Improvement of the efficacy an WHO alcohol-based hand rub formulation by reducing isopropanol content and associating *Syzygium aromarticum* and *Piper nigrum* essential oils

ABSTRACT:

Aim: This work was carried out in order to reduce the isopropanol contain of a World Health Organization (WHO) basic alcohol based hand rub (ABHR) formulation by substituting part of it with ethanol and essential oils.

Study design: A quasi-experimental design was used, based on WHO basic formulation modification antimicrobial assessment followed by a panel test and challenge test of the best formulation.

Place and duration of the study: The study was carried out in different laboratory of the University of Yaoundé 1 during March 2017 to July 2018

Methodology: This study was performed by producing different formulations nested from the WHO basic formulation and testing their antimicrobial capacity on selected strains based on the microbial percentage reduction. Following this, the best formulation was compared to two commercial products trough a panel test and later challenged with selected organism (*Staphylococcus aureus* SR196, *Salmonella* Typhi 15SA, *Escherichia coli ATTC25922* and *Pseudomonas aeruginosa* PA01) inoculated in the product after different storage periods for one year.

Results: The partial substitution of isopropanol with ethanol and *Piper nigrum* and *Syzygium aromaticum* essential oils proved to possess more antimicrobial properties than the original WHO formulation. The best formulation caused a 6 Log cells/ml reduction of the initial population compared to the 4 Log cells/ml of the WHO formulation. The product also proved to maintain its activity for one year and to be able to deactivate possible contaminations by *Salmonella* Typhi 15SA, *Staphylococcus aureus* NCTC10652, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PA01 .

Conclusion: The present work is a contribution to the improvement of ABHRs and could permit the reduction of hand hygiene associated infections in industries and health care facilities.

1.INTRODUCTION:

One of the main source of contamination of human with infectious microorganism is the use of unclean hands. According to the World Health Oragnization (WHO) guidelines for hand hygiene **[1]**, health care associated infections (HCAIs) affect hundred of millions of patients worldwide every year, and hand hygiene is the primary measure to prevent this. In developed countries, HCAI concerns about 5 to 15% of hospitalised patients and may rise up to 9 to 37% of those admitted into intensive care **[2, 3]**. More than 50% food poisoning cases have been reported to be associated with improper food handling by food practitioners **[4].** They are able to transit agents of foodborne diseases due to incorrect personal hygiene practices **[5]** moreover, according to **[4]**, hand hygiene is the most basic yet critical criterion for ensuring safe foods. There are two types of microbes colonizing hands: the resident flora, which consists of microorganisms residing under the superficial cells of the stratum corneum and the transient flora, which colonizes the superficial layers of the skin, and is more amenable to removal by routine hand hygiene. Transient microorganisms survive, but do not usually multiply on the skin. They are often acquired by health care workers (HCWs) during direct contact with patients or their nearby contaminated environmental surfaces and are the organisms most frequently associated with HCAIs **[6, 1, 7]**. It is reported that hand can carry microorganism like, *S. aureus* (MRSA), vancomycin resistant *Enterococcus* (VRE), MDR-Gram Negative bacteria (GNBs), Candida spp. and *Clostridium difficle*, which can survive for as long as 150 h, *Klebsiella* sp **[1, 6, 8]**. According to the results of a study on doctors hands performed by **Paul *et al.* [9]**, There was a significant contamination of the doctors' hands at entry (59.1%) and at exit (90.9%). Overall, *Staphylococcus* was the predominant organism (59% at entry and 85% at exit); coagulase-negative ones were more prevalent at entry (32%) and coagulase-positive ones were more prevalent at exit (54%). Among the gram negative organisms, *Escherichia coli* (4.5%), *Pseudomonas* (4.5%), Enterococci (13.6%) and *Klebsiella* (9%) were the main ones isolated. Based on these facts, the main recommendation is to perform hand washing when hands are visibly dirty and to routinely use alcohol-based hand rub products for the decontamination of hands **[8, 6]**.

