

HUMANITARIAN INNOVATION FUND

WASH Grant Final Report

Organisation Name	Action Against Hunger-Spain and London School of Hygiene and Tropical Medicine (LSHTM)
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Project Title	Moringa leaves as handwashing product for water and soap scarce contexts
Partner(s)	National Public Health Reference Laboratory (NPHRL) of Ghana
Problem Addressed / Thematic Focus	<p>Handwashing with soap is crucial to intervene in the disease transmission cycle. The short supply of soap and water in some contexts, affects drastically hygiene practices, mainly handwashing. These contexts refers to arid environments or to scenarios where water supply or soap are discontinuous.</p> <p>Based in previous studies, the idea was to assess the potential of Moringa oleifera as a handwashing product. Being an alternative to soap in water and soap scarce scenarios, such as humanitarian emergencies.</p>
Location	Ghana
Start Date	1 st July 2017
End Date	<p>30th Nov 2017</p> <p>Plan would have finished at the end of July 2018 if the first phase had showed results for the second phase to be developed. The second phase was intended to explore the acceptability of using Moringa as hand-washing product within the community.</p>
Total Funding	£47,520 for the first phase (£85,000 was the total project funding).
Total Spent	Pending to report as the financial reporting deadlines has been extended for this project.

Reporting Period	02/06/17 to 30/11/17
Type of Innovation	<i>Moringa oleifera</i> is a tree distributed in tropical and subtropical regions. It has antimicrobial activity against human pathogens. It has the potential to be a handwashing product with same efficacy

	as soap, with no need of being used with water, being of interest for water and soap scarce contexts.
Project Impact Summary	<p>Diarrhoea is the second leading cause of death in children under five years old, with children in developing countries being disproportionately affected. Hand-washing with soap has been found to be an important and cost-effective preventive measure against diarrheal disease. In an attempt to provide a novel solution to this problem, the efficacy of <i>Moringa oleifera</i>, a fast-growing, drought-resistant plant found throughout much of the Indian subcontinent and Africa, was recently investigated. The results of this study were promising as they established that dry <i>M. oleifera</i> leaf powder, in both dry and wet preparations, was similarly efficacious to regular, non-medicated soap in its ability to remove bacteria from artificially contaminated hands.</p> <p>The purpose of the current study, therefore, is to investigate whether different preparations of <i>M. oleifera</i> sourced locally in Ghana function as an antimicrobial in a minimally processed form, whether the plant is bactericidal or bacteriostatic in aqueous solution, such as rinse water used for handwashing and if the plant is as effective as soap as handwashing product.</p> <p>The results showed that:</p> <ol style="list-style-type: none"> 1) The locally-sourced <i>M. oleifera</i> (fresh leaves, fresh seeds, dry leaf powder) used in the study was found to be contaminated with bacteria. We assessed levels of contamination of Moringa products from different providers and all showed the same results. 2) Moreover, it was found that <i>M. oleifera</i> promoted the growth of bacteria in both, sterile and rinse water solutions. 3) Moringa dried form and boiled leaves reduced bacteria in contaminating hands from volunteers but the reduction was significantly lower than the one obtained with soap (mean log₁₀-reduction 2.02, 2.56 vs 3.36 respectively (p<0.01)). <p>As a conclusion, this study suggest that in its unprocessed or minimally processed form, <i>M. oleifera</i> is not an effective antimicrobial against faecal indicator bacteria in aqueous and rinse water solution and its use as a hand-washing product should not be advocated due to the plant's promotion of bacterial growth, this being of particular concern in resource-limited settings.</p>

PROJECT ACTIVITIES AND OUTPUTS

Please go to **Appendix 1** and attach the final workplan, showing all work that was actually completed.

1. With reference to the final workplan, what have been the key achievements of the project?

- We have managed to complete all the laboratory tests on time as planned, because we started to work in the project on advance, despite all the delays in contracts and transfer of funds. This has been thanks to all the preparatory work than Action Against Hunger, LSHTM and the NPHRL in Ghana have done previously to get the contracts.
- We not only tested our hypothesis but also added new experiments with Moringa that will be very helpful in the WASH sector.
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INNOVATION OUTCOMES

Whether this innovative project was successful, not successful, or a mix of both, the HIF would like you to report as much detail as possible, so that success can be built on and failures can be learned from. By 'success' we mean that the innovation has achieved the planned positive impact/outcome, or that it has performed better than the current process, product or system.

