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An enhanced, statistically repeatable method for screening antimicrobial formulations in laundry applications, an adaptation of BSI EN 17658

KortneJo Anello[1], Albert E. Parker[2,3], Lauren Porter[1], Laura Purevdorj-Gage[1]✉ & Qilong Xu[1]

There is an increasing need for reliable methods to measure the reduction of microorganisms during the domestic laundering process. An enhanced throughput (ETP) methodology, an adaptation of the European standard BS EN 17658:2022, was developed as a screening tool for evaluating experimental laundry formulations with improved capacity and efficiency. The modifications include the replacement of a lab-scale tumbling device with a more compact and affordable instrument along with additional procedural modifications to simplify the test procedure without compromising the critical parameters such as mechanical action, bioburden, liquor ratio and soil. The ETP method was utilized to replicate the rinse cycle test from an international ring trial, where the participating laboratories assessed the efficacy of 0.04% Dodecyl dimethylammonium chloride (DDAC) as a benchmark standard. In addition, an experimental formulation was evaluated according to ETP and the full EN standard against the five required microorganisms including *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus hirae* and *Candida albicans*. Overall, the average log reduction values obtained with both the benchmark and experimental formulation did not differ between the two methods. Furthermore, based on the benchmark data, the ETP method consistently showed lower variability within a lab than the EN method, as evaluated in the ring trial. The advantages and weaknesses of ETP as a research screening tool are also discussed in this work.

Keywords Laundry, Sanitization, Antimicrobials, Throughput methodology, Rotawash

Domestic laundry practices are undergoing significant transformations with the global strive towards the reduction of energy consumption and use of chemicals with negative environmental and public health impact. Innovative washing devices that use less water, shorter wash cycles and lower wash temperatures are becoming a norm rather than an exception. In parallel, the demand for novel, eco-friendly laundry detergents and additives that provide efficient removal of soil and pathogen reduction under these new standards is also increasing. Thus, reliable methodologies that can assess antimicrobial performance under the conditions that are relevant to domestic laundering are needed for both regulatory, research, and screening purposes.

On August 15th, 2022, the European Committee for Standardization (CEN) released a standard methodology BS EN 17658:2022, and the minimum performance requirements for establishing if a chemical product used in the domestic laundry procedures have antimicrobial activity. In prior years, an international ring trial involving seven laboratories from various firms, including Arxada LLC., was organized to evaluate the robustness and reproducibility of this method according to the ISO 5725–2 and ISO 13528 standards[1–3]. The participant laboratories evaluated 3 levels of active substances and 7 microbial parameters in both main wash and rinse cycle, demonstrating good reproducibility and repeatability across different laboratories[1]. This allowed the CEN to finalize the method as an EN standard and which can be used to generate efficacy data as required by the Biocidal Product Regulation (BPR), (EU) 528/2012.

The EN test procedure as depicted in Fig. 1a utilizes lab-scale devices such as the Rotawash (Atlas, Rock Hill, SC, USA) which was previously described and validated for domestic application[4]. Overall, the EN17658

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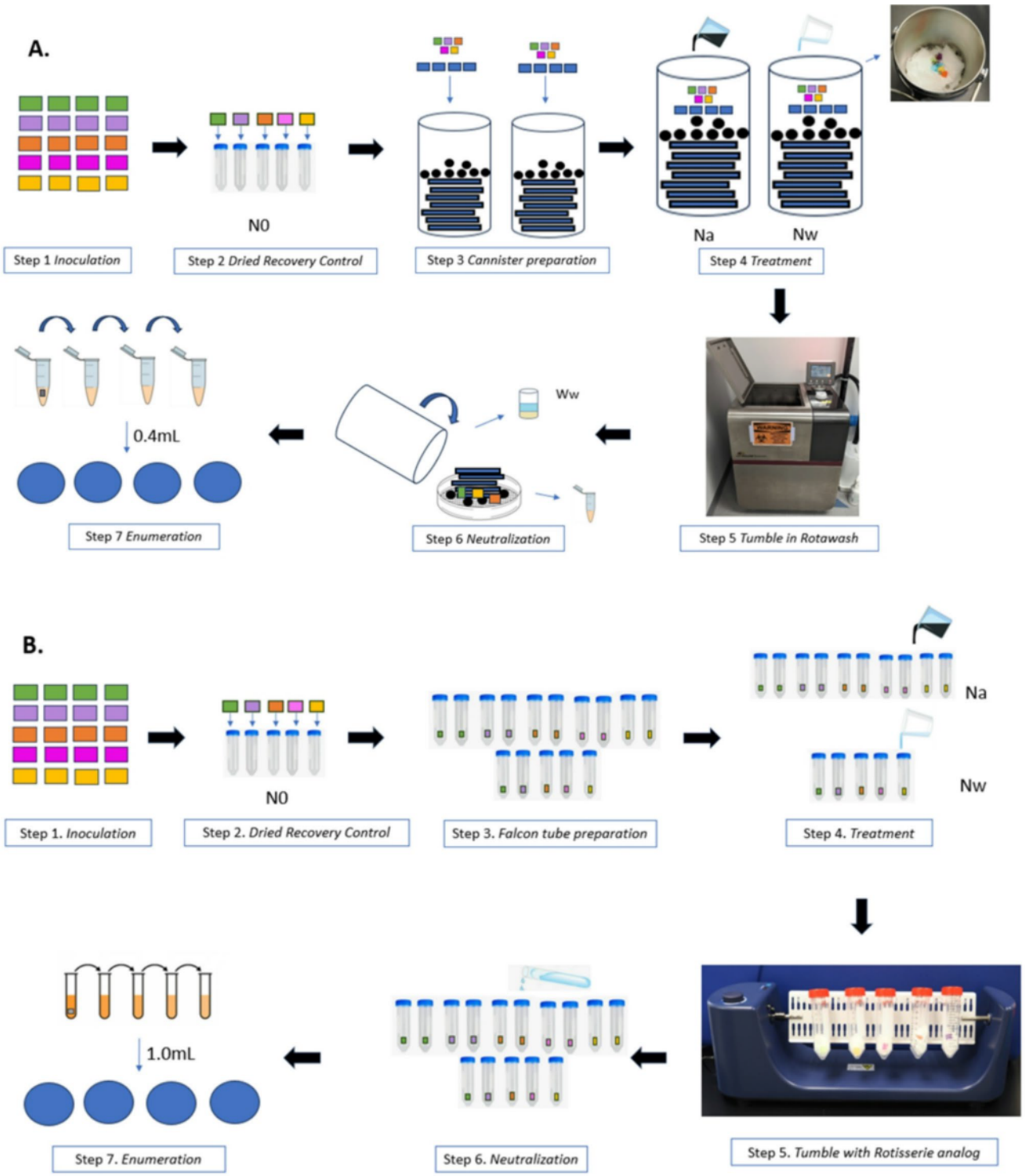

