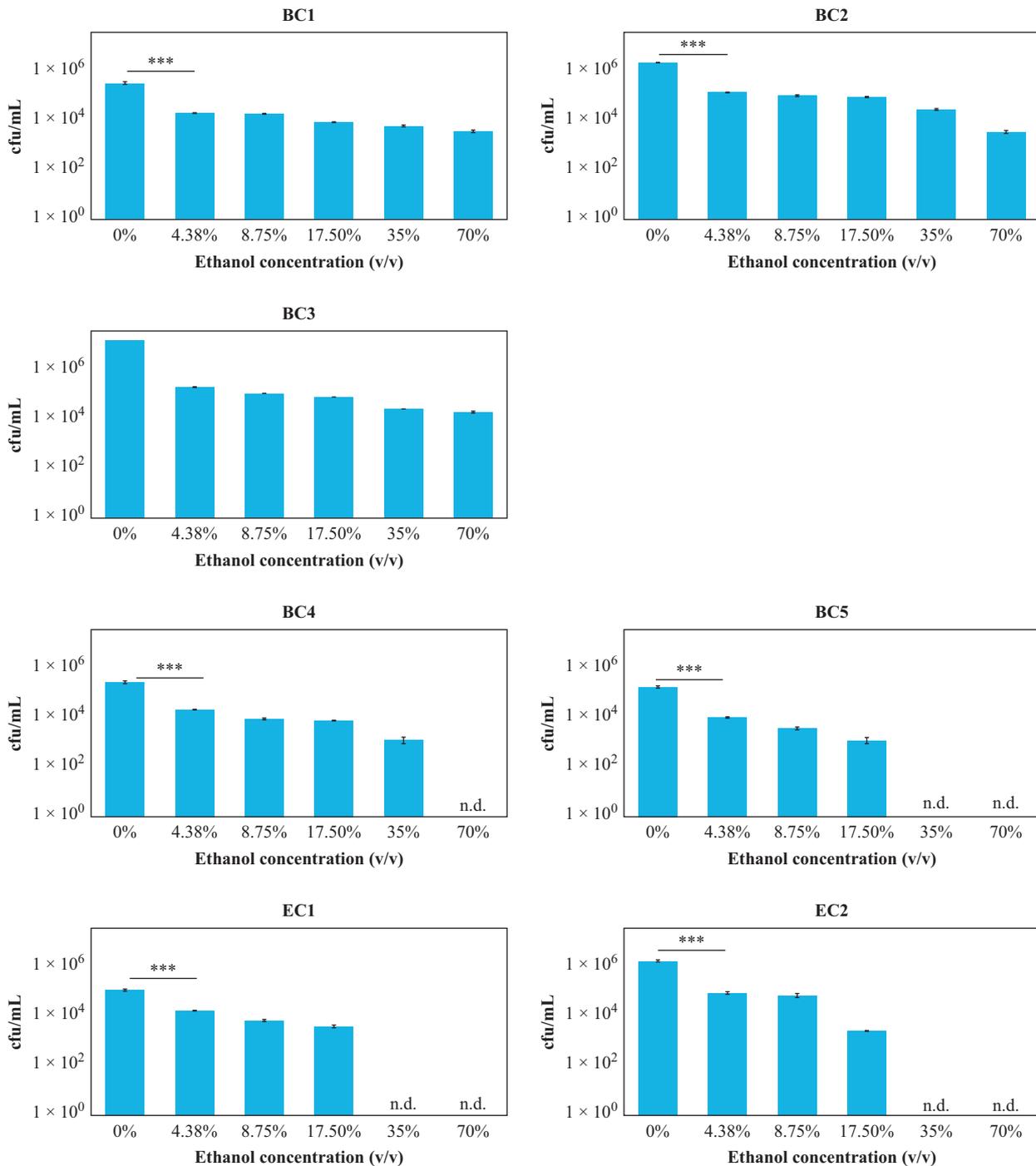


Figure 3. Microbial biofilms are tolerant to low levels of alcohol. Means and SD from triplicate experiments are shown. BC, *Bacillus cereus*; EC, *Enterobacter cloacae*. *** $P < 0.001$; n.s., not significant; n.d., not detectable.

B. cereus; *Staphylococcus* species were next most frequently isolated (Table I). Interestingly, the only Gram-negative bacterial species isolated was *E. cloacae* at 2%, which is an opportunistic pathogen associated with urinary tract infections and pneumonia in immunocompromised individuals [17]. Since *B. cereus* can form endospores which are resistant to alcohol, we assessed whether the HSD samples contained any endospores by using the endospore staining assay. Intact bacterial cells were observed, but no endospores in the samples,

indicating that the bacterial cells were vegetative (Supplementary Figure S1).