Alcohol-based hand robs (ABHRs) are biocides used in human hygiene, in urgent cases and in situations where there is not the possibility to perform hand washing protocols. They are mainly used outdoor and in medical environment. They are very useful in deactivating microorganisms present on the hand **[1, 10]**. An EPIC study showed the superiority of alcohol based antiseptic hand rubs having more than 70 per cent alcohol **[11, 12]**. In these products, isopropanol is frequently used compared to ethanol. Isopropanol ingestion is the second most common alcohol ingestion following ethanol but is the most common toxic alcohol ingestion

reported to the United States poison control centers each year **[13]**. In 2009, more than 20,000 cases were reported to the American Association of Poison Control Centers, with more than 80% of these cases being unintentional. Accidental exposures have been reported when large amounts of rubbing alcohol were used transdermally or children ingested it accidentally. But one of the main concern today is skin dryness and irritation associated to the high alcohol contain of this product **[13]**. isopropyl alcohol toxicity is associated with rapid inebriation followed by hemorrhagic gastritis. Due to having a higher molecular weight than ethanol, isopropanol is more intoxicating than ethanol and can produce an altered sensorium, hypotension, hypothermia, and even cardiopulmonary collapse. Hypotension is associated with a severe overdose and is related to a mortality rate of nearly 45% **[13]**. About 80% absorption of isopropanol, which has and half-life of 3 to 7 hours, is metabolized within 30 minutes and 3 hours and is transformed by the liver to acetone by the enzyme alcohol dehydrogenase **[14-16]**. It hence becomes necessary to search for means to reduce the exposition to isopropanol during the use of ABHRs. Some natural products like spices essential oils have shown to possess good antimicrobial properties. In fact, *Piper nigrum* and *Syzygium aromaticum* essential oils tested on our previous work **[17]** have shown good properties on some Gram+ and Gram- bacteria. Furthermore, many authors have confirmed these properties **[17-22].** Based on these rational our research hypothesis was the following: using essential oils *Syzygium aromaticum* and *Piper nigrum* and substituting part of isopropanol with ethanol in the WHO alcohol- based hand rub formulation can improve the antimicrobial potential of this ABHR.

Following these concerns, the main objective of this work was to reduce the isopropanol contain of and WHO basic ABHR formulation [**23**] by substituting it with ethanol and essential oils.

2. MATERIAL AND METHODS

2.1 Chemical and microbial material

Regarding chemical material, Ethanol, isopropanol, peroxyde hydrogen and glycerol were all obtained from Sigma Aldrich trough a local supplier and were all of analytical grade. The essential oil (EOs) of *Piper nigrum* and *Syzygium aromaticum* were obtained by hydrodistillation as described in Ismail *et al.* 2017. *Staphylococcus aureus* SR196, *Staphylococcus aureus* NCTC10652, *Salmonella* Typhi 15SA, *Salmonella* Enteritidis 155A, *Escherichia coli ATTC25922* and *Pseudomonas aeruginosa* PA01 were used as target strain in this work.

2.2 Preparation of the Alcohol-based hand robs (ABHRs)

Three different formulation were prepared based on previous preliminary works including that of the WHO(F3) [**23**]. These formulations were prepared in other to compare those including the essential oil to those not containing them. In Table 1, the different formulations tested are presented and the essential oil concentrations used are based on the results published in Ismail *et al.* [**17**].

Table 1 : Different ABHRs formulated

Composition	**Formulation F1+**	**Formulation F2+**	**Formulation F3+**	**Formulation F1-**	**Formulation F2-**	**WHO Formulation F3**
Ethanol	**40%**	**20%**	**0**	**40%**	**20%**	**0**
Glycerol	**1,5%**	**1,5%**	**1,5%**	**1,5%**	**1,5%**	**1,5%**
Hydrogen peroxyde	**1,5%**	**1,5%**	**1,5%**	**1,5%**	**1,5%**	**1,5%**
Isopropanol	**40%**	**60%**	**75%**	**40%**	**60%**	**75%**
EO Sa	**700PPm**	**700PPm**	**700PPm**	**0**	**0**	**0**
EO PN	**1200PPm**	**1200PPm**	**1200PPm**	**0**	**0**	**0**
Stérile distillated water	**17%**	**17%**	**22%**	**17%**	**17%**	**22%**