Fig. 1. A schematic representation of BSI EN 17658:2022 (**A**) and ETP method (**B**). Textile carriers are represented by small rectangles, color-coded for each test microorganism type. Dark circles are metal beads.

standard[5]affords improved capacity and efficiency as compared to the preceding EN16616 protocol[6] which requires a life-sized washing machine for healthcare applications. In addition, the new standard provides the option to test prewash, main wash, and rinse phases separately, allowing the assessment of a broad range of antimicrobial additives offered in domestic markets. However, the Rotawash device is costly; large (25"L x 26"W x 43"H), requiring a dedicated electrical circuit along with floor drainage depending on specific building code requirements. Additionally, the new EN 17658 is labor-intensive and time-consuming and therefore not ideal in research settings where multiple formulations and test conditions need to be assessed quickly and economically (see Supplemental Information for further details on the EN standard). In a single unit of Rotawash device,

8–12 canisters can be processed in parallel under the same parameters. However, running 8–12 canisters under realistic conditions presents a significant challenge. In our experience for example, it was feasible to test only one formulation with the untreated hardwater control (Nw), if we were to fully replicate the test with one operator. Preparation of test ballast requires considerable preparatory work which includes cutting of the ballast fabric into 10 cm x 10 cm squares and boiling the materials in distilled water twice before letting them air dry before testing. This preparation step alone requires at least 1–2 days to complete ballast materials sufficient for a single set of experiments consisting of a control and test sample. During the test method execution, multiple operators' involvement in parallel may be required to meet the procedural timelines specified by the method. For example, the inoculated carriers are required to be assayed immediately following the drying process while the test products diluted in the hardwater need to be tested within 2–3 hours, therefore limiting the number of replicates and experimental time available to the operator(s). In addition, after the tumbling is completed, there is a step termed as "fishing procedure" which needs to be completed within 5 minutes of post-tumbling time. Fishing is a procedure where an operator must sort through the ballast materials and retrieve the individual test (Na), control (Nw) and cross contamination (RI) carriers and place them into separate neutralizing solutions. Depending on the number of test replicates, this step can limit the throughput, often requiring more than one operator working in parallel to meet the 5-minute deadline for each sample and control. Once the test and control carriers are placed into separate tubes containing a neutralizing solution, each tube will need to be processed and plated within 30 minutes; and this introduces further stringency to the workflow.

In this work, we developed a modification of the EN 17658:2022 standard by replacing the Rotawash device with a bench-top device, Analog Rotisserie Tube Rotator (Scilogex®, Fig. 2) along with some additional, procedural modifications (Fig. 1b) that increase throughput while maintaining the mechanical action (tumbling) and ballast-to-liquor ratio consistent with the original standard. Weighing approximately 6 pounds, this commercially available device is more compact (21"L x 6"W x 8"H) and significantly more affordable compared to the Rotawash device. The device accommodates up to 12 individual centrifuge tubes, which are clamped onto a rectangular platform attached to a horizontal rotisserie rod. It operates on 110–220 Voltage and can rotate the sample tubes along the horizontal axis at adjustable speeds ranging from 10 to 80 revolutions per minute (rpm). It can be safely used within a temperature range of 5 to 40°C, complying with EN standard guidelines, which specify a maximum temperature of 40°C during the main wash cycle and allow for ambient temperature during the rinse stage cycle.

The new method, referred to as Enhanced Throughput Method (ETP), was used to evaluate the efficacy of the DDAC at 0.04% as tested in the ring trial. Previously, the dose of DDAC showed borderline effectiveness and had higher standard deviations compared to treatments using water or the effective concentration of DDAC that was tested in the ring trial[1]. Since disinfectant concentrations with intermediate efficacy often led to greater variability[7], we chose the 0.04% DDAC dose for this study as a more rigorous benchmark to evaluate the repeatability of the ETP method. The test was replicated on three independent days with duplicate exposure vessels for each test microorganism. The mean LR and repeatability standard deviation (SD) across tests for DDAC 0.04% were compared between ETP and EN17658. A research formulation was tested according to both ETP and the full method, and statistical comparisons of the mean LR values were conducted across the two test data sets to validate the method further. Finally, the impact of our modifications introduced to the EN method in parallel with the strengths and weaknesses of ETP as a screening tool were also discussed.

Results

Study replication and exposure conditions

Three independent tests simulating the rinse cycle[1] were conducted with each of the mandatory test organisms including *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus hirae* and *Candida albicans* in duplicate exposure vessels. The treatment solution was tested at a concentration of 400 ppm (0.04%) of active DDAC applied in a 1:5 ballast-to-liquid ratio with a tumbling time of 15 min. After the incubation period, 30 ml of the neutralization solution was added directly to each exposure vessel and held for at least 10 min before performing the microbiological enumeration assay, as described in the materials and methods section.

