ABSTRACT
Chemical disinfectants pose risks to live cells. In a closed cell handling system, temperature and relative humidity (RH) were examined as a means to control microbes. Compared with biological safety cabinet conditions, lower RH and physiologic temperatures increased log reductions on polypropylene and steel surfaces. Environmental control can reduce the need for disinfectants.

Material and Methods
Organisms and media
Bioballs (Biomerieux) were used. Colony forming units (CFU) were assessed using culture plates with tryptic soy agar (Sigma). A. brasiliensis plates were cultured at 25 °C for 36–48 h while the other microorganisms were cultured at 35 °C for 20–24 h before counting. Surface environmental monitoring (EM) was observed with contact plates with tryptic soy agar (BBL, BD). Contact plates were incubated at 35 °C for at least 20–24 h.
Disc inoculation

Discs (10 mm diameter) were made of the same materials as used to manufacture NGI: polypropylene (made in-house) or stainless steel (Beadthoven Jewelry, Guong Dong, China). The discs were triple-cleaned/disinfected with TexQ (Texwipe) then SporKlenz (Steris), followed by 70% ethanol for 30–60 min each soak, with a triple sterile water rinse between each. Each Bioball inoculum in Bioball Rehydration Solution was diluted in Dulbecco’s phosphate-buffered saline (DPBS) (Sigma) with 0.05% Tween-80 (Sigma). 1 x 10^5 CFU were applied to each coupon as a single 20-μL droplet.

Surface microbial release assay

To assess how well the different surfaces rinse free of microbes, methods were adopted from Pappas et al. (3). Discs were inoculated with 1 x 10^5 CFU of B. subtilis and incubated at 37 °C/15% RH. Discs at time zero were collected immediately and processed as the baseline. Two hours later dried discs were collected, immersed in 1 mL DPBS-T twice for 10 seconds each time. Percentage of released bacteria was calculated using the equation: released bacteria (%) = 100 x [1-log (Yi)/log (Y0)], where Y0 = microbes at time 0, and Yi = microbes at time i.

Microbial reduction assays

Prior to these studies, the NGI processing chamber (PC) surfaces were disinfected with SporKlenz, and gases were replaced with fresh triple-filtered dry compressed gases (20% O2, 0.1% CO2, balance N2) to eliminate disinfectant fumes. Discs were inoculated in place on the PC floor, as if a drop of the inoculum had contaminated the work surface; exposed to PC conditions, and retrieved at intervals. After vortexing in 1 mL DPBS-T five times for 10 seconds each time, microbial suspensions were diluted further before being spread on agar plates. Log reductions were calculated using the equation: R = log (Y0) – log (Yi). R is the log reduction for each time point, where Y0 = microbes at time zero, and Yi = microbes at time i.

All statistical analyses were performed using GraphPad Prism (Version 8.4.2, GraphPad Software). Data are expressed as the mean + standard error of mean (SEM). If a set of means were compared, Sidak was used for multiple comparisons. When comparing each mean to a control, Dunnett was chosen for multiple comparisons. Significance was assessed at the p < 0.05 level.
NGIs, configured as in BSC conditions or NGI conditions with PCs was set to mimic either room air conditions (37 °C/15% RH) for up to seven days.

Figure 2. Polypropylene discs have the same rinsability as stainless steel. No significant differences were found in the release of dried-on B. subtilis spores from polypropylene or stainless steel exposed to next generation isolator conditions (37 °C/15% RH) for up to seven days.

RESULTS

Physical atmospheric conditions were continuously controlled in a closed cell processing system NGIs, configured as in Figure 1(a) and (b), were used to control environmental conditions. A laminar flow hood protected the entry and exit doors with HEPA filtered air. Airlock-like buffer chambers exchanged entering room air with dry, tanked gases. The atmosphere in the PCs, composed of dry compressed gases, was continuously HEPA filtered. RH was introduced as 150 mm Petri dishes of sterile distilled water. Fresh dry gases automatically infused if RH exceeded set limits. The floor temperature of each processing chamber matched chamber atmosphere temperature.

As NGI surfaces are stainless steel and polypropylene, discs made from the same materials were inoculated (Figure 1[c]) and harvested in duplicate or triplicate at intervals to test for remaining viable CFU. Chamber conditions were held constant at set RH and temperature (Figure 1[c–f]). Continuous particle monitoring during exposures showed 0.5 micron or greater particle levels well below the ISO 5 limit of 3520/m³ (Figure 1[g]). The environment in the PCs was set to mimic either room air BSC conditions or NGI conditions with different RH levels.