2. Has the project demonstrated the success of the innovation? (Please choose only one answer.)

- ☐ Completely successful
- ☐ Significantly successful
- ☒ Partially successful
- ☐ Completely unsuccessful

2b. Please select the successes that your project have achieved:

(You may choose more than one)

- ☐ There is real evidence that the project achieved the planned outcome(s)
- ☒ There were perceived contributions or improvements to the planned outcome(s)
- ☒ Learning was achieved within the project cycle
- ☐ 'Lessons learned' were gathered and circulated to humanitarian stakeholders and actors
- ☐ The completion of this project has led to another innovation
- ☒ Other (please comment) _____

Unexpected findings with relevance for the scientific community about the properties of the plant. The locally-sourced *M. oleifera* (fresh leaves, fresh seeds, dry leaf powder) used in the study was found to be contaminated with faecal bacteria indicator (E.coli). Moreover, it was found that *M. oleifera* promoted the growth of bacteria in both sterile and rinse water solutions. We believed that this observation can be due to the high nutritious levels of the Moringa plant. *M. oleifera* is highly nutritious and carbon rich meaning that it provides a good medium for bacterial growth. Moringa dried form and boiled leaves reduced bacteria in contaminating hands from volunteers but the reduction was significantly lower than the one obtained with soap (mean log10-reduction 2.02, 2.56 vs 3.36 respectively (p<0.01)).

2c. Please select the challenges your project has encountered:
(You may choose more than one)

- ☐ The project did not complete its planned activities
- ☒ There is no real evidence that the project achieved the planned outcome(s)
- ☐ There were few perceived contributions or improvements to the planned outcome(s)
- ☐ Learning was not achieved within the project cycle
- ☐ 'Lessons learned' were not circulated to humanitarian stakeholders and actors
- ☒ Other (please comment)

The challenge that we found is that the sources of Moringa received in the laboratory were contaminated with faecal indicators. Being contaminated with faecal indicators, the plant was not effective in reducing the E.coli same as soap. These finding in real situations, made us realised that we cannot recommend beneficiaries to use this product as hand-washing product.

2d. If there is any evidence for the successful performance of the innovation, please describe it further:

We discovered that Moringa sources received in the laboratory were contaminated with faecal indicators, but we still wanted to test if 4 gr of dried Moringa powder presented the same efficacy as soap as a hand-washing product. When the experiment was performed among 16 volunteers (following an adaptation of European Committee for Standardization protocol (EN 1499)), we found that the efficacy of Moringa oleifera was smaller than the one observed with soap. Moringa product reduced bacteria in pre-contaminated volunteers hands, however the reduction of statistical significantly smaller than the one obtained by using soap.

We also tested the effect of Moringa products to kill bacteria in water contaminated with a faecal indicator (E.coli). Bottles containing sterile distilled water were artificially contaminated with *Escherichia coli* (*E. coli*) and varying amounts of different *M. oleifera* preparations (fresh leaves, boiled seeds, dry leaf powder) were added. The colony forming units (cfu/mL) of each bottle was measured each day for five days using membrane filtration to assess the efficacy of each different preparation in reducing cfu/mL compared to a control. We found that not only Moringa forms did not killed bacteria but also it was found that *M. oleifera* promoted the growth of bacteria in both sterile and rinse water solutions (from the water obtained from rinses of participants). This effect could be due to the nutritious properties of *M. oleifera*. These second results also indicate that more attention and caution should be given in other applications that have been given to Moringa products as a water disinfectant.

3. Please show the components of the project which contributed the most to any successes:
(where 1 = most influence 3 = least influence)

Component	1	2	3	N/A
Design and placement of the innovation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
The methodology or approach to collecting evidence	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Context	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
The availability of resources and capacities (financial, human, technical etc.)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Success in identifying and responding to different project and innovation risks

☒ ☐ ☐ ☐

Strength of relationships and collaborations within the team and with other stakeholders

☒ ☐ ☐ ☐

The process was flexible and responsive to emerging results

☒ ☐ ☐ ☐

Ability to draw on experience and expertise of existing practice, codes and standards

☒ ☐ ☐ ☐

Other:

☐ ☐ ☐ ☐

Other:

☐ ☐ ☐ ☐

4. Please show the components of the project which contributed the most to any unsuccessful elements of the project

Component	Yes- contributed to failures
Weaknesses in the design and placement of the innovation	<input type="checkbox"/>
The methodology or approach to collecting evidence	<input type="checkbox"/>
Context	<input checked="" type="checkbox"/>
A lack of access to resources and capacities (financial, human, technical etc.)	<input type="checkbox"/>
Difficulty in identifying and responding to different risks	<input type="checkbox"/>
Lack of good relationships and collaboration within the team and with other stakeholders	<input type="checkbox"/>
Having a process that was not flexible or responsive to emerging results	<input type="checkbox"/>
No ability to draw on experience and expertise of existing practice, codes and standards	<input type="checkbox"/>
Other:	<input checked="" type="checkbox"/>
Starting of the project without having finalized the signature of contracts and submission of funding between donor and lead organization and between partners organizations.	

5. What are the top three, key lessons learnt relating to the innovation? *This should relate to the innovation or the sector in which it operates, rather than project implementation.*

- Unprocessed or minimally processed forms of *M. oleifera* are not an effective antimicrobial against faecal indicator bacteria in aqueous and rinse water solution and it is not as efficient as soap as a hand-washing product.

- The presence of Moringa in water with E.coli promoted the bacterial colonies growth. For this reason, its use as a hand-washing product should not be advocated. Being this of particular concern in resource-limited settings.
- More attention should be paid in using the Moringa seeds as a method to disinfect/clean water (used as flocculent agent), as this study has proved that seeds also can increase the levels of bacteria in water (coming from the seeds or present in the water). We recommend more research in this matter.