EOSa= Essential oïl of *Zysygium aromaticum* ; EO PN=essential oil of *Piper nigrum*

2.3 Antibacterial activity of the different ABHRs formulated

The antibacterial activity test was performed in 2 phases, using microbial suspensions of 10^7cells/ml. primarily, 1 ml of this microbial broth was spread on the surface of a Mueller Hinton agar (MHA) plate and incubated for 24 h at 37°C. Following this, 6 disks of 5 mm diameter of the agar surface covered by the colonies (1g each) were removed and two portions included in sterile tubes containing 18 ml Mueller Hinton broth for the evaluation of the level of decimal concentration. in order to assess this, the broth containing the two disks were vortex, then diluted in MH broth using a 10 ratio dilution from 10^{-1} to 10^{-7}. These dilutions were incubated at 37°C for 24 h and the highest dilution tube presenting microbial growth was used to indicate the decimal concentration of the culture and represented the cell density on two disks after 24 h growth. The 6 disks permitted to have three repetitions and in case of different results, the mean was calculated and rounded to the highest integer.

Secondly, 1 ml of the microbial suspensions of 10^7cells/ml. was spread on the surface of a Mueller Hinton agar plate already covered by 1 ml of one of the ABHRs formulations and 6 disks exported after 3 minutes of contact, then the decimal load of viable cells evaluated as

previously described. The control was represented by microbial spread without exposure to ABHRs (Figure 1). The percentage reduction after 3 minutes exposure due to the ABHRs was calculated using the following formula:

$$percentage \cdot reduction = \left(\frac{(N_i - N_f)}{N_i} \right) * 100 \quad \text{Equation 1}$$

where N*i* and N*f* are respectively the initial load obtained from the control experiment and final load form the test experiment

After 24 hours, when possible, the number of colonies on the MH agar plates were counted and expressed as percentage reduction after 24 h with respect to the initial load.