Microbes are tolerant to killing by low levels of alcohol

Based on their unique locations, such as hospital, clinic, school, supermarket and restaurant, five *B. cereus* and two *E. cloacae* isolates were chosen for further microbiological evaluation. For *B. cereus*, these isolates were tolerant to

Table I
Microbial species composition of isolates

Bacterial species	Occurrence (%)
<i>Bacillus cereus</i>	29
<i>Staphylococcus warneri</i>	9
<i>Bacillus pumilus</i>	6
<i>Staphylococcus saprophyticus</i>	5
<i>Micrococcus luteus</i>	3
<i>Staphylococcus capitis</i>	2
<i>Enterobacter cloacae</i>	2
<i>Kocuria kristinae</i>	2
Other	42

alcohol, where three isolates (BC1, BC2, and BC5) survived 70% ethanol treatment (Figure 2). Since most commercial hand sanitizers contain at least 60% alcohol, this explains why *B. cereus* can colonize directly on the dispensing nozzle [18]. Moreover, vegetative *B. cereus* remained intact even with alcohol treatment (Supplementary Figure S2), indicating that the bacteria are tolerant to alcohol even in the absence of endospores. On the other hand, neither of the *E. cloacae* isolates (EC1 and EC2) survived high concentrations of ethanol (Figure 2), where they were only unaffected by 17.5% alcohol. This could be attributed to prolonged ethanol evaporation from the nozzle, enabling microbes to survive there [19].

Since bacteria spend most of their lives as biofilms on most biotic and abiotic surfaces with a high potential to contaminate environmental and food surfaces, we also assessed whether their biofilms could tolerate higher levels of alcohol [20,21]. All bacterial isolates grew biofilms on the peg lid of the MBEC assay, where the *B. cereus* biofilms were in general tolerant to 70% alcohol (Figure 3). However, *E. cloacae* biofilms remained susceptible to high alcohol concentrations (Figure 3), corroborating our data on planktonic cells (Figure 2). This implied that *E. cloacae* may have been colonizing HSDs with evaporated hand sanitizers.

Antibiotic resistance profiles of bacterial isolates

To assess whether the HSD-associated bacteria are important in the context of public health and clinical settings, we first determined the antibiotic resistance profiles of the bacterial isolates, as per the CLSI guidelines [11]. *B. cereus* were treated with the representative antibiotic of each class widely

used in clinical settings, where the isolates possessed resistance to β -lactams and macrolides, but remained mostly susceptible to rifampicin, aminoglycosides and fluoroquinolones (Table II). However, *E. cloacae* isolates were resistant to most common antibiotic classes, such as macrolides, β -lactams, and rifampicin (Table II), indicating that HSDs could harbour multidrug-resistant bacteria.

Bacterial isolates are virulent against *Caenorhabditis elegans* infection assay

The ability to cause disease is a major concern in public health. We evaluated the ability of the HSD-associated bacteria to infect and kill *C. elegans*, which is frequently used as an animal infection model to evaluate bacterial virulence [14,15,22]. For *B. cereus*, only BC1 and BC5 isolates were more virulent than the ATCC *B. cereus* strain, whereas the rest of the isolates were not virulent (Figure 4). Both EC1 and EC2 isolates were highly virulent against *C. elegans* (Figure 4), indicating that the HSD-associated bacteria could cause diseases in humans.

Discussion

HSDs are important for hygiene maintenance in clinical and public settings. Without proper hygiene and frequent maintenance of HSDs, HSDs might serve as potential breeding grounds for widespread dissemination of pathogens and antibiotic resistance, resulting in the spread of diseases across unknowing users. This has significant impact on human health as nearly half of all HSDs sampled from different locations, including hospitals and restaurants, possess bacteria in the dispensing nozzle. It is a surprising finding as the HSDs are automatic and contact-free with few opportunities for direct contact by users and hence microbial contamination. This is in contrast to manual HSDs that are highly susceptible to microbial contamination due to direct hand contact of the lever. Furthermore, contrary to assumptions that only spores could survive under harsh alcohol treatments, intact vegetative bacterial cells were present in the swabbed samples despite constant exposure to hand sanitizer in the nozzle spout. Hence, we suggest that there could be other factors that enable microbial colonization on automatic HSDs, such as prolonged ethanol evaporation from the nozzle, and misuse of HSDs, such as direct hand contact of nozzle spout [19].

Next, our work showed that HSD-associated bacteria acquired some degree of alcohol tolerance, albeit restricted to

Table II
Antibiotic resistance profiles of *Bacillus cereus* (BC) and *Enterobacter cloacae* (EC) isolates

	Amp		Gm		Levo		Rif		Ery		Amox	
	$\mu\text{g/mL}$	S/I/R										
BC1	>1.00	R	2.00	S	0.31	S	0.60	S	5.00	I		
BC2	>1.00	R	2.00	S	<0.15	S	0.60	S	5.00	I		
BC3	>1.00	R	4.00	S	0.60	S	0.08	S	>10.00	R		
BC4	>1.00	R	4.00	S	0.30	S	0.30	S	>10.00	R		
BC5	>1.00	R	2.00	S	5.00	I	0.60	S	5.00	I		
EC1					>0.08	S	>5.00	R	>5.00	R	>10.00	R
EC2					>0.08	S	>5.00	R	>5.00	R	>10.00	R

Minimum inhibitory concentrations (MICs) of ampicillin (Amp), gentamycin (Gm), levofloxacin (Levo), rifampicin (Rif), erythromycin (Ery), and amoxicillin (Amox) are listed, where their profiles are classified as: S, susceptible; I, intermediate; R, resistant.