6. Do the final outcomes support the initial rationale for the innovation?

- ☐ Yes, completely
☐ Yes, significantly
☐ Partially
☒ No, not at all

Please describe further:

The results show that *Moringa oleifera* obtained from local sources cannot be used for handwashing purposes without further treatment, as it exists a high probability that the Moringa products are contaminated with other bacteria. The findings also showed that all three different preparations of the plant promote bacterial growth in aqueous solution and that when used as a hand-washing product it was less efficacious in reducing bacteria from volunteers pre-contaminated hands than soap. If *M. oleifera* accelerates or otherwise aids the growth and proliferation of bacteria that have a low infectious dose, such as is the case with many gastrointestinal pathogens, its use around sinks, this being an important site of infection, or in humanitarian settings could increase the risk of infection. In fact, many important diarrheal pathogens are known to persist on dry inanimate, and particularly moist, surfaces for extended periods of time. Thus, should they encounter something that can promote their growth, like *Moringa*, it is very possible and in fact likely that what would have otherwise been a few bacteria can soon turn into a dangerous reservoir of infection.

Moringa cannot be used in emergency context, as processing of Moringa (to get a clean and microbiological accepted product) will take time and we don't believe that this will be adequate in these contexts.

7. How has your understanding of the innovation changed through the project period?

The observed results made us think that the innovation was not as useful as we expected, because Moringa products obtained from local sources can be contaminated with bacteria, and then beneficiaries will need to process it before using it. The reason why this innovation was attractive was because the plant could be grown and available for beneficiaries easily, but we did not account for the possibility that the product could be contaminated and also that the plant did not have enough bacteriostatic power to fight this contamination, then we don't think that this solution could be appropriate for emergency contexts or situations where not many resources/time are available for the process of the product before its use.

8. Did the innovation lead to any unexpected outcomes or results? How were these identified and managed?

Yes, we did not expect the Moringa products to be contaminated with faecal bacteria indicators. We still wanted to test if the product could reduce bacteria in hands, and it did, but the reduction effect was significantly smaller than the one obtained with soap. Then we could not replicated the same results obtained in laboratories in London by the same group of researchers. In London the Moringa products were obtained from an European company, which probably had to pass different safety and controlled tests to produce the Moringa products. We also tested if Moringa products could kill bacteria in water pre-contaminated, but we observed that bacteria not only did not reduce but it also increased bacteria levels (*E. coli*) along the different testing days (5 days testing).



METHODOLOGY

9. Was the methodology successful in producing credible evidence on the performance of the innovation?

- ☒ Yes, completely
- ☐ Yes, significantly
- ☐ Partially
- ☐ No, not at all

Please describe further:

We used the following methods (summary of methods):

- -Test contamination of Moringa products: Bottles containing sterile distilled water were prepared for each Moringa form, and then Moringa was added to each of the bottles. A dilution of the mixture were filtrated and cultivated in Briliance plates (to detect E.Coli).
- -Test if *M. oleifera* sourced locally in Ghana, is bactericidal or bacteriostatic in aqueous solution: Bottles containing sterile distilled water were artificially contaminated with *Escherichia coli* (*E. coli*) and varying amounts of different *M. oleifera* preparations (fresh leaves, boiled seeds, dry leaf powder) were added (Two different amounts of each preparation were assessed (10g and 50g fresh ground leaves; 10g and 50g boiled ground seeds; 10g and 25g dry leaf powder). The colony forming units (cfu/mL) of each bottle was measured each day for five days using membrane filtration to assess the efficacy of each different preparation in reducing cfu/mL compared to a Control. A brief experiment set up using the same methods was also done with *Enterococcus faecalis* to allow for a comparison of the efficacy of *M. oleifera* between Gram-positive and Gram-negative organisms.
- -A handwashing trial was conducted to assess the efficacy of different forms of *M. oleifera* (boiled leaves, dry leaf powder) in removing *E. coli* from artificially contaminated hands compared to regular, non-medicated soap. The water used to rinse hands after hand-washing with each different product was collected and the cfu/mL of each collected rinse water sample was measured each day for three days and differences in cfu/mL were calculated.

(More details in Appendix 2)



PARTNERSHIPS AND COLLABORATION

10. How and why did the partnership change during the course of the project?

Our partnership worked well, we had a bit of problems with contracts (as they got all delayed) which also affected the relationship with the partners in Ghana. This created few problems, and also we observed that the communication was not so fluent with our laboratory partners.

11. Are there plans to continue your partnership, either while scaling up this innovation or on other projects?

- ☐ Yes, with this innovation
- ☐ Yes, with another project
- ☒ Maybe
- ☐ No

Please describe further:

Plans to continue the partnership between Action Against Hunger-Spain and LSHTM may be possible for the dissemination among the scientist community and humanitarian aid agencies.