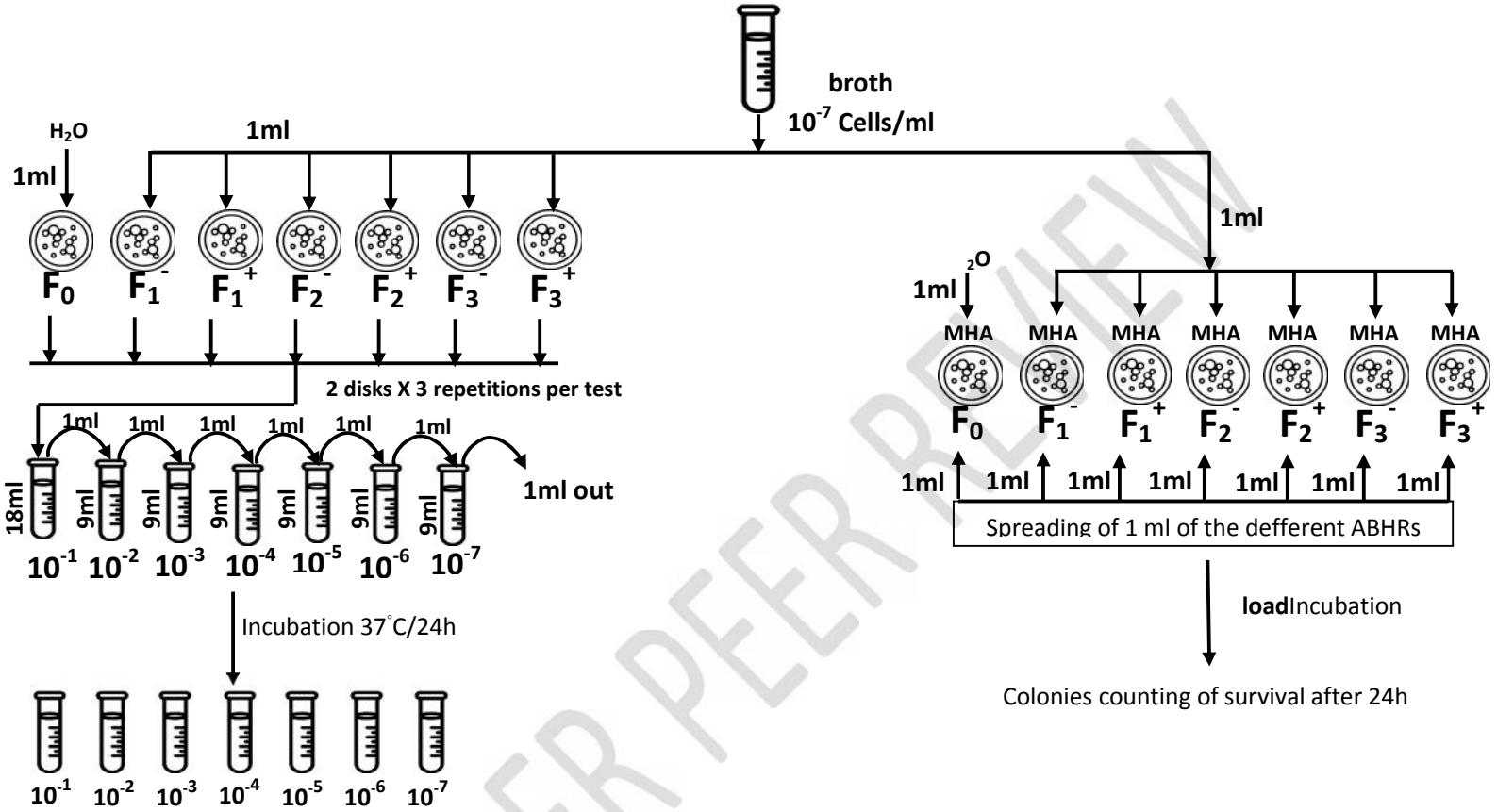

Fig1 : Method of evaluation of the effect of the ABHRs after 3 min and 24 h of action

F1, F2, F3 (cf table 1), - = without essential oil, += with essential oil, MHA=Mueller Hinton Agar

2.4 Adjustment of glycerol in the best formulation obtained from previous tests

A common observation with the previous formulations irrespective of their antimicrobial potential was their extreme fluidity. In order to improve on this aspect, the best formulation obtained from the previous experiments was adjusted with different levels of glycerol (1.5%, 6.5%, 9.5% and 13.5%), reducing distilled water. A verification of the level of decimal reduction after 3 min and 24h was then performed as described previously using the same 03 strains. The best improved formulation was then used for panel and challenge tests.

2.5 Panel test

The best formulation of this work (FA) was compared to two other commercial formulations (FB and FC). FB and FC had respectively the following composition: FB (water, Denaturated alcohol, glycerine, perfume, aminomethyl propanol, carbomer, benzophenone-4, *Aloe barbadensis* leaf juice powder) and FC (Ethyl alcohol, purified water, triethanolamine, benzalkonium chloride, glycerin, carbomer). And evaluation sheet was handed to each of the 29 selected frequent users of ABHRs with the following descriptive: colour, odour, skin contact sensation, texture and overall appreciation. All the participant of the panel test were instructed to only use their palms to assess skin contact sensation and texture, and to wash their hands immediately after the test. The evaluation was performed within a scale of 0 (not appreciated) to 5 (highly appreciated). Data collected were analysed by performing the mean and standard deviation appreciation per criteria followed by a Tukey HSD post-hoc analysis of the significant differences between the formulations, and within the same criteria.