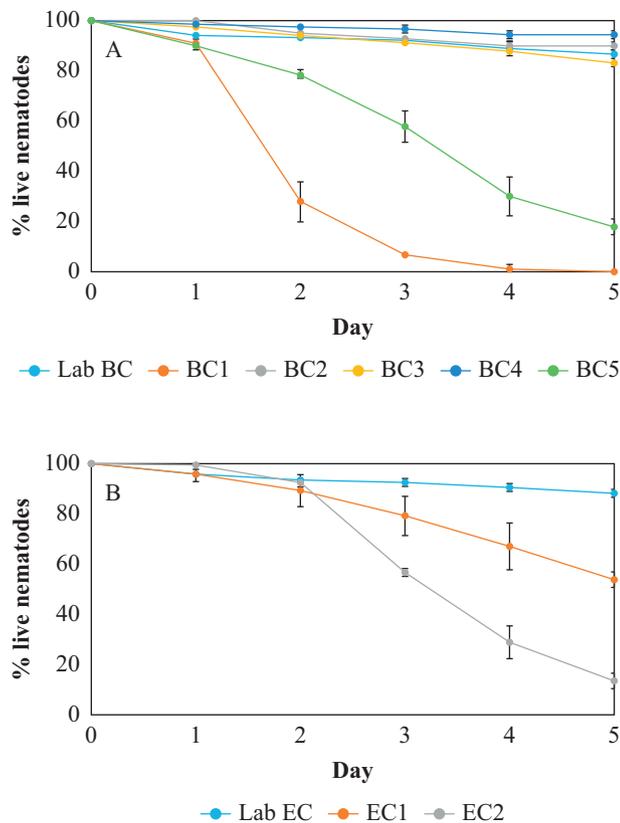


Figure 4. *Bacillus cereus* (BC) (A) and *Enterobacter cloacae* (EC) (B) are virulent against *Caenorhabditis elegans* infection assay.

a few bacterial isolates. While there were no signs of alcohol resistance in our study, some *B. cereus* isolates could survive the rapid killing of 70% alcohol at low viable numbers. Nonetheless, they were still a cause for concern, as *B. cereus* isolates were susceptible to low ethanol concentrations decades ago and only their spores were resistant to ethanol [23,24]. This indicated that bacteria may evolve alcohol resistance in future with prolonged and excessive use of alcohol disinfectants. Moreover, they were resistant to various antibiotic classes, with a heightened ability to cause disease. This showed that bacterial pathogens from HSDs possess alcohol tolerance, antibiotic resistance, and virulence potential.

Our study has several limitations, where we employed culture-based techniques, instead of culture-independent methods, such as metagenomics, to identify HSD-associated bacteria. While there is a possibility of missing out on unculturable bacteria with fastidious nutrient requirements and anaerobic bacteria, many human pathogens can grow in microbiological agar and direct exposure to air enables the survival of aerobic bacteria. It is important to note that the HSD nozzles in direct contact with hand sanitizer were also exposed to the external environment with constant air circulation, indicating that anaerobic bacteria may not colonize well in such environments.

As we collected the samples over the course of one month, we also did not account for the changes in temperature and humidity of the surrounding environment, where such factors may alter the HSD-associated microbiome. Lastly, it is unclear how frequently the HSDs were utilized and maintained. A poorly

maintained HSD which is rarely used may encourage growth and colonization of microbes. Nonetheless, our work raises the need to consider how microbes can adapt to alcohol in infection prevention. From the manufacturers' point of view, the hand sanitizer formulations may require modifications to retain their effectiveness, for example using different alcohols such as propanol or adding other antimicrobial compounds [25,26]. The HSD manufacturers may also consider using antimicrobial surfaces in the nozzle or incorporating ultraviolet light features to disinfect the nozzle after every use.

In conclusion, HSDs are widely assumed by the public to be sterile, but our work surprisingly showed that alcohol-tolerant microbes do exist on HSDs, even with direct contact with hand sanitizer. These microbes are pathogenic in nature, where they possess resistance to various antibiotic classes and virulence potential. This indicates that HSD-associated microbes may cause diseases in users, especially immunocompromised patients, the elderly, and children. Hence, we propose frequent cleaning and replacing with fresh hand sanitizers, if left unfinished over prolonged time. Public education is also key to proper use of such devices. These precautions will ensure protection of public health and sustainable use of sanitizing alcohols, thereby preventing the emergence of alcohol-resistant pathogens.

Author contributions

Y.W.S.Y., Y.M., S.Y.L., and W.H.P. performed the experiments and analysed the data; S.L.C. planned the experiments and wrote the article. All authors discussed the results and commented on the manuscript. All authors had access to all the data in the study and had final responsibility for the decision to submit for publication.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhin.2022.05.017>.

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