DISSEMINATION

12. Please describe any steps taken to disseminate the outcomes of the project.

Please include all completed and forthcoming, as well as all planned and unplanned products (for example, research and policy reports, journal articles, video blogs, evaluations).

We have created a summer project thesis with the results of the study. And we are planning to write a publication and a report to be shared among NGOS and other implementing agencies.

13. Has the project received any third party coverage during the project (from news media, third party blogs, researchers or academics etc.)?

It has been shared among academics at LSHTM in different seminars and in the HIF blog.

SCALE UP AND DIFFUSION – WHAT NEXT?

14. Is the project or innovation to be replicated or scaled up?

- ☐ Yes, we will scale up in the same or similar context
- ☐ Yes, we will scale up within our organisation (including running more pilots or trials)
- ☐ Yes, we will replicate the innovation/project in another context or country
- ☐ Yes, the innovation/project will be replicated or scaled up by another organisation or stakeholder
- ☐ Yes, other



☒ No

If you answered yes to question 14, please answer 14b:

14b. What model are you pursuing to scale up or sustain your innovation?

- ☐ Applying for more donor funding
- ☐ Selling the innovation or patent
- ☐ Cost recovery (for example, selling your service or being paid as a consultant to implement the innovation)
- ☐ Innovation to be taken up by organisation or government as standard and included in standard planning and core funding by them
- ☐ Other _____

Please describe further:

15. If the project or innovation could be replicated or scaled up, please list the three most important issues or actions that will need to be considered:

(where 1 = most important and 3 = least important)

Suggestion/issue	1	2	3
1	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

[illegible]

[illegible]

[illegible]



		Risk analysis for the other uses of Moringa												
		Assessment of the knowledge changes and impact												
		Market and Value Chain analysis												
	Monitoring and Evaluation													ACF
	Sustainability and viability analysis	Design of a Business model												ACF
	Conclusions and reporting Phase 3	Endline survey												ACF
		Writing documentation												ACF/LSHTM
		Capitalization and lessons learnt												ACF/LSHTM

Table 2: Changes to Workplan

For every change in the final workplan that is different to your original worktable AND that has not already been reported to the HIF, please add a record in this table. Changes can include alterations to the methodology, project process or innovation design, for example.

No changes to the initial workplan during the first phase but cancellation of the second phase of the project based in the results obtained from the laboratory during the first phase.

Change (as referenced in workplan above)	Reason for change	Overall impact of change
1.		
2.		
3.		
4.		

Appendix 2. Materials and methods

Objective 1

Experiment to determine extent of *M. oleifera* contamination

An experiment was set up to determine if:

- (1) The fresh *Moringa* leaves were contaminated with *E. coli* in addition to the other bacteria.
- (2) If the *M. oleifera* seeds and dry leaf powder were also contaminated.

To answer these questions, six 125mL sterilised, glass-screw cap-reagent bottles (Wheaton) containing 99mL of sterile, distilled water were set up. A different preparation of *M. oleifera* was then added to each of the bottles. The different preparations used included:

- 1mL of a non-boiled fresh *M. oleifera* leaf mixture
- 1mL of a boiled *M. oleifera* leaf mixture (boiled using the same method as for boiled seeds in Section 1.2.6)
- 1mL of a non-boiled ground seed mixture
- 1mL of a boiled ground seed mixture (prepared as in Section 1.2.6)
- 1g of dry *M. oleifera* leaf powder (the leaf powder was used as is in the form provided from the supplier)
- Control (no *Moringa* was added)

The bottles were then placed in the incubator at 37°C±1. After 18-24 hours of incubation the cfu/mL of each bottle was assessed using membrane filtration. To verify the results, another order for fresh *M. oleifera* leaves, seeds, and dry leaf powder was placed from the same supplier (Ghana Permaculture Institute). The same experiment was carried out again with the new order.

Determination of bacterial species present on *M. oleifera*

To determine the species of bacteria present on *M. oleifera*, a 125mL sterilised, glass-screw cap-reagent bottle (Wheaton) containing 100mL of sterile, distilled water was prepared. 5g of dry *M. oleifera* leaf powder was added to the bottle. The bottle was incubated at 37°C±1 for 18-24 hours. The next day, a 10-6 and

a 10-8mL dilution of the mixture were processed using membrane filtration. Membranes were placed onto Brilliance plates which were incubated at 37°C±1 for 18-24 hours. A sterile pipette was also used to drop several drops of the mixture directly onto a separate Brilliance plate.

After removal from the incubator all different coloured bacterial colonies were sub-cultured onto separate Brilliance and TSA plates to obtain pure cultures. These plates were incubated at 37°C±1 for 18-24 hours. Upon removal from the incubator, the different bacteria were streaked onto the following agars and subjected to following biochemical tests:

- Triple Sugar Iron Agar (TSI)
- Simmons' Citrate Agar (Citrate)
- Urea Agar Slant
- Indole Test (including a motility test)
- Gram stain

The results of the different tests were read the following day. Four drops of Kovac's reagent was added to the Indole Tests to detect positive or negative results.

The same procedure was repeated for testing fresh *Moringa* leaves (5g, unground) and fresh *Moringa* seeds (5g, unground).

