

Evaluation of three isolation experiments for Campylobacter bacteriophages from chicken skin: a comparative study

ABSTRACT

Background: Campylobacter strains are of the leading pathogens causing bacterial gastroenteritis, whose infections are generally considered to be one of the most common foodborne illnesses of animal origin. The etiology of this infection often goes back to eating contaminated raw meat or infected poultry. The bacteria are present in abundance in chicken skin. The use of appropriate bacteriophages is one of the most effective experiments in eliminating Campylobacter strains. Phage therapy refers to the use of bacteriophages to treat bacterial infections. **Aim:** Accordingly, the present study aimed to compare three experiments of bacteriophage isolation in chicken skin. **Experiments:** Thus, 15 samples of chicken skin were collected from five different fresh chicken suppliers in Ghaemshahr, Iran. The samples were transported to the laboratory aseptically in the vicinity of ice, and then cultured in blood agar medium, and the isolates were identified by various tests including gram staining, catalase and oxidase tests. **Results:** the results were compared before and after three bacteriophage isolation experiments. Out of 15 chicken skin samples tested in all three experiments, 6 (40%) strains were identified in the first experiment, 8 (53.4%) strains in the second experiment and 12 (20%) strains in the third experiment after bacteriophage therapy. **Conclusion:** The bacteriophage isolation experiments alone or in combination with other intervention strategies are recommended as promising tools for greater food safety. These experiments can be useful to increase food safety and reduce the risk of infection in humans through the consumption of potentially infected edible parts of chicken. According to the results of this study, among the three proposed experiments, the experiment of chicken skin enrichment in Bolton selective media containing target isolates was the most efficient approach, which showed a high limit of detection at low concentrations and the highest rate of phage recovery. This can be a more reliable way to isolate the Campylobacter bacteriophages and eliminate the Campylobacter strains.

Keywords: *Campylobacter, Campylobacter Bacteriophage, Chicken skin, Food Contamination.*

1- INTRODUCTION

Campylobacter strains have been the most common cause of human bacterial diarrhea in many developed countries over the past two decades. In general, infection of chicken carcasses with Campylobacter strains is common and plays an important role in human infection. The EU reports have confirmed the rate of human Campylobacteriosis outbreak at around 50 cases per 100,000 people in more than 17 countries. It is estimated that approximately 9 million people experience human Campylobacteriosis annually in the EU regions [1,2]. For health, economic and nutritional reasons, chicken skin and meat are of the main sources of animal protein needed by communities today, so that more than 50% of this need is met through these products. Despite its low incidence, Campylobacter is a significant infectious agent due to the high volume of poultry meat consumption and potential risk of this pathogen. Despite sanitary measures in poultry farms, almost all of the bacterial infections studied are found in samples of processed poultry meat in industrial slaughterhouses. The prevalence of Campylobacter strains in raw poultry products is in the range of 0 to 100% and on average of 62% [3]. Various mitigation strategies, such as competitive exclusion, the use of chemical or antibiotic additives, and strict health protocols, have been implemented with relative success in the EU [4]. There has been a worrying elevation in antibiotic resistance in farm animals in recent years [5]. The World Health Organization (WHO) has included fluoroquinolone-resistant Campylobacter strains in its list of global antibiotic-resistant bacteria that pose the greatest risk to humans [6]. An important challenge for global public health is the search for new alternative ways to control Campylobacter infection by reducing the use of antibiotics in food production [7]. A promising candidate for reducing Campylobacter outbreak in the farm to fork process is the use of bacteriophages (phages) as biological control agents. Phages are viruses specifically capable of infecting and killing bacteria widely distributed in the environment and often exist as normal microbiota in the diet, including poultry products [8]. Bacteriophages have properties that seem attractive to those looking for new solutions to control foodborne pathogens and spoilage microorganisms. These phages have a history of safe use, and can be host-specific and replicated in the host. Campylobacter, Salmonella, *Listeria monocytogenes* and various spoilage microorganisms have responded to phage control in some food products. However, the employment of phages as microbial biological control agents can be complicated by factors such as the apparent need for the host threshold level before further replication and with sub-optimal performance, at best, under sub-optimal temperatures for the host. Razei et al. (2017) dealt with the rapid detection of *Campylobacter jejuni* based on

