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(RESEARCH ARTICLE)

Effect of variations in heterotrophic plate count methods on bacterial estimation in some sachet water in Calabar Metropolis

Mmuoegbulam Augusta Oluchi *, Unimke Augustine Agorye, Andy Iniobong Ebenge, Anika Ogemdi Chinwendu and Macaulay Rachael Teniola

Department of Microbiology, Faculty of Biological Sciences,, University of Calabar, PMB 1115, Calabar, Cross River State, Nigeria.

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Abstract

Heterotrophic plate count (HPC) or standard plate count is a standardized method for determining the density of aerobic and facultative anaerobic heterotrophic microorganisms in water. In this study, water samples were analyzed to ascertain the effects of temperature, media, and incubation time in HPC methods of bacterial estimation. The samples were analyzed using the membrane filtration and direct plating methods. No faecal coliform was detected in all the samples and at all temperature. However, some coliforms and heterotrophic bacteria were present in the water samples. The organisms were isolated at 4 °C, 28 °C and 37 °C after 24-72 h. The heterotrophic bacterial isolates include: *Enterobacter* spp., *Staphylococcus aureus, S. epidermidis, Bacillus sphericus, B. cereus, Micrococcus roseus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* It was observed that yeast extract agar gave a higher heterotrophic plate count at 28 °C while EMB agar yielded the highest total coliform count at 37 °C. The result of the physicochemical analysis showed that the water samples met the World Health Organization (WHO) and Standards Organization of Nigeria (SON) standards for drinking water while the bacteriological quality based on the total coliform count result indicates the need for improvement in the treatment systems.

Keywords: Heterotrophic plate count; Total coliform; Bacterial estimation; Sachet water; Physicochemical

1. Introduction

Heterotrophic plate count is a method that measures colony formation on culture media of heterotrophic bacteria in drinking water; it can be used to measure the overall bacteriological quality of drinking water in both public, semi-public and private water systems [1]. Routine microbiological testing of drinking water supply, recreational waters, and environmental waters is essential for the protection of public health [1].

According to World Health Organization [2], more than 3.4 million people in the world die each year from water-borne diseases. In addition, it is estimated that around 50% of the population in developing countries suffer from water-related diseases at one point or the other [3]. Moreover, Shayo *et al*. [4] reported that evaluation of water quality has gained worldwide attention because majority of diseases causing morbidity and mortality are water-related. These occurrences has made infections contracted from contaminated water supplies a leading cause of illness and death worldwide and heterotrophs are the major pathogens of water [3] which includes some enterotoxigenic enterobacteria [5]. Mmuoegbulam *et al*. [6] also stated that drinking water is very important in determining the health condition of people in an environment thereby making evaluation of the quality of every public-water pertinent so as to determine their portability.

* Corresponding author
E-mail address: oluchidinma@yahoo.com

Heterotrophs which are a group of microorganisms (bacteria, moulds and yeasts) that use organic carbon sources to grow can be found in all types of water and account for the majority of bacteria found in drinking water systems [7]. Basically, the use of agar plates to recover a wide range of microorganisms from water is referred to as "heterotrophic plate count" (HPC Test) or standard plate count [8] and it attempts to provide a standardized means of determining the density of aerobic and facultative anaerobic heterotrophic microorganisms in water. HPC test can be used to measure the overall quality of drinking water in public, semi-public and private water systems [1]. Generally HPC test is studied for microorganisms from natural (typically non-hazardous sources) microbiota of water, however, in some cases used for microorganisms derived from various contamination sources [1]. This study was geared towards determination of the effect of variations on the heterotrophic plate counts of some selected brands of sachet water produced and sold in Calabar metropolis and their physicochemical qualities.

2. Material and methods

2.1. Collection of samples

Sachet drinking water (pure water) samples were collected in the morning after packing from four (4) sachet water producing companies in Calabar Metropolis and transported to the laboratory for heterotrophic plate count and physicochemical analysis.

2.2. Microbiological analysis

The water samples were analyzed microbiologically according to the method of APHA [9], mostly for bacteria group using the plating technique (pour plate) and membrane filtration technique to check for the total viable count of microorganisms.

2.2.1. Pour plate technique

Each of the water sample was diluted with appropriate diluents (distilled water). A tenfold serial dilution was carried out on each of the sample, using the pour plate method where 1 ml of 10^{-5} dilution was pipetted into sterile Petri - dish and 15 ml of molten agar (nutrient agar and yeast extract agar for isolation of bacteria). The plates were gently swirled by rolling them on the bench. Inoculation of the samples was done in triplicate and the agar was allowed to solidify before incubation in an inverted position at different incubation temperatures and periods of 4 °C, room temperature and 37 °C, 24 hours, 48 hours and 72 hours respectively.

2.3. Maintenance of isolates

After incubation discrete bacterial colonies were subcultured on fresh nutrient agar plates to obtain pure cultures. The plates were incubated again and the distinct colonies of bacteria were picked and stocked on nutrient agar for necessary identification.