For the calculation of CFU recoveries, data from any dilution for which CFU < 14 were ignored unless that was the only data available in which case the CFU was substituted with 14 as per EN norm[5] to best compare to the

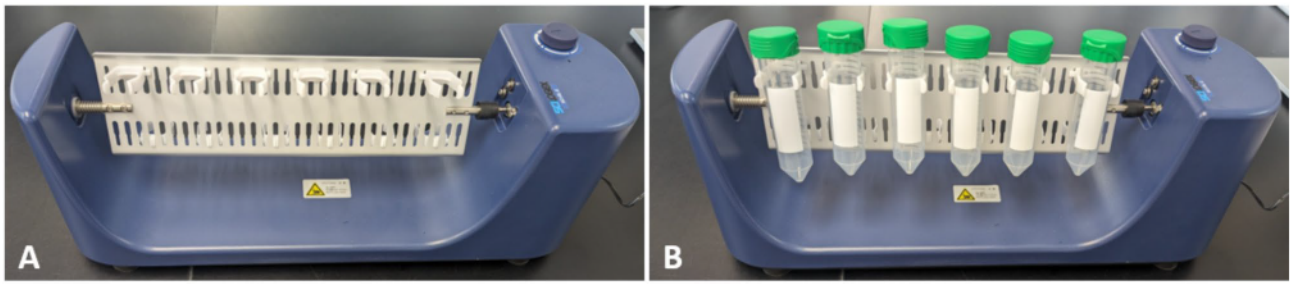

Fig. 2. Scilogex rotisserie analog device (A) and the device loaded with 6 falcon tubes on each side (B).

	Inoculum (N Log_{10} CFU/ml)					Carrier (N_0 Log_{10} CFU/carrier)					N_0 - Nw				
	PA	EC	SA	EH	CA	PA	EC	SA	EH	CA	PA	EC	SA	EH	CA
EN Standard	≥9.18		≥8.18		≥7.18	≥6.60				≥5.60	≤2.00				
ETP (0.04% DDAC)	9.27	8.87	8.46	8.84	7.13	7.44	7.04	6.87	7.12	5.62	0.12	0.01	0.25	−0.07	0.07
ETP (RD Formulation)	9.22	9.04	8.76	9.01	7.63	6.93	6.84	6.79	6.80	5.82	0.05	0.03	0.02	−0.39	0.14
EN17658 (RD Formulation)	9.41	9.15	9.21	8.78	7.76	7.53	6.78	7.11	7.28	6.10	1.37	1.01	0.60	0.78	0.72

Table 1. The control numbers recovered from ETP, and the full EN test methods as compared to corresponding EN standard requirements as shown across the first row. Each point on the table is a mean Log_{10} CFU concentration measured in replicate samples as described in the materials and methods section. Pa = *P. aeruginosa*, Ec = *E. coli*, Sa = *S. aureus*, Eh = *E. hirae*, Ca = *C. Albicans*. N = microbial suspensions, N_0 = dried recovery control, Nw= untreated control with EN hardwater of approximately 375 ppm hardness as calcium carbonate.

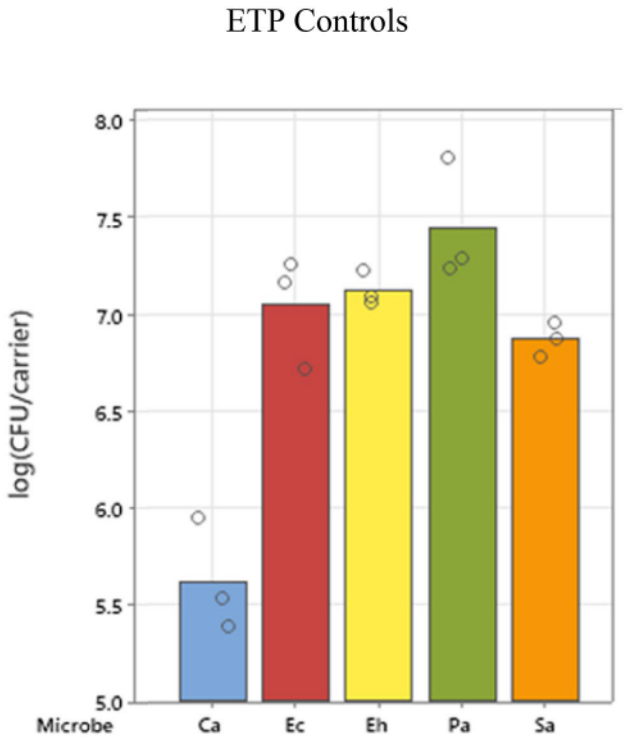

Fig. 3. Mean population density recovered from the dried recovery controls (N_0) from each test microorganism (bars) and each point (open circles) in the figure is the Log_{10}CFU/carrier for a single carrier in a single ETP test.

ring trial[1]. A substitution rule of 0.5 CFU only for zero CFUs was also used that better demonstrates the method's limit of detection (see supplemental information).

Population density control numbers

In general, the EN standard requires minimum population density targets for the test microbial suspensions (N) and minimum target recovery from the dried recovery control carriers (N_0), which are provided in Table 1. The EN test also requires the difference between the Log_{10} N_0 and Log_{10} Nw to be equal to or less than 2 Log_{10} CFU/carrier to ensure a population density sufficient for demonstrating a 3–4 Log_{10} CFU/carrier inactivation effect on test carriers (Na). Overall, the control numbers for N, N_0 and N_0-Nw obtained in our ETP tests were within the required range according to the EN standard, except for *C. albicans* and *E. coli* inoculum. The differences between Log_{10} N_0 and Log_{10} Nw were within the set limit across all species. The N_0 control data associated with the 0.04% DDAC treatment are plotted in Fig. 3. The repeatability standard deviations (Sr) of the N_0controls ranged from 0.087 to 0.317 (Table 2) indicating excellent reproducibility of the controls[8] (Fig. 3).