58 PCR technique and assessment of its sensitivity and specificity. Their aim was to design a specific PCR process
59 to identify *C. jejuni* [9]. Numerous studies have been conducted on the use of bacteriophages to strengthen
60 various food products. In this study, bacteriophages have been used successfully to control the growth of
61 pathogens in food. They are supposed to play an important role in food safety in the future. However, many
62 foods and particulate matter processes in the intestines of animals inactivate phages and reduce their virulence
63 capacity. Encapsulation technologies have been successfully used to protect phages against extreme
64 environments and have been shown to maintain their activity and release in targeted environments [10]. The use
65 of Campylobacter-specific bacteriophages seems to be a promising tool in the food safety for the biological
66 control of this pathogen in the poultry meat production chain. However, the isolation of bacteriophages is a
67 complex challenge because they appear to be low on chicken skin or meat. Isolation of Campylobacter
68 bacteriophages is the first challenge in developing a bacteriophage-based product to control Campylobacter.
69 They are isolated wherever their hosts are present and also from environmental samples and poultry products
70 [11]. However, the presence of Campylobacter bacteriophages is very low even in these samples [12]. On the
71 other hand, the isolation rate of Campylobacter bacteriophages varies in published articles, probably due to
72 differences in isolation experiments or the type and origin of the sample [7]. Various isolation experiments have
73 been proposed to date, but no standard experiments for the isolation of Campylobacter bacteriophages have yet
74 been developed. To optimize existing experiments and suggest the best experiment, the present study selected
75 three different experiments introduced in several previous articles as appropriate protocols in terms of phage
76 recovery rate, with the aim of comparing the three isolation experiments of Campylobacter bacteriophages from
77 chicken skin. In this study, in addition to determining the effectiveness of Campylobacter bacteriophages on the
78 bacteria separately, finally, three experiments of isolating Campylobacter bacteriophages from chicken skin
79 were compared and the best experiment was introduced.

80 81 2- MATERIALS AND EXPERIMENTS

82 In this study, 15 samples of fresh chicken skin, thighs or wings or neck were randomly collected from five
83 different chicken suppliers in Ghaemshahr (Iran). The samples were transferred to Rai Azma Food Hygiene and
84 Health Laboratory aseptically in the vicinity of ice, immediately followed by performing the necessary tests.
85 First, the chicken skin pieces inside the Falcon tubes were completely vortexed with normal saline. The skin
86 pieces were removed by forceps under sterile conditions, and the remaining fluid was transferred to sterile
87 Falcon tubes. The Falcon tubes were centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded, and
88 the remaining precipitate was added with 30 ml of Preston enrichment broth containing antibiotics. The Falcon
89 tubes were incubated in a special jar under micro aeration conditions at $42 \pm 1^\circ\text{C}$ for 24 hours. It should be
90 noted that the micro aeration conditions were created by lighting a candle inside the jar. All steps were
91 examined with standard strains to ensure the accuracy of the isolation process. After 24 hours, the samples were
92 taken out of the incubator and immediately cultured in four regions onto blood agar media containing
93 antibiotics. Re incubation was performed at $42 \pm 1^\circ\text{C}$ for 48 h under micro aeration conditions. Finally, the
94 plates were examined macroscopically. According to the morphology of the grown colonies and biochemical
95 tests (including gram staining, oxidase, catalase, nitrate reduction and nalidixic acid resistance tests), suspicious
96 Campylobacter colonies were isolated and their purification was performed for all three subsequent isolation
97 experiments [13]. Campylobacter isolates were used as host bacteria in this study. For isolation, chicken skin
98 samples were diluted at a ratio of 1: 4 (w/v) in SM buffer [50 mM tris-xcl (pH = 7.5), 0.1 M NaCl, 8 mM
99 MgSO_4 , 0.01% (w/v) gelatin], and were cultured by Rapid experiment of Campylobacter Detection. The plates
100 were dried at ambient temperature and stored at 37°C for 72 h under micro aeration conditions (5% oxygen,
101 10% carbon dioxide and 85% nitrogen). The Campylobacter isolates were stored at -80°C in Brain Heart
102 Infusion (BHI) Broth with 10% Glycerol. To prepare the final phase cultures, the frozen-thawed samples (200
103 μl) were cultured onto Columbia Blood Agar (oxid) with Defibrinated Sheep Blood (5% v/r, oxid) under
104 micro aeration conditions at 37°C . After overnight incubation, the cells were harvested up to $0.6 (10^8 \text{ CFU/ml})$
105 in BHI Broth until reaching OD_{600} , and kept again at 37°C for 4 hours [7].