Polypropylene and stainless-steel surfaces release dried-on microbes equally well

As some differences have been previously reported (3), the ability of polypropylene and stainless-steel surfaces to release dried-on B. subtilis spores was tested first. No significant differences were found in the ability of B. subtilis spores to release from polypropylene and stainless steel, even after seven days dried onto the surface (Figure 2).

NGI conditions produce larger microbial reductions than room air BSC conditions in a microbe-dependent manner

The working hypothesis was that there would be differences between microbial persistence in the closed NGI and room air BSC conditions. Five different representative microbes with varying sensitivities to desiccation were used: P. aeruginosa (Gram-negative bacterium), C. albicans (yeast), S. aureus (Gram-positive coccus), A. brasiliensis (mold), and B. subtilis (Gram-positive, spore-forming bacterium). Discs (polypropylene or stainless steel) were inoculated with a known number of microbes in each chamber and maintained in either NGI PC conditions (37 °C/15% RH), or a processing chamber set to room air BSC conditions (21 °C/40% RH), both with continuous HEPA filtration. Data from three or more independent experiments were combined. Statistically significant CFU log reductions were found for four of five microorganisms (Figure 3). These reductions were only from the physical atmospheric conditions, not from any chemical disinfection.

On both materials, P. aeruginosa experienced significant log reductions within 1 h under both conditions, with greater log reductions under NGI conditions (Figure 3[a]). No significant time-dependent (within-group) reduction was found with C. albicans under BSC conditions with either material, while reductions compared with time zero were highly significant under NGI conditions after 0.5 h (Figure 3[b]). S. aureus had no significant time-dependent log reductions under BSC conditions on either material. In contrast, significant time-dependent log reductions were observed under NGI conditions with both materials. Although trends showed greater log reductions in NGI conditions, this was significant only at 8 h on polypropylene (Figure 3[c]). A. brasiliensis exhibited significant time-dependent reductions in both conditions, but NGI conditions showed greater log reductions compared to BSC conditions (polypropylene at 4 h and 8 h) (Figure 3[d]). Consistent with known resistance to desiccation, little log reduction was seen with B. subtilis spores under any conditions (Figure 3[e]).

Reduced RH increases microbial reduction

To examine the effect of NGI PC RH on microbial resistance, discs were inoculated with S. aureus in four different conditions: room air BSC (21 °C/40% RH) as a control and NGI temperature (37 °C) at three different RH levels in a typically low range: 5, 15, and 25% RH. With decreasing RH, there was a trend toward greater log reductions on both materials. At 37 °C/5% RH, polypropylene discs showed significantly greater log reductions compared with BSC
Figure 3. Typical next-generation isolator (NGI) conditions reduce microbial viability more than room air biological safety cabinet (BSC) conditions.

A. P. aeruginosa

B. C. albicans

C. S. aureus

D. A. brasiliensis

E. B. subtilis

Differences between discs exposed to room air BSC conditions and typical NGI conditions with five microbes: P. aeruginosa (a), C. albicans (b), S. aureus (c), A. brasiliensis (d), and B. subtilis (e) were tested. Asterisks (*) denote significant within-group differences (compared to 0) determined by two-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test, while pound (#) indicates significant between-group differences determined by two-way ANOVA followed by Sidak’s multiple comparisons test. (* or #, p<0.05; ** or ##, p<0.01; *** or ###, p<0.001; ****, p<0.0001)

Figure 4. Lower relative humidity (RH) facilitates microbial reduction.
Polypropylene or stainless-steel discs were inoculated with S. aureus in four closed PCs under different conditions: 21 °C with 40% RH, or 37 °C with 5%, 15%, or 25% RH. Data were expressed as mean of log reduction + standard error of mean (SEM) from four independent experiments. Asterisks (*) denote significant between-group differences between 37 °C/5% RH group and 21 °C/40% RH group determined by two-way analysis of variance followed by Sidak’s multiple comparisons test. (*, p<0.05).
conditions at 2 h and 4 h of exposure (Figure 4). This suggests that lower RH contributes to the microbial reductions seen in the NGI.

Higher physiologic temperature in a closed system enhanced microbial reduction

RH, by definition, is temperature dependent. Follow-up experiments examined the effect of temperature on microbial resistance in the processing chamber. *S. aureus* was inoculated on both polypropylene and stainless-steel discs in processing chambers with an RH of 15% and a temperature of 21 °C, 31 °C, or 37 °C. CFU were recovered at time zero and 4 h. Discs exposed to 37 °C had greater CFU reductions on both materials (Figure 5) than at room temperature. These data suggest that in addition to the lower RH, the higher physiologic temperature in an NGI enhances microbial reduction, reducing risks to cells and tissues.