2.4. Incubation

The various inoculated labelled petri dishes were incubated at 4 °C, 28 °C and 37 °C for 24 h, 48 h and then 72 h. The plates were examined at the end of incubation.

2.5. Growth enumeration

After incubation of plates, the plates were observed carefully for count of colonies. The total bacteria count was determined from nutrient agar and yeast extract agar. Total coliform count was obtained using MacConkey Agar, Endo Agar and EMB agar.

2.6. Physicochemical analysis

The physical parameters analyzed include temperature, pH, conductivity, BOD, total dissolved solids (TDS), total hardness and turbidity. These were determined using HACH pH, conductivity and BOD meters. The chemicals and heavy metals like sulphide, nitrite, nitrate, iron, zinc, chromium, nickel and cobalt were analyzed using a spectrophotometer (HACH-DR 5000 model). Each parameter was determined with the corresponding reagent.

3. Results and discussion

3.1. Heterotrophic bacterial count (HBC)

3.1.1. Growth on nutrient agar at 4 ℃ with varied incubation periods

It was observed that at 4 ºC after 24 h incubation, no bacterial growth occurred in all the water samples analysed using both nutrient and yeast extract agar. However, after 48 h and 72 h of incubation, samples A and D still had no growth while samples B and C had I CFU/ml. The result is as presented in figure 1.

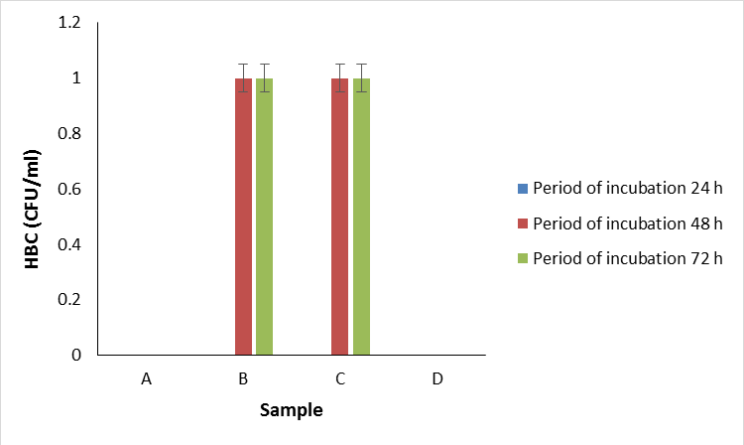

Figure 1 Comparison of HBC at 4 °C on nutrient agar

3.1.2. Growth on yeast extract agar at 4 ℃ with varied incubation periods

The result in figure 2 showed that at 4 °C on yeast extract agar, sample A had a heterotrophic bacterial count of 0 CFU/ml after an incubation period of 24 h but had an HBC of 2 CFU/ml after 48 h and 72 h incubation period. Sample B had an HBC of 0 CFU/ml after 24 h but with 6 CFU/ml HBC after 48 h and 72 h period of incubation. Sample C with 0 CFU/ml HBC after an incubation period of 24 h had a bacterial count of 7 CFU/ml after 48 h and 72 h of incubation. Also at 4 °C, sample D had no growth on yeast extract agar but had 2 CFU/ml after 48 h and 72 h of incubation (Figure 2).

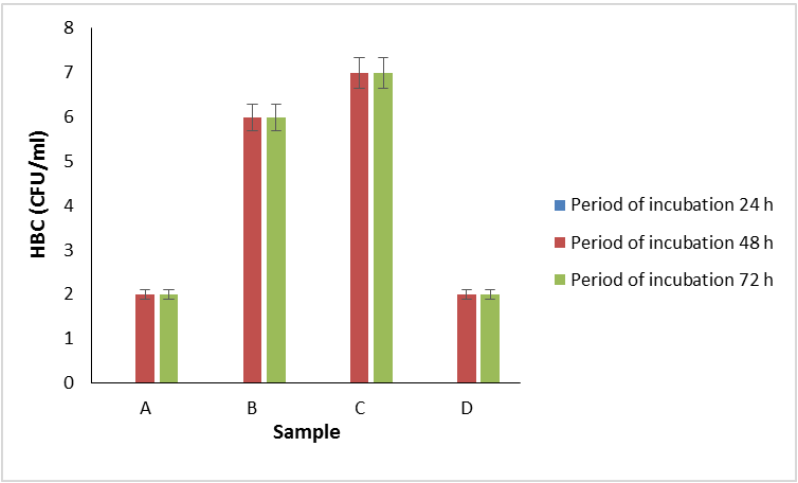

Figure 2 Comparison of HBC at 4 °C on yeast extract agar

3.1.3. Growth on nutrient agar at 28 ℃ with varied incubation periods

Figure 3 showed that at 28 °C, samples A and B had no growth on nutrient agar after 24 h and 48 h incubation periods but had HBCs of 1 CFU/ml and 2 CFU/ml after 72 h respectively. It was observed that samples C and D both had counts of 1 CFU/ml after 24 h but with counts of 2 CFU/ml and 1 CFU/ml after 48 h respectively. However, sample A had an

HBC of 1 CFU/ml after72 h of incubation while samples B, C, and D had counts of 2 CFU/ml, 4 CFU/ml and 9 CFU/ml respectively.