Objective 2

The purpose of Objective 2 was to assess the efficacy of different preparations of locally-sourced *M. oleifera* (fresh leaves, seeds, and dry leaf powder) as antimicrobial agents in aqueous solution.

Seven 2000mL bottles containing 1190mL of sterile, distilled water were inoculated with *Escherichia coli* and the colony forming units (cfu/mL) was assessed using membrane filtration. Different *M. oleifera* mixtures were then prepared (fresh leaves were ground with sterile water; seeds were ground with sterile water and then boiled; dry *M. oleifera* leaf powder was used as purchased from the supplier). Two different amounts of each preparation were assessed (10g and 50g fresh ground leaves; 10g and 50g boiled ground seeds; 10g and 25g dry leaf powder). The different preparations of *Moringa* were then added to the bottles containing the *E. coli* suspension. Bottles were placed in the incubator at 37°C±1. The cfu/mL of each bottle was reassessed using membrane filtration every 18-24 hours for four more days (to give a five-day total) to determine any changes in cfu/mL. Controls containing only the *E. coli* suspension were also set up. All preparations were tested in triplicate (n=3). A similar experiment was set up using *Enterococcus faecalis* to compare results obtained for a Gram-negative and Gram-positive organism.

1. Preparation of media

Tryptone Soya Agar (TSA) (Oxoid, CM0131) and Brilliance *E. coli*/Coliform Selective Agar (Brilliance) (Oxoid, CM1046) were prepared according to the manufacturer's instructions.

After preparation, plates were checked for purity by incubating at 37°C±1 for 18-24 hours. The absence of bacterial growth was interpreted to mean that plates were sterile.

2. Bacterial strains

Escherichia coli (ATCC 25922) (hereafter referred to as *E. coli*) and *Enterococcus faecalis* (*E. faecalis*) (ATCC 29212) (hereafter referred to as *E. faecalis*) were used for this study. The *E. coli* strain used was obtained from the London School of Hygiene & Tropical Medicine and *E. faecalis* was obtained from the National Public Health and Reference Laboratory in Accra, Ghana.

3. Bacterial stock maintenance

Several isolated *E. coli* colonies (grown on a Iso-Sensitest Agar plate overnight) were inoculated in 2mL of sterile, distilled water in a 7mL sterile bijou (Thermo Scientific, Sterilin™ 7mL Polystyrene Bijou Container, 129A) until a turbid suspension was reached. The suspension was sub-cultured onto a TSA and Brilliance plate to check for growth and proper preparation of media. The plates were incubated for 18-24 hours at 37°C±1.

Bacterial stock of the *E. coli* strain was maintained by sub culturing from Brilliance agar every other day onto another Brilliance and TSA plate. Given that Brilliance plates distinguish *E. coli* from other bacteria by its purple colour (22), no further confirmatory tests were required.

4. Inoculation of sterile, distilled water

A suspension of *E. coli* equal to 1.0 McFarland (confirmed by visual comparison to 1.0 McFarland standard used for reference¹) was prepared in sterile, distilled water. Two of such 5mL bacterial suspensions were then aseptically poured into a 2000mL sterilised, glass-screw cap-reagent bottle (Simax, reagent bottle with screw GL 45 acc. to DIN, code 1632414321950) containing approximately 1190mL of sterile, distilled water. Seven such bottles were prepared to allow experiments to be run in triplicate (two differing amounts of each preparation would be added to three bottles each) and for inclusion of a Control².

- A 1.0 McFarland standard was prepared by combining 0.1mL of 1.0% Barium chloride (BaCl₂) and 9.9mL of 1.0% Sulfuric acid (H₂SO₄) in a 13mL sterile bijou (53). After thorough mixing, 7mL of the prepared mixture was transferred to a Sterilin 7mL bijou which was used as a reference.



- Experiments assessing different preparations of *Moringa* were run one at a time. As such, seven bottles were set up each week for assessing two different amounts of one *Moringa* preparation. This process was repeated twice more to assess the efficacy of all three different *Moringa* preparations.

The same process was repeated for preparing bottles used in experiments assessing boiled ground *M. oleifera* seeds and dry *M. oleifera* leaf powder but with slight modification. Instead of adding two 5mL *E. coli* suspensions of a 1.0 McFarland standard to each 2000mL reagent bottle (Simax), one 125mL sterilised, glass-screw cap-reagent bottle (Wheaton media bottles, glass, size 125 mL, with rubber lined phenolic caps) containing 100mL of sterile, distilled water was inoculated with *E. coli* until a 1.0 McFarland standard was reached. 10mL of this suspension was then added to each of the seven bottles. This was done to have a comparable cfu/mL in each bottle from inception.