**DISCUSSION**

Reported here are microorganism-dependent log reductions of CFU due to atmospheric conditions in closed-cell processing chambers in the absence of disinfectants. Consistent with their phenotypes, challenge species more sensitive to desiccation (e.g., *P. aeruginosa*) were found to be more susceptible to the warmer, drier NGI atmosphere. Many clinical-driven studies have reported extended microbial survival times on inanimate surfaces in the healthcare environment (4,5). However, these studies were performed in the higher RH and lower temperature of uncontrolled room air conditions. Likewise, the temperature/humidity effects on virus survival were done in room air (6).

*P. aeruginosa* and *C. albicans* showed significantly greater log reductions within one hour in the closed NGI compared to the conditions of a BSC. This conclusion supports the use of compressed, dry gases in closed cell processing chambers to help control microbial risks for cell and tissue production. The advantage of the dry NGI environment goes beyond the inherent barrier protections of closed systems in reducing the introduction of microorganisms from personnel.

It was not unexpected to see little reduction of *B. subtilis* under study conditions. Although desiccation resistant microbes, such as spore-forming bacteria, are a small proportion of ambient bioburden, sporicidal disinfectants still play a strategic role in a balanced disinfection regimen for cell production. Wiping down items prior to import with a sporicide can also reduce the incidence of viable spores inside the system without increasing fume risks to the cell products inside.

Temperature and humidity are major limiting factors of microbial growth (7). Below certain RH limits, microorganisms can survive but not proliferate. Bacteria will not grow at RH lower than 85% (8) nor will mold at RH lower than 76% (9). In a closed system that uses dry compressed gases, RH in the cell processing chamber can be controlled to well below these levels. Also, lower RH reduces the transfer of bacteria from fomite surfaces to gloved hands (10). Low RH in a closed system may reduce contamination risk by not only killing microbes and keeping them from reproducing, but also affixing them to chamber surfaces where routine wipe-downs can remove them. The use of dry gases in cell handling chambers, where contamination risks to cultures are highest, does not preclude the use of higher RH levels in attached incubation chambers, where cell culture vessels are closed, to reduce evaporation of cell culture media over the long term.

Temperature has been known for over 70 years to influence the viability of microbes in aerosols (11). In our studies, higher temperatures reduced recovery of viable *S. aureus* from processing chamber surfaces. Fortuitously, physiologic temperature (37 °C), which is optimal for human cells, significantly enhanced microbial kill in conjunction with low RH. Continuous control at 37 °C also

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**Figure 5.** Controlled temperature facilitates microbial reduction in BioSpherix next-generation isolator (NGI). Polypropylene or stainless steel discs were inoculated with *S. aureus* in processing chambers having constant 15% RH with varying temperature. Discs were collected at 0 and 4 hours. Data were expressed as mean of log reduction + standard error of mean (SEM) from three independent experiments. Asterisks (*) denote significant within-group differences determined by two-way analysis of variance followed by Sidak’s multiple comparisons test. (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

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eliminates suboptimal temperature effects on growth of the cell and tissue cultures, which can be stressed by even brief temperature changes (12). So the same environmental conditions that disadvantage microorganisms also are advantageous to cell and tissue products.

Stainless steel has been one of the most popular bioprocessing materials in the industry; however, it has been losing ground to the plastics in single-use technologies for decades (13). Unlike other reports in the literature (3), these studies here found that polypropylene performed as well as stainless steel in terms of both resistance and microbial resistance. Higher log reductions of S. aureus were shown at higher physiologic temperature on polypropylene compared to stainless steel. Polypropylene, which is also more expensive than steel, deserves further investigation for bioprocessing chambers, particularly in the walls and ceiling, which have lower abrasion risks.

If disinfectants in a closed cell processing system can pose a risk to live cell and tissue products (14–16), reducing the microbial risk via other means reduces the need for disinfectants. The ability to mitigate microbial risk through physical attributes of the cell handling environment may also reduce the need for antibiotics within the cell cultures as well, which have been linked to mitochondrial toxicity (17,18), and changes in metabolism (19), growth (20), and differentiation (21).

Atmospheric conditions (gas composition, temperature, and RH) are consistently circulated throughout and maintained in a closed system. Atmospheric conditions also do not rely upon the consistent performance of a technician to disinfect the processing chamber. Conditions inside the chamber can be monitored remotely, recorded automatically, and do not depend upon disinfectant expiration dating and coverage, and contact time for effectiveness. The controlled atmospheric attributes in a closed cell processing chamber offer real advantages for reducing microbial risk over the traditional room air BSC conditions for cell production processes.

CONCLUSION
To support emerging cell therapy products, both bioburden control and optimal cell growth conditions need to be considered. The combination of a consistent, higher physiological temperature and low RH within a closed NGI processing chamber offers improved control of vegetative microorganisms using control of the physical atmospheric parameters.

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REFERENCES