2.6 Challenge test of the ABHR formulated

Challenge test is an experiment by which a process and a product are evaluated on their capacity to prevent the expression of a danger deliberately introduced at a level higher than normality. Microbial challenge testing hence consist of inoculating the product during formulation with specific pathogens and or spoilage organism and to assess their vitality within the formulation and during conservation. Single cultures of selected organism (*Staphylococcus aureus* SR196, *Salmonella typhi* 15SA, *Escherichia coli ATTC25922* and *Pseudomonas aeruginosa* PA01) were inoculated at a final concentration of 10^7cells/ml each in a different ABHR during formulation and 3 min after the end of the formulation, the number of decimal reduction was assessed through dilution of the ABHR in MH broth as described previously. A control of the lost of the ABHR antimicrobial efficacy after dilution was performed by adding 1ml of ABHR to 9ml broth of 10^5cells/ml cell concentration and performing dilution before incubating at 37°C for 24h. the level of decimal growth equivalent

to the initial concentration was an indication that the ABHR lost its antimicrobial efficacy after 10 times dilution and hence could not interfere during the challenge test evaluation of residual cells. Figure 1 indicates the flow sheet of the challenge test. Moreover, in order to assess the antimicrobial stability, ABHR were prepared and conditioned in many containers and used in triplicate for each microorganism during the different challenge test periods (day1, 2 days, 1 week, 1 month, 3 months, 6 months and 1 year).briefly, at each testing period, the product was inoculated and 3 minutes later, sampled as described above for Day1.

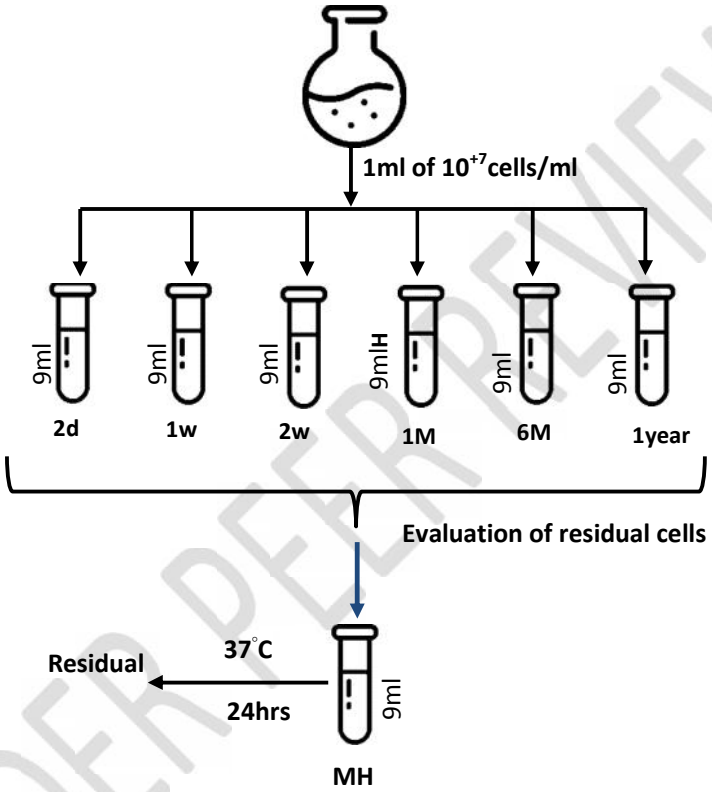

Fig 2 : Challenge test flow sheet performed at 1 day, 2days, 1 week, 2 weeks, 1 month, 3 months, 6 months and 1 year after production

3. RESULTS

3.1 Antibacterial activity of the different ABHRs formulations

The level of decimal reduction of the different ABHR formulations is presented in Table 2 for the bacterial strains, namely, *Staphylococcus aureus* SR196, *Salmonella* Typhi 15SA, *Salmonella* Enteritidis 155A. The results are presented as percentage of reduction with respect to the initial cell load. Regarding *Staphylococcus aureus* the results indicate that formulations with essential oil F1+ and F2+ permitted a cell reduction of 6log cells/ml after 3 min exposure

compared to the same formulations without essential oil that only caused a 5 log cells/ml reduction. No difference of deactivation level of *Staphylococcus aureus* was observed between WHO formulation (F3-) and the same formulation including the essential oil (F3+). Regarding *Salmonella Enteritidis* 155A, F3+ induced a 6 log cells/ml compared to the same formulation in the absence of essential oil that permitted a reduction of 4 log cells/ml. regarding *Salmonella Enteritidis* 155A, the presence of EOs caused and increased reduction after essential oil addition in F3+. *Salmonella Typhi* 15SA was in general less sensitive to the ABHR. ABHR formulations F1 and F3 were improved with essential oil addition, while F3 which is the WHO basic formulation was less effective as antibacterial on the strain tested and was also the ABHR less improved after EO addition. Formulation F2+ was the most active on all the strain tested with 6log cells/ml reduction irrespective of the strain.