106 Three different isolation experiments for Campylobacter bacteriophages were evaluated and applied to all 15
107 chicken skin samples.

108 **Experiment 1:** The chicken skin samples were placed in sterilized filter bags and enriched in 15 ml of BHI
109 broth by culturing the final phase of host Campylobacter strains to reach a final concentration of 10^6 CFU/ml .
110 After enrichment, the mixtures were kept at 37°C for 48 h under micro aeration conditions.

111 **Experiment 2:** 10 g of chicken skin samples were added to SM buffer (50 Mmol/l Tris-HCl [pH = 7.5], 0.1
112 mol/l NaCl, 0.008 mol/l MgSO_4) and stored for 4 hours at 4°C [8]. The suspension was centrifuged at 8600 gr
113 for 10 min and the resulting aqueous phase was treated with chloroform (4: 1, v/v) and re-centrifuged at 8600 gr
114 for 10 min [14].

115 **Experiment 3:** The chicken skin samples were placed in sterile filtered bags containing 10ml of Campylobacter
116 Selective Bolton Broth (oxid), selective antibiotics (oxid) and 5% lysed horse blood and 10 ml of fresh Bolton
117 broth supplemented with 400 Mg/ml of CaCl_2 and 400Mg/ml of MgSO_4 . The mixture was vortexed and stored

118 at 42°C for 18 h under micro aeration conditions and treated with chloroform. The prepared mixture was
 119 enriched by the host *Campylobacter* strains within the log phase until a final concentration of 6 CFU/10ml. The
 120 mixture was kept at 42°C for 48 hours under micro aeration conditions [7].

121 **Phage identification:** One drop of each phage sample (10 µl) was added to each of the *Campylobacter* strains
 122 and the plates were incubated. The lysis area was scratched and suspended in 100 µl of SM band, and re-plate
 123 on the third level of *Campylobacter*. Different dilutions were prepared and individual phage plaques were
 124 obtained and this test was performed in triplicate to ensure purity. Fresh phage lysates were stored in sterile
 125 tubes at 4°C and finally at -80°C in SM buffers with 20% glycerol [7]. All tests were performed in triplicate.
 126

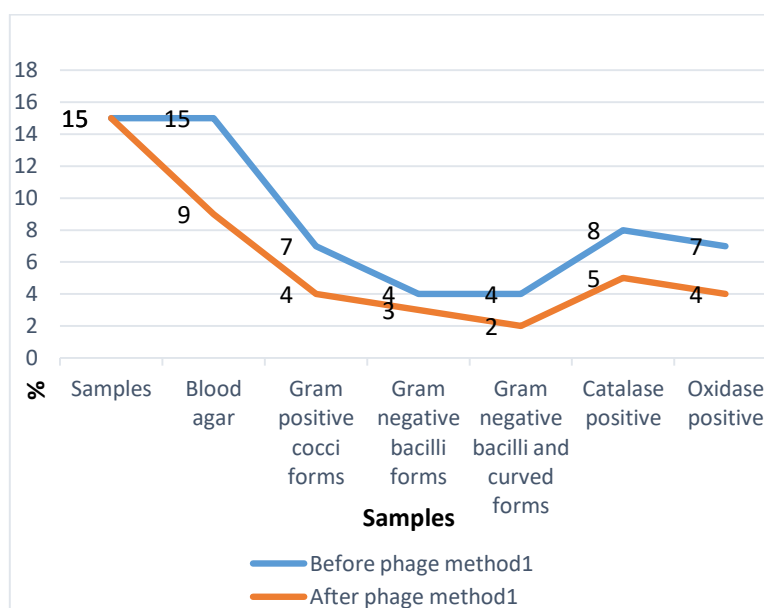
127 3- RESULTS AND DISCUSSION

128 Since the data are quantitative (with a sample size of n <30), the three dependent groups were compared by
 129 pairwise comparisons (pre/post) through the statistical experiment of mean comparison to show the difference
 130 and one-factor repeated measures analysis of variance (ANOVA) using SPSS software. The results are shown as
 131 tables and line graphs for each experiment.
 132