5. Estimation of Day 1 cfu/mL

The Day 1 cfu/mL of each bottle was obtained after inoculation with *E. coli* using membrane filtration. The suspension in each bottle was serially diluted using 125mL sterilised, glass-screw capreagent bottles (Wheaton) containing 99mL of sterile, distilled water. Given that the exact cfu/mL of each bottle was unknown, two different dilutions were made in case the lesser dilution showed confluent growth. As such, separate serial dilutions were carried out until concentrations of the suspension of 10⁻⁶mL and 10⁻⁹mL were achieved (see Membrane Filtration Protocol in Appendix). Each serial dilution was filtered using a sterilised DelAgua filter and sterile membrane filters (EMD Millipore MF-Millipore HAWG04700 Mixed Cellulose Ester Filter Membrane, Hydrophilic, 0.45µm Pore Size, 47mm Filter Diameter, White, Gridded Surface; LabExact 1200004 MCE Membrane Flats, Sterile, Gridded, Hydrophilic, 0.45µm, 47mm). After filtration, the membrane was aseptically placed onto a Brilliance plate and incubated at 37°C±1 for 18-24 hours. Upon removal, colonies on plates were counted within 30 minutes. The estimated cfu/mL of each bottle was then calculated using the follow-ing formula:

Formula for calculating estimated cfu per mL using membrane filtration

$$= \frac{\text{Number of colonies on membrane}}{\text{Volume of original sample filtered (mL)}}$$

6. Preparation of *M. oleifera* mixtures

After assessing the initial cfu/mL of the *E. coli* suspensions in each bottle, a specified amount of *Moringa* was added to each bottle. The *M. oleifera* was purchased locally in Accra from Ghana Permaculture Institute. The *M. oleifera* is picked in the morning and washed with saline solution (NaCl). The leaves are then placed inside a plastic bag with holes for aeration before distribution. To produce dry leaf powder, the same process is followed, however after washing leaves with saline they are placed in a drying oven before being made into a powder. Seeds are harvested in the same manner as leaves.

To make the fresh ground *M. oleifera* leaf mixture, 300mL of sterile, distilled water and 500g of fresh *M. oleifera* leaves, including the stems, were added to a sterilised, stainlesssteel laboratory blender. The leaves were then blended for approximately two minutes until a homogenous mixture was reached. The seven bottles that were prepared were then split into three groups:



Control (n=1)

10g fresh ground leaves (n=3)

50g fresh ground leaves (n=3)

Boiled ground *Moringa oleifera* seeds³ were prepared by blending 500g of fresh seeds with 300mL of sterile, distilled water in a sterilised, stainless-steel laboratory blender for approximately two minutes until a homogenous mixture was reached. The mixture was then aseptically transferred to a 600mL sterilised, glass, open-top beaker (Pyrex). The beaker was covered with aluminium foil and set a top a metal wire box under which a Bunsen burner was set a flame. The mixture was monitored until it came to a rolling boil which was maintained for five minutes. The mixture was then allowed to cool at room temperature ($26^{\circ}\text{C}\pm 2$) for three hours after which it was placed into the refrigerator and stored at 6°C until ready for use. Once the Day 1 cfu/mL was assessed, bottles were split into three groups before the boiled seed mixture was added:

Control (n=1)

10g boiled ground seeds (n=3)

50g boiled ground seeds (n=3)

The dry leaf powder was used as is without any modification. Bottles were again split up into three groups:

Control (n=1)

10g dry leaf powder (n=3)

25g dry leaf powder (n=3)

A lesser amount (25g versus 50g) was added to each bottle compared to the fresh leaf and boiled seed preparations as the dry leaf powder was undiluted unlike the leaves and seeds which were both diluted with water.

After the *Moringa* mixtures were added, bottles were mixed by inversion and incubated at $37^{\circ}\text{C}\pm 1$. The cfu/mL of each bottle (including the Control) was reassessed every 18-24 hours for the next four days. Dilutions were adjusted (more or less diluted samples were pro-cessed) in hopes of capturing accurate estimates of cfu/mL according to the results of the previous day.

7. Controls

Given that the same experimental framework would be used for assessing the efficacy of the three different preparations of *M. oleifera* (fresh ground leaves, boiled ground seeds, dry leaf powder) and that the Control used in each experiment would be prepared in the same manner, only one Control was included when testing each different preparation. Accordingly, however, the Control was also assessed in triplicate.



8. Quality control

To ensure the accuracy and reliability of results, a blank control was run at the beginning, middle, and end of every other day throughout the duration of the study. 100mL of sterile, distilled water was processed as a control. This control was chosen given that it would ensure a few different things:

(1) Through and complete sterilisation of the 125mL sterilised, glass-screw cap-reagent bottles (Wheaton) and sterile, distilled water used for serial dilutions

(2) Thorough and complete sterilisation of the DelAgua kit between each filtration

(3) Initially non-boiled seeds were going to be assessed however, before the efficacy of the *M. oleifera* seeds and powder were assessed, it was found that the *M. oleifera* leaves were contaminated with bacteria. In light of this finding, an experiment (described in Section 7.1.10) was undertaken to determine if the seeds and powder were also contaminated and it was found that they both were. Considering these results, boiled *M. oleifera* seeds were selected for further investigation. The dry leaf powder, however, was used as is to evaluate its potential as a hand-washing product as proposed in the study by Torondel, et al.