Table 2 : Microbial decimal reduction expressed in percentage and Log cells/ml due to exposure of different bacteria to the different ABHRs formulations for 3 minutes

ABHRs Formulations	Microbial culture (10^7cells/ml)	Percentage reduction	Reduction expressed in log_{10} cells/ml
TEMOIN (BMH seul)	*S.a*	0	0
	S.e	0	0
	S.t	0	0
F1+	*S.a*	99,9999	6
	S.e	99,9999	6
	S.t	99,999	5
F1-	*S.a*	99,999	5
	S.e	99,9999	6
	S.t	99,9999	6
F2+	*S.a*	99,9999	6
	S.e	99,9999	6
	S.t	99,9999	6
F2-	*S.a*	99,999	5
	S.e	99,9999	6
	S.t	99,999	5
F3+	*S.a*	99,99	4
	S.e	99,9999	6
	S.t	99,9	3
F3- (WHO) control	*S.a*	99,99	4
	S.e	99,99	4
	S.t	99,99	4

S. a = Staphylococcus aureus ; S.e = Salmonella Enteritidis *; S.t = Salmonella* Typhi ; "+" = with essential oil ; "- "= without essential oil

The results of the percentage inactivation after 24 h exposure to the different ABHRs, are presented in Table 3. It can be observed that in the control without ABHR, growth was not

prevented, whereas in the tests experiment, the minimum reduction percentage was 99.99975% corresponding to 5.6 log cells reduction of the initial population of about 7 log ufc/ml. ABHR formulation F2+ still remained the best after 24 h contact as it assured a 100% reduction (no colony observed) after exposure on *Staphylococcus aureus and Salmonella* Enteritidis and 99.9999% reduction on *Salmonella* Typhi.

Table 3 : Effect of different ABHRs formulations on the three strains after 24 h exposure, expressed in percentage with respect to the initial cell load.

ABHRs formulations	Percentage reduction after 24h (%)		
	S. a	***S.e***	***S.t***
F0	NC	NC	NC
F1+	100	100	99,99975
F1-	99,99975	100	99,99975
F2+	100	100	99,9999
F2-	99,99975	100	99,99975
F3+	99,99975	100	99,99975
F3-	99,99975	99,99975	99,99975

S. a = Staphylococcus aureus ; S.e = Salmonella Enteritidis *; S.t = Salmonella* Typhi *;* NC = not countable, Pretri disk overloaded.

3.2. Adjustment of glycerol in the best formulation obtained from previous tests

In order to reduce the extreme fluidity and the sensation of dryness, the best formulation F2+ obtained from the previous experiments was adjusted with different levels of glycerol (1.5%, 6.5%, 9.5% and 13.5%), reducing distilled water. The final formulations and results of antibacterial activity after 3 min and 24h exposure is presented in Table 4. As it can be seen, the substitution of part of distilled water with glycerol did not impact on the bacterial activity. the same levels of reduction were observed after 3 min and 24 h exposure. Based on these results, Formulation F2+C was chosen for the glycerol content.