133 **Table 1- Frequency and percentage of samples with *Campylobacter* strains observed before and after**
 134 **performing the first experiment in blood agar medium, gram staining and diagnostic tests**
 135

| Samples | Positive growth in blood agar medium (%) | Gram staining | | | Diagnostic tests | |
|--|--|---------------------------------------|---|--|------------------------------|-----------------------------|
| | | Gram-positive cocci form bacteria (%) | Gram-negative bacilli form bacteria (%) | Gram-negative curved and bacilli form bacteria (%) | Catalase positive result (%) | Oxidase positive result (%) |
| Before phage therapy in the first experiment (frequency) | 15 | 7 | 4 | 4 | 8 | 7 |
| After phage therapy in the first experiment (percentage) | 60.0±4.60 | 26.60±0.82 | 20.0±0.56 | 13.30±1.16 | 33.30±0.79 | 26.60±0.56 |

136 According to the table above, after phage therapy in the first experiment, no *Campylobacter* strains were
 137 observed in 40% of the samples.
 138 First experiment
 139



142 **Figure 1- Line graph of Campylobacter strains observed before and after the first experiment in blood**
 143 **agar medium, gram staining and diagnostic tests**

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145 According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after
 146 phage therapy in the first experiment.

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148 **Table 2- Frequency and percentage of samples with Campylobacter strains observed before and after**
 149 **performing the second experiment in blood agar medium, gram staining and diagnostic tests**

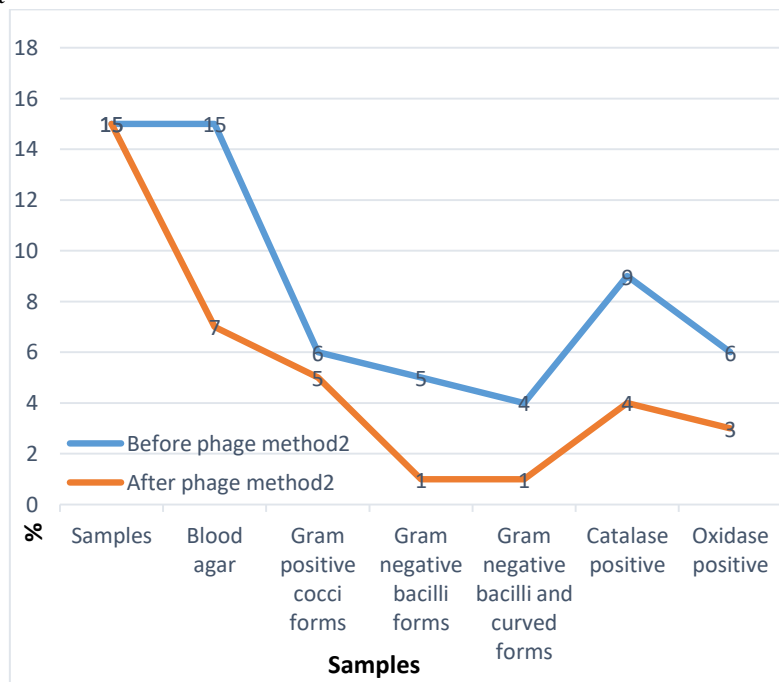
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| Samples | Positive growth in blood agar medium (%) | Gram staining | | | Diagnostic tests | |
|---|--|--------------------------------------|--|---|------------------------------|-----------------------------|
| | | Gram-positive cocciform bacteria (%) | Gram-negative bacilliform bacteria (%) | Gram-negative curved and bacilliform bacteria (%) | Catalase positive result (%) | Oxidase positive result (%) |
| Before phage therapy in the second experiment (frequency) | 15 | 6 | 5 | 4 | 9 | 6 |
| After phage therapy in the second experiment (percentage) | 46.60±0.68 | 33.30±0.79 | 6.60±0.15 | 6.60±0.15 | 26.60±0.56 | 20.0±0.52 |

151

152 According to the table above, after phage therapy in the second experiment, no Campylobacter strains were
 153 observed in 53.4% of the samples.