Mechanical action

Mechanical action can be defined as physical forces that move textiles within a laundry drum while creating shear and friction between the fabrics. This friction facilitates the removal of soil and microorganisms from

Microbe	Mean (log_{10} CFU/carrier)	SE Mean	Sr	95% LCL (log_{10} CFU/carrier)	95% UCL (log_{10} CFU/carrier)
Ca	5.62	0.1700	0.2944	4.89	6.35
Ec	7.04	0.1642	0.2844	6.34	7.75
Eh	7.12	0.0504	0.0873	6.91	7.34
Pa	7.44	0.1830	0.3169	6.66	8.23
Sa	6.87	0.0520	0.0901	6.64	7.09

Table 2. The mean log density (Log_{10} CFU/carrier), standard error (SE) of the mean, repeatability SD (Sr) and the lower (LCL) and upper (UCL) confidence limits of N_0 based on three independent studies with 0.04% DDAC in ETP.

	ETP at one lab				EN17658					COMPARISON (ETP – EN17658)				
Microbe	Mean LR	S_r	LCL mean LR	UCL mean LR	Mean LR	S_r	S_R	LCL mean LR	UCL mean LR	Diff Mean LR	LCL Diff	UCL Diff	p	Ratio of S_r's
Pa	2.80	0.23	2.22	3.38	2.63	0.71	0.91	1.68	3.58	0.17	−1.77	2.10	0.83	0.33
Ec	4.09	0.11	3.81	4.38	3.14	0.55	1.07	2.02	4.26	0.96	−1.59	−3.80	0.34	0.21
Sa	4.12	0.09	3.90	4.34	3.06	0.44	1.12	1.89	4.24	1.06	−1.84	4.06	0.38	0.21
Eh	4.08	0.43	3.04	5.13	2.94	0.66	1.23	1.64	4.23	1.15	−1.94	4.23	0.38	0.64
Ca	2.87	0.29	2.14	3.60	3.14	0.73	1.00	2.10	4.19	−0.27	−2.49	1.94	0.76	0.40

Table 3. The LRs and repeatability SDs (S_r, across tests in the same lab) of the LRs for all 5 species using ETP tested at one lab at Arxada to the EN method tested in a 6-lab ring trial. LCL diff and UCL diff is a 95% confidence interval for the difference in mean LRs. Based on the ring trial, S_R is the reproducibility SD of the LRs across multiple labs (which was only possible to calculate for the EN method; reproducibility of the ETP is unknown).

the textile fibers through the combined action of water and detergent. Several factors influence the level of mechanical action, including the volume of water, the fabric-to-liquid ratio (also known as the liquor ratio), and the speed of drum agitation[4].

In our study, we utilized 50 mL size falcon tubes because it allowed the reduction of the ballast and liquor amount by 50-fold while maintaining the same 1:5 liquor ratio (2 g of ballast and 10 g of either control hardwater or test solution) with a comparable size of headspace that is achieved in the actual 1 L canister. Furthermore, we set the rotational speed of the rotisserie analog device to 40 rpm, matching the speed of rotation employed in Rotawash. The test tubes were positioned perpendicular to the rotational axis, allowing the vessels to rotate in a manner similar to how canisters are rotated in Rotawash.

The effectiveness of mechanical action can be assessed by measuring the number of microorganisms removed from textiles during water treatment. This removal can be quantified by subtracting the mean Nw values (untreated hardwater control samples) from the mean N_0 values (dried recovery control samples). As previously mentioned, the EN test specifies that the difference between Log_{10} N_0 and Log_{10} Nw should be equal or less than 2 Log_{10} CFU per carrier. A larger difference may indicate excessive removal or reduction of cells from the control carriers, influenced by physical factors such as temperature, liquor ratio, and, in this case, the variations in the mechanical action resulting from different test vessels. Since we are adding the neutralization solution directly into the vessel, this difference cannot be measured according to our approach. However, we conducted an additional set of tests where the Nw carriers were transferred to separate neutralization tubes and the differences between Log_{10} N_0 and Log_{10} Nw were calculated for each bacterial species (1.57 ± 0.01 PA, 1.37 ± 0.19 EC, 0.97 ± 0.15 SA and 2.01 ± 0.13 EH) and compared against the appropriate set of data collected during the ring trial (see Discussion).

Comparative efficacy of benchmark 0.04% DDAC treatment

Table 3 compares the mean LRs and repeatability SDs of the LRs for all five species using ETP tested just at Arxada's one lab to the EN method tested in a 6-lab ring trial. In terms of mean LR values, *P. aeruginosa* achieved 2.80 LR, *E. coli* achieved 4.09 LR, *S. aureus* achieved ≥ 4.12 LR, *E. hirae* achieved ≥ 4.08 LR and *C. albicans* ≥ 2.87 LR. The two data sets were statistically compared in Table 3 and Supplemental Table S1. There were no statistically significant differences ($P > 0.05$) in the mean LR for any microbe, although the mean LR for the ETP were higher for all microbes except *Candida*. Importantly, the repeatability of ETP was better than EN17658 (that is, the SD S_r was smaller for ETP).