Table 4 : Adjustment of glycerol content in the best formulation

ABHRs Formulations	F2+A	F2+B	F2+C	F2+D
Ethanol	20%	20%	20%	20%
Glycérol	1,5%	9,5%	13,5%	6,5%
Peroxyde d'hydrogène	1,5%	1,5%	1,5%	1,5%
Isopropanol	60%	60%	60%	60%
HE CG	700PPm	700PPm	700PPm	700PPm
HE PN	1200PPm	1200PPm	1200PPm	1200PPm
Eau distillée stérile	17%	9%	5%	12%
% reduction after 3 min for S.a	99.9999	99.9999	99.9999	99.9999
% reduction after 3 min for S.E	99.9999	99.9999	99.9999	99.9999
% reduction after 3 min for S.T	99.9999	99.9999	99.9999	99.9999
% reduction after 24h for S.a	100	100	100	100
% reduction after 24h for S.E	100	100	100	100
% reduction after 24h for S.T	99.9999	99.9999	99.9999	99.9999

3.3 Comparative panel test of the formulation obtained with two commercial products

29 panellists chosen on the bases of their frequent use of ABHRs (at least ones a day) were selected and asked to give appreciation on ABHR F2+C (named FA) and two commercial products FB and FC which composition have been presented previously. According to Figure 3, product FA and FB were similar but were significantly different ($p<0.05$) form FC regarding the colour, with FB being most appreciated. FC was most appreciated for odour and all the products were significantly different ($p<0.05$) between each other, for this aspect. Regarding the sensation given by the product on the hands, the formulation proposed in this work (FA) was rated in between the two commercial products, while for the texture it was statistically rated in the same way as ABHR FC. All the three products were statistically equivalent in term of general appreciation ($p<0.05$)-

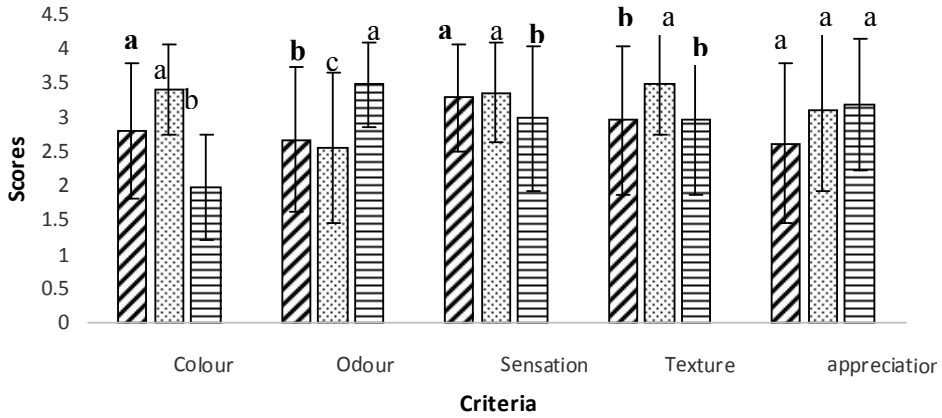

Fig 3 : Comparison of the ABHR formulation obtained in this work FA (black oblique lines histograms) with two commercial samples FB (dotted histogram) and FC (horizontal lines histogram)

3.4. Challenge test of the ABHR formulated

Addition of 1 ml of the ABHR in 9 ml broths containing 5 log cells/ml did not prevent the different microorganisms from growing. This indicated that the ABHR diluted 10 times can not prevent growth of the selected microorganisms. ABHR formulation F2+C, inoculated with the different strains *Salmonella* Typhi 15SA, *Staphylococcus aureus* NCTC10652, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PA01 at 7 Log ufc/ml did not presented any vital residual cells after 3 min at D1 of production. Moreover 1ml of the

formulation after being stored for 2 days, 1 week, 1 month, 3 months, 6 months and 1 year did not allowed the survival of cells after 3 minutes of contact with the selected pathogen as indicated in Table 5.

Table 5: residual cells evaluation obtained during the challenge test after deferent periods of product storage

Storage period	*Salmonella* Typhi 15SA	*Staphylococcus aureus* NCTC10652	*Escherichia coli* ATCC25922	*Pseudomonas aeruginoa* PA01
Growth control	+	+	+	+
Day 1	-	-	-	-
2 days	-	-	-	-
1 week	-	-	-	-
2 weeks	-	-	-	-
1 month	-	-	-	-
3months	-	-	-	-
6 months	-	-	-	-
1 year	-	-	-	-

-= no growth ; + = growth

4 DISCUSSION

The efficacy of ABHRs is mostly related to their capacity of reducing the highest number of microbial cells in less time possible. In fact these products are generally used in conditions where hand washing using water and antiseptic soaps is not possible for their non availability or the limited time at disposal. A contact test on agar Mueller Hinton for 3 minutes and 24h was used in this work in order to compare different ABHRs.