9. Statistical analysis

To determine if there were significant differences between the change in log₁₀ cfu/mL between each day for Controls versus the different preparations of *M. oleifera*, paired t tests were conducted in Stata 15. The mean log₁₀ difference between consecutive days (Day 1-2; Day 2-3; Day 3-4; Day 4-5) in addition to differences between Day 1 to 3, Day 3 to 5, and Day 1 to 5 were analysed.

Objective 2 (2nd part). Investigation of different *M. oleifera* preparations against a Gram-positive faecal bacterium

To test the theory that *M. oleifera* may only be active against Gram-positive organisms, a brief experiment was set up using the same methods outlined above but using *E. faecalis*. *E. faecalis* was selected as it, much like *E. coli*, is considered a faecal indicator bacteria.

For this experiment, however, the *M. oleifera* leaves were boiled in sterile, distilled water and were not ground. The seed and powder preparations were as for Objective 2. For the sake of time and simplicity, the mixtures were prepared using 98mL of sterile, distilled water in a 125mL sterilised, glass-screw cap-reagent bottle (Wheaton). As such, the amount of *Moringa* added to each bottle was adjusted to 1mL for the boiled leaves and the boiled, ground seeds and 1g for the dry *M. oleifera* leaf powder. Additionally, only 1mL of a 1.0 McFarland standard bacterial suspension of *E. faecalis* was added to each bottle,

including the Control. The cfu/mL of the *E. faecalis* suspension was measured before being added to each bottle. As Brilliance plates are selective and do not support the growth of Gram-positive organisms, TSA plates were used for this experiment. Given time constraints, the experiment was not run in triplicate (n=1) and the cfu/mL of each bottle was assessed for a total of three days instead of five. Accordingly, the results of this experiment were not statistically analysed and are instead merely meant to provide an idea of the efficacy of the different preparations of *M. oleifera* tested against a Gram-positive faecal organism.

Objective 2 (3rd part)

The purpose of Objective 2 (3rd part) was to assess the efficacy of different preparations of locally sourced *M. oleifera* (fresh boiled leaves and dry leaf powder) as an antimicrobial agent in collected rinse water samples.

A hand-washing trial was set up to evaluate the efficacy of locally sourced *M. oleifera* as a handwashing product. After artificially contaminating the hands of participants with *E. coli*, participants were asked to wash their hands for 1 minute according to the standard handwashing protocol using one of three different products: 5mL regular, non-medicated soap, 5mL boiled *M. oleifera* leaves, or 4g dry *M. oleifera* leaf powder. After washing their hands for 1 minute, participants were asked to rinse their hands with 300mL of sterile, distilled water. At this point, large 2000mL sterilised bottles and large, sterilised funnels were set up to collect the rinse water. After collection of the three rinse water samples (rinse water of regular, non-medicated soap, boiled leaves, and dry leaf powder) from the participant, the initial cfu/mL of each sample was assessed by membrane filtration. The cfu/mL was reassessed every 18-24 hours for two more days (to give a three-day total). This process was repeated twice more to allow tests to be run in triplicate (n=3).

1. Preparation of media and supplies

The preparation of media was as in previous Section.

Nine 2000mL glass-screw cap-reagent bottles (Simax) were sterilised in the autoclave in addition to nine large, plastic funnels covered with aluminium foil (both large and small mouths).

2. Rinse water experiment set-up

In a separate study, the efficacy of different preparations of *M. oleifera* were being assessed as handwashing products in comparison to a reference soap. In total, three different hand-washing products were assessed: (1) 5mL of regular, non-medicated soap (RNS) (Boots Cream Hand Wash 350mL), (2) 5mL of a dilution of fresh *M. oleifera* leaves in water (100g of fresh *M. oleifera* leaves were added to 500mL of sterile, distilled water. The mixture was then boiled for five minutes and left to diffuse for three days.), and (3) 4g of dry *M. oleifera* leaf powder. Participants were asked to wash their hands for 1 minute according to the standard handwashing procedure to remove transient bacteria (25). After drying hands using paper towels, the left and right hand of each participant was immersed in a contamination fluid (Tryptone Soya Broth (TSB) containing non-pathogenic *E. coli*) up to the mid-metacarpal for 5 seconds. Hands were then allowed to air dry for three minutes before the pre-value was estimated. This was done by having participants rub the fingers and thumbs of each hand in the bottom of separate sterile petri dishes containing 10mL of sterile TSB for 1 minute. The appropriate amount of the different

handwashing preparations (regular, non-medicated soap, boiled leaves, or powder) was then dispensed onto the hands of participants and they were again asked to wash their hands according to the standard handwashing procedure for 1 minute. After 1 minute participants were asked to rinse their hands. At this point the sterilised 2000mL reagent bottle (Simax) and funnel were set up in the sink where the participants were to rinse their hands. 300mL of sterile, distilled water was used to rinse the hands of participants. Care was taken to collect the entirety of the water used for hand-rinsing.