154 Second experiment



155

156

157 **Figure 2- Line graph of Campylobacter strains observed before and after the second experiment in blood**
 158 **agar medium, gram staining and diagnostic tests**

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160 According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after
 161 phage therapy in the second experiment.

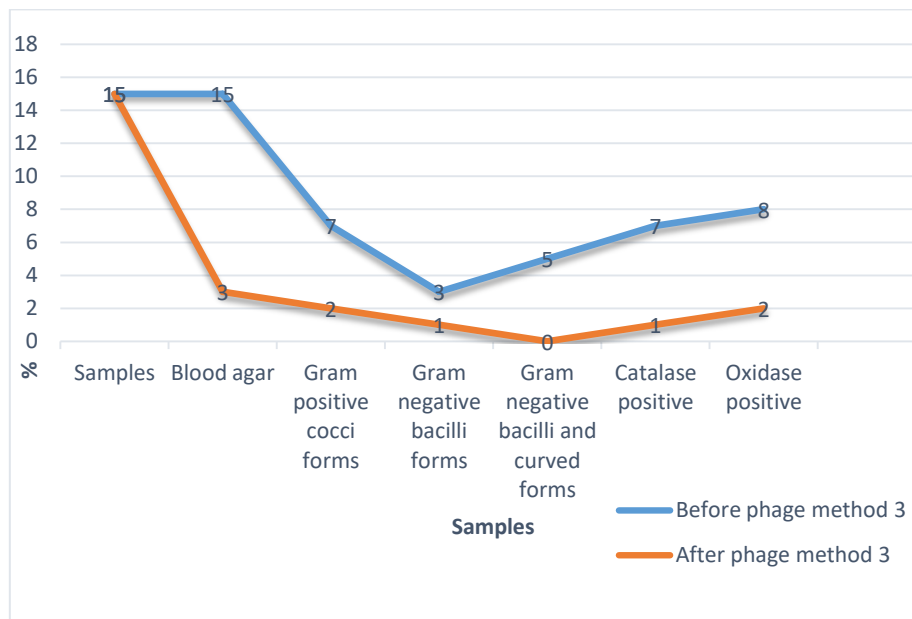
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Table 3- Frequency and percentage of samples with Campylobacter strains observed before and after performing third experiment in blood agar medium, gram staining and diagnostic tests

| Samples | Positive growth in blood agar medium (%) | Gram staining | | | Diagnostic tests | |
|--|--|---------------------------------------|--|---|------------------------------|-----------------------------|
| | | Gram-positive cocci form bacteria (%) | Gram-negative bacilliform bacteria (%) | Gram-negative curved and bacilliform bacteria (%) | Catalase positive result (%) | Oxidase positive result (%) |
| Before phage therapy in the third experiment (frequency) | 15 | 7 | 3 | 5 | 7 | 8 |
| After phage therapy in the third experiment (percentage) | 80.0±1.20 | 13.30±0.22 | 6.60±0.15 | - | 6.60±0.15 | 13.30±0.22 |

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According to the table above, after phage therapy in the third experiment, no Campylobacter strains were observed in 80% of the samples. Third experiment



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Figure 3- Line graph of Campylobacter strains observed before and after the third experiment in blood agar medium, gram staining and diagnostic tests

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According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after phage therapy in the third experiment. Failure to treat these infections with antibiotics has led researchers and scientists to use more efficient and alternative experiments to eliminate and control these bacteria. One of these successful alternatives or supplements is the use of bacteriophages (phages) to treat infections in many refractory infections [15]. This phenomenon, or phage therapy, means the use of bacteriophages to treat bacterial infections, and especially the combination of two or more phage types called phage cocktails has been used to increase the host spectrum of a particular genus against various bacterial infections [16,17]. Unlike most antibiotics, phages are smart weapons that act specifically and thus exert little harm to beneficial bacteria in the body, such as gut bacteria, while