Comparative efficacy of research formulation

To further validate the method, a research formulation was tested according to ETP and the full EN approach. The mean LR data along with the control limits are presented in Fig. 4; Table 1, respectively. Overall, mean LR data for ETP and the full method ranged from 2.26 to 4.33 and 2.45–4.54, respectively (Fig. 4). *P. aeruginosa* yielded CFU values of >330 or TNTC (too numerous to count) in all dilutions assayed in both ETP and EN methods, indicating the formulation was not effective against this organism.

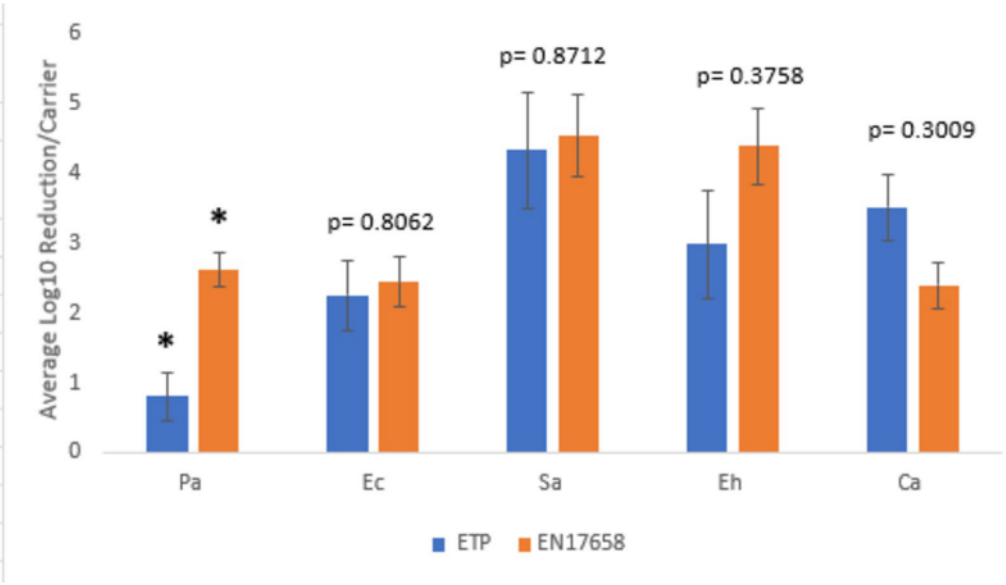

Fig. 4. Antimicrobial performance of RD formulation expressed as the mean LR values for each test microorganism in ETP (blue bars) and the full EN method (orange bars). The tops of the bars indicate a mean LR, the error bars indicate ± standard error of the mean LR calculated from $n = 1$ test for ETP, and $n = 2$ replicate tests for the EN method. *Log reductions, while accounting for the LODs of ETP and EN17658, are < 0.81 and < 2.61, respectively, indicating that Pa did not result in noticeable reduction in both methods.

Discussion

Laundry sanitization is a dynamic process involving a range of interdependent parameters including soil, temperature, contact time, mechanical effect, and chemistry. A reliable screening method should incorporate such parameters in a cost- and time-efficient manner while yielding endpoint data that are statistically comparable to those generated by the original test method.

In this work, we developed a simplified version of the EN 17658:2022 standard while maintaining the mechanical action (tumbling) and ballast-to-liquor ratio consistent with the original standard. Our adaptations include the replacement of Rotawash with a smaller, less expensive device called rotisserie tube analog (Fig. 2). A single unit of rotisserie analog allows up to 12 sample tubes with size capacities ranging from 12 to 50 mL volume. Depending on the experimental parameters, the sample number can be scaled up to 24 if two units of rotisserie analog are used in parallel. In our case, we miniaturized the test vessel down to a 50 mL size falcon tube while maintaining the same 1:5 liquor-to-ballast ratio with a comparable size of headspace that is achieved in the actual 1 L canister. Secondly, to streamline the downstream processing as well as to maintain the total bioburden per ballast and per liquor volume as close to the full test as possible, we placed each test swatch separately within a single falcon tube instead of placing all 5 pre-contaminated swatches in one vessel. In addition, we eliminated the addition of metal beads and the addition of cross contamination (RI) swatches to simplify the testing procedure. Finally, immediately after the tumbling time is complete, the neutralization liquid was added directly into the falcon tube to eliminate the fishing procedure. The elimination of "fishing" procedure increased the throughput of our approach, but it increased the limit of our detection since the total volume of liquid present in the exposure vessel is 40 mL (neutralizing solution 30 mL + liquor 10 mL). The total volume plated in the EN method is 0.4 mL and 1 mL in ETP, resulting in a LOD of 1.15 log and 2.75 log in EN and ETP, respectively.

Elimination of metal beads did not appear to impact the mechanical action generated in our ETP vessels. According to our mechanical action confirmation test, the differences calculated between the mean N_0-Nw values were comparable to the values obtained in the Ring trial data (1.86 ± 0.31 for PA, 1.50 ± 0.39 for EC, 0.92 ± 0.46 for SA and 1.38 ± 0.38 for EH) suggesting that the mechanical action in our ETP vessel was also comparable to the one generated in the Rotawash canister. Due to low recovery efficiency, CA was omitted from the mechanical confirmation study.

In keeping the remaining test parameters consistent with the EN standard, we utilized our ETP approach to evaluate the efficacy of 0.04% DDAC substance which was the same substance tested in the ring trial study (personal communication with Lauren Porter). We also compared ETP to the EN standard using an internal research formulation.