The WHO basic formulation (F3-) provided after 3 min of contact, 99.99% reduction of *Staphylococcus aureus* SR196, *Salmonella* Typhi 15SA, *Salmonella* Enteritidis 155A. The fact that this formulation was the less active than those with less isopropanol but containing 20 to 40 % ethanol is an indication that the combination of the two alcohols is more active than using isopropanol alone. **Man *et al.* [24]** after testing low molecular weight alcohols on bacterial viability concluded that n propyl alcohols were more active followed by isopropanol,

n butyl and iso butyl alcohols, and all the precedent compounds were more active that ethanol. In fact alcohols can affect microorganism vitality with two main mechanism **[25]**; membrane lipid layer solubility and protein alcohol solubility. It is generally accepted that alcohol efficacy increases in the presence of water and that 60 to 90 % v/v alcohol concentration is the most active range. In this work, at the contrary, formulations combining ethanol and isopropanol which had less water than the control WHO base formulation were more active. These observation may be the result of the alcohol combinations or the slight overall alcohol contain. The addition of essential oils in the different formulations permitted an increase of the antimicrobial activity in all cases. In fact the EOs of *Piper nigrum* and *Syzygium aromaticum* were demonstrated to be active on these strains by **Ismail *et al.* [17]**. It can be hypothesized that there was a synergic effect between the alcohols and the EOs. In fact the membrane disrupting effects of alcohols can increase the diffusion of the essential oils and hence potentiate their action.

The increase of glycerol contain by substituting part of distilled water in the most active ABHR (F2+) did not impact on the activity. This result indicates that glycerol as water could be good carriers of the different antimicrobials. According to the French society of clinical hygiene **[10]** citing the European norm EN 12054, ABHRs should provide at least a 5 log reduction after a contact between 1 to 5 min on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus hirae* strains. The ABHR formulated here had a 6 log reduction in 3 minutes indicating its good efficacy. Comparison of the formulation (F2+C) containing 13.5% of glycerol with two commercial products indicated that they were not statistical different in terms of general appreciations. Nonetheless, statistical differences were observed in terms of colour and odour among the three products. These variations can be associated to non-antimicrobial components added to the commercial products like perfume and leaf juice in commercial product FB and carbomer in FC.

In terms of product stability, the challenge test demonstrated the capacity of the developed formulation to eradicate possible contaminations by *Salmonella Typhi* 15SA, *Stphylococcus aureus* NCTC10652, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PA01 during the production and to preserve the same antimicrobial efficacy after one year of storage at ambient temperature. This stability may be due to the fact that the antimicrobial components do not react with each other during storage by producing non active compounds.

5-CONCLUSION

In conclusion, the present work formulated an ABHR with a reduced isopropanol content known to be toxic based on WHO basic composition. Moreover, the new formulation improved by the addition of *Piper nigrum* and *Syzygium aromaticum* essential oils had an increased antimicrobial activity, reducing 6 log cells/ml of pathogens in 3 minutes. No difference in overall appreciation was observed when comparing the formulation of this work with two commercial products by the panellists. The product also proved to maintain its activity for one year and to be able to deactivate possible contaminations by *Salmonella Typhi* 15SA, *Staphylococcus aureus* NCTC10652, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PA01 .

6-RECOMMENDATIONS AND LIMITATIONS

Based on this study, the authors recommends that formulations of ABHRs be made with reduced isopropanol content and that producers should exploit the rich diversity of plants antimicrobial. This study has however some limitations that need to be covered in order to obtain consent for application. In the future, the level of skin cells toxicity of the formulation proposed need to be assessed as well as antifungal and antivirus potential.

CONSENT

All the participant gave a written consent

ETHICAL APPROUVAL

As per University standard guidelines and according to the laboratory research board ethical approval was limited to participant consent that was collected and preserved by the authors

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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