185 antibiotics eliminate them. Phages, on the other hand, act in a limited way, entering their inactive life cycle after
186 destroying harmful bacteria, and show virtually no activity against non-host bacteria [7]. The use of phages is an
187 attractive strategy for producing safe food, because they specifically affect the pathogen. They are harmless to
188 humans, animals and plants and have no negative effect on normal microbiota or other beneficial properties of
189 food. Campylobacter-specific bacteriophages can be applied in poultry farms to prevent or reduce
190 Campylobacter contamination of birds [18]. According to the analysis of the results obtained from the present
191 study, the first experiment showed bactericidal effects, but was not very satisfactory (40%). This experiment
192 appears to reduce the growth of other bacteria present in chicken skin samples, resulting in reduced growth of
193 Campylobacter strains. The results of this study are consistent with studies by Nafarrate et al. in 2020 [7] and
194 Hungaro et al. in 2013. This experiment can exhibit the effect of bacteriophages as an alternative factor to
195 reduce the contamination of poultry carcasses in industrial conditions [19]. The present study demonstrated that
196 the use of the second experiment can also affect the bactericidal rate (53%). This experiment was performed by
197 Atterbury et al. in 2005 on chicken fecal samples [8] and then by Janez et al. in 2014 on fresh chicken meat
198 samples [15]. The results of their study also showed a decrease in bacterial density after phage inoculation, so
199 that Atterbury et al. (2005) reported a 56% bactericidal rate [8]. Comparison of the results from the first and
200 second experiments indicated that since Campylobacter strains have inactive metabolism at temperatures below
201 4°C, storage of samples at this temperature increases the efficiency of the experiment [7]. However, the results
202 obtained in the third experiment revealed that 80% of the samples had no Campylobacter strains and in a way it
203 can be said that bactericidal activity was much more effective in this experiment. The highest isolation rate of
204 Campylobacter bacteriophages was observed in the third experiment, compared to the lower isolation rates
205 through the first and second experiments. This higher rate appears to be related to the proliferation of strains
206 during storage of the samples in Bolton selective broth medium, which has led to the growth of Campylobacter
207 strains on chicken skin. Increased growth of Campylobacter strains enhances the likelihood of phage attachment
208 to host cells. In general, the third experiment was the most efficient in phage isolation, and showed the best
209 phage recovery rate from the sample surface and the lowest presence of Campylobacter strains in the samples.
210 Apart from the fact that the difference in results between repetitions was minimal, this experiment was
211 introduced as a reliable and repeatable approach. The results of this study are completely in line with the
212 findings reported by Nafarrate in 2020, which considers the above experiment as the most effective experiment
213 of bacteriophage isolation among the existing experiments and also introduces this experiment as a reliable
214 approach compared to others. The findings of this study confirm the fact that poultry products, especially
215 chicken skin, are a rich source of Campylobacter strains, as previously reported by other researchers [3,10].

216 4- CONCLUSION

217 Phages are normally present in food products and may be consumed in our diet. This is very important for food
218 safety because reducing the density of Campylobacter strains in food-producing animals or disinfecting
219 carcasses and other raw products during food processing through the use of bacteriophage does not mean adding
220 a foreign element to our diet. On the other hand, phages can be recruited as biological control tools for
221 Campylobacter strains. These bacteriophages can be utilized alone or in combination with other intervention
222 strategies as a promising tool for food safety applications. Diversity in Campylobacter phage treatment
223 experiments can be effective in developing new approaches to promote food safety. These experiments can be
224 useful to increase food safety and reduce the risk of infection in humans through the consumption of potentially
225 infected edible parts of the chicken. Given that most people use packaged chicken, which contains the skin and
226 other parts of the chicken and can lead to contamination of the chicken's food and various parts of the kitchen,
227 the third experiment, among the three proposed experiments, could be a more reliable approach to eradicate
228 Campylobacter strains.

229 230 **COMPETING INTERESTS DISCLAIMER:**

231
232 **Authors have declared that no competing interests exist. The products used for this research**
233 **are commonly and predominantly use products in our area of research and country. There is**
234 **absolutely no conflict of interest between the authors and producers of the products because**
235 **we do not intend to use these products as an avenue for any litigation but for the**
236 **advancement of knowledge. Also, the research was not funded by the producing company**
237 **rather it was funded by personal efforts of the authors.**

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