For the test to be valid, the EN standard requires minimum concentration limit for both the inoculum suspension (N) and dried carrier (N_0) counts for each microorganism (Table 1, first row). In our study, the mean N and N_0 values for each replicate test met the minimum criteria for each test bacterial species, except for *E. coli*. The mean N value for *E. coli* was 0.31 Log_{10} CFU/ml lower than the 9.18 Log_{10} CFU/ml requirement. However, N_0 consistently achieved the ≥ 6.60 Log_{10} CFU/carrier requirement despite the shortcoming. Additionally, the mean N value for *C. albicans* was about 0.05 Log_{10} CFU/ml lower than the limit due to a low N value measured in one of three replicate tests and the mean density recovered from the control carriers (N_0) were slightly lower

in two replicate studies (Supplemental Table S2). These slight differences in the control carrier counts did not appear to have influenced the efficacy outcome of DDAC against the yeast since the mean LR values between the two studies were not statistically different ($P=0.76$). Nevertheless, lower CFU counts observed with the yeast should be addressed in future studies. Based on the difference between the median N and N_0 values, it appears that approximately 1.51 Log_{10} CFU per carrier of *C. albicans* cells either died during the drying process of the carriers or were not recovered due to low recovery efficiency. Recovery could be improved by reducing the drying time of the carriers post-inoculation (personal communication, Lauren Porter) or by using more effective cell recovery techniques, such as longer vortex mixing, sonication, or a combination of both.

In terms of mean LR differences, our method tended to give bigger LR values, particularly for *E. coli* (LR = 4.09), *S. aureus* (LR ≥ 4.12 LR) *E. hirae* (LR ≥ 4.08 LR) and *C. albicans* (LR ≥ 2.87). Also, the range of LR values in ETP was wider (2.80–4.12) as compared to the range of LR generated by the ring trial (2.63 to 3.14). It should be noted that in the EN method, all carrier types are placed in the same vessel during exposure and if antimicrobial treatment is not effective, cross-contamination can occur. In fact, according to the ring trial data, the average population density recovered from the cross-contamination (RI) carriers were 2.02 and 3.60 Log_{10} CFU/carrier in the 0.04% DDAC treatment and control groups, respectively. It is, therefore, highly likely in the case of an ineffective level (0.04%) of DDAC treatment, species such as *P. aeruginosa* may have cross-contaminated other test carriers, resulting in more uniform LR values across the other species. Therefore, the effect of cross-contamination observed in the full EN test may, in part, explain the lower LR values measured in *E. coli*, *S. aureus* and *E. hirae* as compared to the ETP study.

Interestingly, among the five microorganisms tested, *P. aeruginosa* yielded the lowest LR values in both methods, indicating that this is the most challenging organism to treat. As compared to Gram-positive species, *P. aeruginosa* is considered more resistant to Quaternary ammonium compounds (QACs)[9]. Since in our ETP approach the microorganisms are added separately, cross-contamination is avoided and the antimicrobial efficacy of a given molecule can be assessed against each test organism, allowing the identification of *P. aeruginosa* as the limiting organism for our screening. Our approach allows for a deeper insight into the mode of action of a given test compound as well as the opportunity to focus on the most challenging organisms for a given product or test condition.

In addition, differences in the exposure time between the international ring trial and ETP should not be discounted. The contact time for ETP was set to 15 mins, compared to the 10 min contact time employed in the ring trial. The longer contact time for ETP accounts for the 'fishing time' in EN17658, where the carriers are removed from the exposure vessel but still covered in test substance for up to five mins before neutralization. The longer contact time set for ETP may explain where LRs were slightly higher for organisms compared to the ring trial. Fishing time is dependent on the analyst's skill and level of experience and therefore may have varied across the participating laboratories. Since the fishing duration is not reported, the exact exposure time during the ring trial is not known, and exposure time should be assumed to be between 10 and 15 min.

Despite these differences, statistical comparisons between the ETP and ring trial data sets did not yield statistically significant differences (Table 3). Furthermore, the ETP method was always less variable within a lab compared to the EN method as assessed in the ring trial (Table 3, Supplemental Table S1), suggesting better repeatability and in our opinion, sufficient correlation with the full EN method to serve as a research screening tool.

Parameters that were not considered in ETP are wash water (Ww) and cross contamination (RI) controls. However, internal screening of the EN17658 method show that formulations that are successful in reducing bacteria on the test swatches are also successful in sanitizing the wash water and therefore prevent cross-contamination in the test canisters (data not shown).

The EN lab-scale devices can simulate laundry prewash, main wash, and rinse phases separately, allowing the assessment of a broad range of antimicrobial additives that could be offered in domestic markets. In our study, only the rinse cycle was assessed by the ETP method. The antimicrobial activity of QACs is deactivated by negatively charged surfactants typically used in laundry detergents. Therefore, QACS are primarily used in rinse stage or pre-soak application processes. Future studies should investigate other biocidal actives and formulations to ensure results between the two methods are translatable. It should be noted, however, that the rotational speed of rotisserie analog is adjustable (10–80 rpm), and the device can be operated at a range of temperatures with varying vessel size and type. Therefore, factors such as mechanical action, temperature and liquor ratio can be controlled, and a range of laundry parameters can be evaluated and adapted for other standards. For example, the rotisserie analog device can be applied as screening for some American standards such as ASTM E2274 or ASTM E2406, which also use lab-scale devices with similar rotation frequency along the horizontal axis. However, the ballast liquor ratio, microbial load per textile carrier and the ballast type must be adjusted accordingly to better reflect the full test. One significant drawback of the ETP method is that wash cycles above 40 °C are not feasible to study due to the operational limitations of the rotisserie device. However, it can be argued that current industrial trends along the antimicrobial efficacy methods such as EN and ASTM standards, are shifting primarily towards the use of ambient to cold water, highlighting the relevance and utility of the ETP device. In conclusion, the data generated with ETP method was not statistically significantly different from the data generated in the international ring trial. The use of a compact and more affordable device such as rotisserie analog in ETP enables a practical and cost-efficient approach to screen experimental formulations for domestic laundry applications. It is important to note that the ETP method was developed for research screening purposes and is not intended to replace the EN standard, which remains the official standard for product testing and qualification under BPR.