3. Estimation of cfu/mL

After collection of rinse water samples, reagent bottles were capped, labelled, and left at room temperature ($26^{\circ}\text{C}\pm 2$) for the next 72 hours. Every 18-24 hours the cfu/mL of each bottle was assessed using membrane filtration. This process was repeated for rinse water collected from two further participants to run tests in triplicate ($n=3$).

4. Statistical analysis

Statistical analysis was conducted in Stata 15 as for Objective 1 (see Section 7.1.12) comparing log₁₀ differences in cfu/mL between days (Day 1-2; Day 2-3; Day 1-3) for regular, non-medicated soap, boiled *M. oleifera* leaves, and dry *M. oleifera* leaf powder rinse

Objective 3

To assess the efficacy of different forms of *M. oleifera* (boiled leaves, dry leaf powder) in removing *E. coli* from artificially contaminated hands compared to regular, non-medicated soap.

The hands of sixteen volunteers were artificially contaminated with *Escherichia coli*. *Moringa oleifera* leaf powder was tested as a hand washing product and was compared with reference non-medicated liquid soap using a cross over design following an adaptation of the European Committee for Standardization protocol (EN 1499).

1. Microorganism

In order to test the efficacy of *Moringa oleifera* leaf powder, we used an adapted protocol of the European Committee for Standardization (EN 1499) which is designed to evaluate the ability of hand-wash agents to eliminate transient pathogens from volunteers' hands without regard to resident microorganisms. This procedure is based on the "post-contamination treatment" of hands, and involves the placement of the test organism (*Escherichia coli* (ATCC 25922)) on the hands of test subjects followed by exposure to the test formulation. These methods are useful in testing the performance of products used in routine hand hygiene in health care centres.



2. Subjects

The study was performed in the Medical Microbiology Laboratory Ghana from July to August 2017, and was approved by LSHTM Ethics Committee and Ghana ethics committee. Sixteen adult volunteers from Ghana were selected for the study, and a formal written consent was received from all of them. The volunteers were physically examined to ensure they were healthy with healthy skin. None had skin disorders like eczema, paronychia, scabies, abrasions, lacerations or skin allergy. They all had short fingernails with no artificial nails. They had no history of drug allergy and had not taken any systemic antibiotic in the two weeks prior to the study, which could otherwise impair the efficacy of the product being tested. All forms of jewellery were removed from their hands prior to hand washing, since it had the potential of retaining some bacteria, which could affect the recovery pre and post values.

3. *Moringa oleifera* preparations.

Moringa products were obtained from a permaculture farm in Ghana.

We tested 5mL of a dilution of fresh *M. oleifera* leaves in water (100g of fresh *M. oleifera* leaves were added to 500mL of sterile, distilled water. The mixture was then boiled for five minutes and left to diffuse for three days.), and 4g of dry *M. oleifera* leaf powder and compare with standard soap.

4. Contamination procedure

The hands of each volunteer were washed with a non-medicated soap, dried and immersed for 5 seconds in a contamination fluid which contained non-pathogenic *Escherichia coli* (ACTC 25922) 8.3×10^8 cfu/ml. Excess of fluid was drained off and hands were air-dried for 3 min.

-Pre value

Bacteria were recovered for the initial pre value by kneading the fingertips of each hand separately for 60 seconds in 10 ml of tryptone soya broth (TSB) without neutralizers. The pre values were estimated using the Miles et al. technique.

5. Hygienic hand washing procedure

The hands of two thirds of the volunteers were treated with the *Moringa oleifera* for one minute; the hands of the other third were washed with the reference solution (5 ml of non-medicated liquid soap) for one minute.

Hands treated with dried *Moringa oleifera* received 4 g, whilst hands treated with *Moringa oleifera* in solution received 5ml of the solution into each pair of hands. In both cases the participants then rubbed their hands with the product for 1 minute. Hands were rinsed under running water for 15 seconds and were allowed to dry for 3 minutes.

-Post values



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After drying of the hands, the thumbs and fingers of both left and right hands were rubbed in separate petri dishes containing 10 ml of TSB for 60 seconds. Post values were determined using the method of Miles et al. After the procedure, the volunteers were given medicated soap to wash their hands before going home.

6. Statistical analysis

For both reference and test products, log counts from the left and right hands of each subject were averaged separately, for both pre and post values. The arithmetic means of all individual log₁₀ reduction values were calculated. Statistical analysis was performed with the Statistical Package STATA version 11.0. First it was checked whether the data were normally distributed using Kurtosis and Skewness test. Since the data did not follow a normal distribution, the Wilcoxon matched pair signed ranks test was used to test for differences between each Moringa oleifera preparation and the reference soap and also between Moringa oleifera and the inert powder. The new product (Moringa oleifera) was considered to have the same efficacy as the reference product (soap) if the mean log₁₀ reduction factor was not significantly smaller for the former than for the latter. Because of the confirmative nature of the test on this application, the level of significance is set at $p = 0.1$. The test is to be used one-sided. The discrimination efficiency of the test procedure described has been set to detect a difference between the two mean log reduction factors of approximately 0.6 log at a power of 95%.