Materials and methods

Test organisms

Pseudomonas aeruginosa NCTC 13359/ ATCC 15442, *Escherichia coli* NCTC 10418/ ATCC 10536, *Staphylococcus aureus* NCTC 10788/ATCC 6538, *Enterococcus hirae* NCTC 13383/ ATCC 10541 and *Candida albicans* NCPF 3179/ ATCC 10231. All micro-organisms were purchased from American Type Culture Collection (ATCC), Manassas, Virginia, U.S.A. Organism cultures were prepared from single use cryovials and passaged according to the EN17658 standard. Bacterial strains were passaged twice onto Trypticase Soy Agar (TSA) slants with each passage incubated for 24 ± 2 h at 36 °C. *Candida albicans* was passaged and incubated at 30 °C for 72 h on Malt Extract Agar (MEA) slants.

Media and test reagents

TSA (Thermofisher, Waltham, MA, U.S.A.); MEA (Thermofisher, Waltham, MA, U.S.A.); Tryptone sodium chloride water, TSC, prepared according to the EN17658 method (Tryptone, pancreatic digest of casein 1.0 g, Sodium chloride (NaCl) 8.5 g, Water to 1000mL, adjust pH to 7.0 ± 2); double strength Dey-Engley broth, 2xDE (Thermofisher, Waltham, MA, U.S.A.); standard EN hardwater of approximately 375ppm calcium carbonate was prepared according to the EN17658 method (Solution A- 19.84 $MgCl_2$ and 46.24 g $CaCl_2$ diluted in 1000 mL deionized water. Solution B- 35.02 g $NaHCO_3$ diluted in 1000mL deionized water. Aliquot 6.0 mL of solution A and 8.0 mL of solution B to 1000 mL of deionized water. The pH was adjusted to 7.0 ± 0.2.

Test system

Rotisserie analog (Scilogex, LLC MS) and sterile 50 ml falcon tubes (VWR Scientific) were used to simulate the lab-scale device and individual laundry drum (exposure vessel), respectively (Fig. 2). The rotisserie analog was set to 40 rpm per the CEN standard and rotational speed was verified using a tachometer (CEN-tech Digital Photo Sensor Tachnometer, Harbor Freight). In total, 15 falcon tubes were used in ETP as exposure vessels (Na and Nw).

Ballast fabric

Polyester cotton blend (65% polyester and 35% cotton) fabric (wfk 20 A, TestFabrics USA), referred to as polycotton ballast, was used to mimic the composition of domestic laundry. To prepare the polycotton ballast, the fabric was cut into 10 × 10 cm squares and boiled in deionized water for 60 min. After boiling, the fabric was hung to air dry overnight. As part of the method adaptation, the 10 × 10 cm squares were then cut into smaller squares used as ballast in 50 mL falcon tubes. Two grams of ballast were added to each falcon tube before autoclaving.

Test swatches

Cotton 1 × 1 cm precut squares (wfk 10 A, TestFabrics USA) were inoculated with the test organism. At least four test swatches were prepared per organism: two swatches for ballasts to be treated with test substance run in duplicate per organism (Na), one swatch for the ballast to be treated with hardwater (Nw), and one swatch for the dried recovery control (N_0). Each test swatch was added to separate falcon tubes. In total, 20 swatches were prepared for testing ETP.

Test substances

Benchmark, 0.04% Dodecyl dimethylammonium chloride (DDAC), was utilized to align with the interlaboratory rinse stage sanitization study[1]. Standard European hardwater of approximately 375 ppm calcium carbonate was used as the untreated control (Nw) and as a diluent to prepare the test substance, DDAC, at the required dose of 0.04%. Similarly, the experimental RD formulation was diluted in hardwater. Each test substance is tested within 2 h of dilution in hardwater. The term liquor refers to the liquid added to the exposure vessel.

Organism culture

On test day, the agar slants were washed with TSC solution, and the density was adjusted (A nm = 620 nm) to target the minimum of $\geq 1.5 \times 10^9$ for Gram negative bacteria, $\geq 1.5 \times 10^8$ for Gram positive bacteria and $\geq 1.5 \times 10^7$ for the yeast. The suspensions were then centrifuged for 5 min at 4700 g, the supernatant was removed and resulting pellets were resuspended in 0.3% Bovine Serum Albumin (BSA, Thermo Scientific Chemicals). The concentration of each test microorganism suspension was validated by enumerating the Colony Forming Units per ml (CFU/ml) by serial dilution and plating with TSA (bacteria) and MEA (for the yeast) in duplicates.

Test carrier drying

Organisms were differentiated by color dyed swatches. Each sterile swatch was aseptically transferred to sterile petri dishes, and 10 µL of organism suspension was pipetted onto each carrier. The test swatches were left to dry for up to 20 min in a biological safety hood. After drying, each carrier was transferred to its own individual falcon tube for testing. Immediately after the drying is complete, the dried recovery control swatches (N_0) were placed into neutralizing solution and assayed for Log_{10} CFU/carrier.

Tumbling procedure

A 10 mL solution of diluted test substance or hardwater was added directly to each prepared ballast containing one inoculated test carrier. The test substance was added to each falcon tube at staggered intervals. The contact time initiates when test substance was added to the first falcon tube. At the end of contact time, 30 mL of neutralizer was added, also in intervals. This staggered approach allows up to 12 samples to be processed within a

single run. The neutralizing solution was then vortexed for 10 min and a 1 mL aliquot of this solution was serially diluted in TSC and pour plated with TSA or MEA in duplicates.

Neutralization validation

To validate neutralization, 10 mL of test substance was added to 30 mL of neutralizer (2x strength Dey-Engley broth, Sigma-Aldrich) in a test tube. After 5 min, 0.1 mL of test suspension at 10^{-5} dilution was added to the neutralized solution and allowed to sit for another 5 min. After the exposure time, 1 mL of the solution was plated in duplicate with the appropriate agar. For comparison, the process is repeated replacing 10 mL of diluted test substance with hardwater. Neutralization verification was performed on each organism, resulting in 10 separate test tubes.

Test carrier dye validation

To validate the use of different dyes (Dylon Hand Dye, Henkel) on test swatches, dyed and undyed swatches were inoculated in the same manner as test swatches and dried in parallel. After drying, the swatches were transferred to individual tubes containing 10 mL neutralizer, vortexed for 10 min, then 1 mL of the solution is plated with the appropriate agar. Lack of toxicity from the dyes were confirmed by comparing the CFU/mL recovered from dyed and undyed swatches (data not shown).

Confirmation of mechanical action

Pre-contaminated test swatches were individually placed into the untreated control exposure vessel containing 2 g of ballast and 10 mL hardwater and allowed to rotate on the rotisserie analog instrument for 10 min. After the tumbling time is completed, each swatch was transferred into a 30 mL neutralizing solution and vortexed for 5 min to dislodge the attached cells from the test swatches. The aliquots of the neutralization solution are then serially diluted and plated onto either TSA or MEA plates. The Log_{10} CFU/carrier recovered from the untreated control swatches (Nw) are then subtracted from the Log_{10} CFU recovered from the dried recovery (N_0) control swatches to estimate the number of cells dislodged due to the mechanical action of tumbling.

Data analysis

The number of colony forming units (CFUs) on each plate were converted to Log_{10} and averaged. Log reduction (LR) of the test swatches was calculated by the following formula:

Log_{10} reduction = Log_{10} CFU N_0 (dried recovery control) – Log_{10} CFU Na (treated swatches).

The CFUs on carriers were calculated by the following formula. The densities were then log transformed to a log density (LD = $\log_{10}$(CFU/carrier)).

(1) $N = \frac{C}{(n1 + 0.1n2 + 0.01n3)\ d} \times A$

N = N_0, Na or Nw.

C = sum of CFU counts taken into account.

n1 = Total plate volume at the lowest dilution (i.e. 10^{-1})

n2 = Total plate volume at the higher dilution (i.e. 10^{-2})

n3 = Total plate volume at the highest dilution (i.e. 10^{-3})

d = dilution factor corresponding to the lowest dilution (i.e. 10^{-1})

A = total volume of test substance and neutralizer

Substitution of 14 CFU: Only CFU data larger than 14 were used to calculate a log density (LD = log (CFU/carrier)). That is, all CFU data less than 14, at any dilution (for example CFU = 10) were ignored, whether for the control or treated carriers. For all of the CFU data at 14 or larger, across all dilutions from the *first* to the *last*dilution the CFUs were weightedly averaged together as explained in Eq. (1) above[10]. For the treated carriers, whenever all CFUs on both plates at all 3 dilutions exhibited CFU < 14, then 14 CFU was substituted into both plates (equivalent to substituting in 28 into one plate) at just at the 0th dilution then scaled up as.

$$\text{CFU/carrier} = \frac{40(14 + 14)/2}{10^0} = 560.$$

An alternate substitution rule of CFU = 0.5 only for zero CFUs is considered in the Supplementary Information.

Test replications

The 0.04% DDAC study was replicated in three independent ETP tests with each test conducted with duplicate treatment (Na) groups for each test microorganism. The experimental formulation was conducted in one independent ETP test and two independent EN tests with duplicate exposure vessels for each treatment group for each test microorganism. Per the EN standard, each test included a singlet of N, N_0 and Nw samples. In all studies, each sample was plated in duplicates and the data is reported as the mean Log_{10} CFU, unless otherwise noted.

Statistical analysis

The mean LR and repeatability SD (S_r) of the LRs (across tests in one lab) for the ETP assay were calculated taking an arithmetic average and SD of the LRs from $n = 3$ tests for each species.

ETP assay's outcomes were compared to the Ring Trial's EN17658 outcomes using ANOVA applied to the mean LR for each lab for each species. From the ring trial, the reproducibility SD (S_R) of EN17658 was calculated (across multiple labs); hence $S_R \geq S_r$. Because test-level EN data were not available for any lab in the ring trial, the *t*-test was applied to Arxada's mean LR (over $n = 3$ tests) and repeatability SD (S_r) for each method and species combination. In Arxada's lab only, ETP assay's outcomes were compared to EN17658 outcomes using an

R&D formulation using 2-sample *t*-test. All ANOVA and graphical analyses were performed in Minitab v21.4.2. Welch *t*-tests were performed in R v4.4.0[11]. Individual value, normal probability and residual versus fits plots were used to assess the assumptions of the statistical models and identify potential outliers.

Data availability

All data are available from the corresponding author upon request.

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Author contributions

KA designed, executed, analyzed and wrote the manuscript. AP performed statistical analysis and review of the manuscript. LP provided technical support and execution of RD formulation and reviewed the manuscript. LP-G designed, analyzed and wrote the manuscript. QX designed, executed, analyzed and wrote the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Impact statement

An antimicrobial screening method was developed utilizing a compact tumbling device which simulates mechanical action and ballast load consistent with domestic laundering. Statistical comparisons of the antimicrobial efficacy data generated by this approach were compared against the data generated by an international ring trial for EN17658. The new method offers multiple benefits as a screening tool by providing increased capacity, reduction of labor and materials used, and ability to assess efficacy against individual organisms including *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus hirae* and *Candida albicans*. The tumbling device offers flexibility to evaluate a range of laundry parameters and can be easily tailored for other standardized methods such as ASTM 2274.

Additional information

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