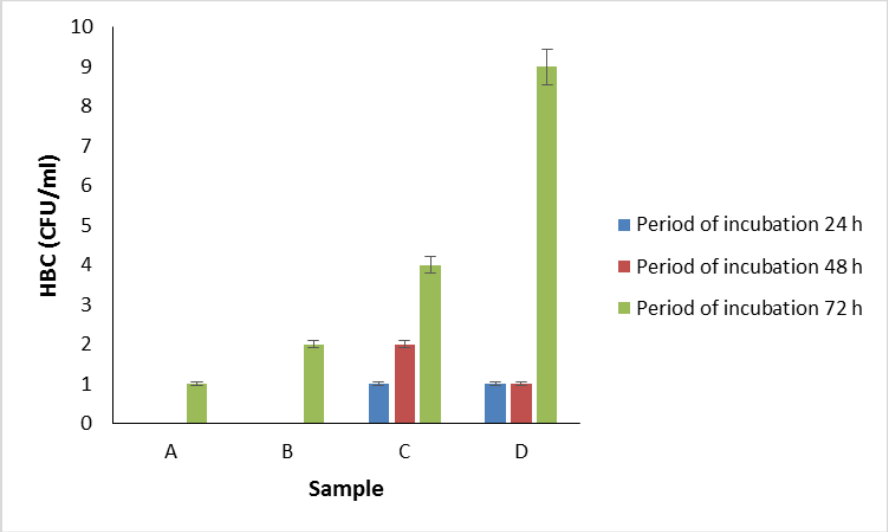

Figure 3 Comparison of HBC at 28 °C on nutrient agar

3.1.4. Growth on yeast extract agar at 28 ℃ with varied incubation periods

As presented in figure 4, after 24 h incubation period, samples A and B had 3 CFU/ml HBC each after 24 h of incubation while samples C and D had an HBC of 2 CFU/ml and 4 CFU/ml respectively after an incubation period of 24 h. For 48 h period of incubation, samples A, B. C. and D gave a bacterial growth yield of 3 CFU/ml, 3 CFU/ml, 2 CFU/ml and 11 CFU/ml respectively on yeast extract agar while after 72 h, the growth yields recorded for samples A, B, C, and D were 3 CFU/ml, 9 CFU/ml, 7 CFU/ml and 11 CFU/ml respectively (Figure 4).

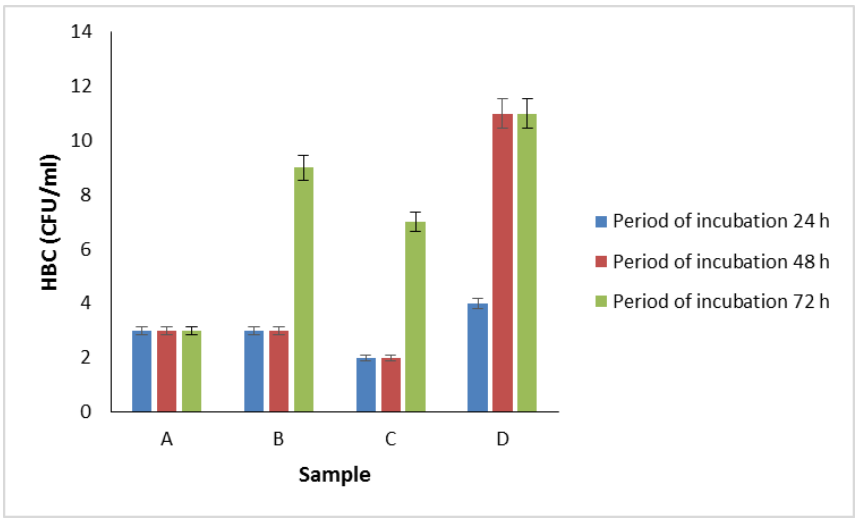

Figure 4 Comparison of HBC at 28 °C on yeast extract agar

3.1.5. Growth on nutrient agar at 37 ℃ with varied incubation periods

The result presented in figure 5 showed that at 37 °C on nutrient agar, samples A, B, C and D all had a heterotrophic bacterial count of 0 CFU/ml after an incubation period of 24 h. However, a bacterial growth count (HBC) of 3 CFU/ml, 0 CFU/ML, 0 CFU/ML and 1 CFU/ml were recorded for samples A, B, C, and D respectively after 48 h and 72 h periods of incubation (Figure 5).

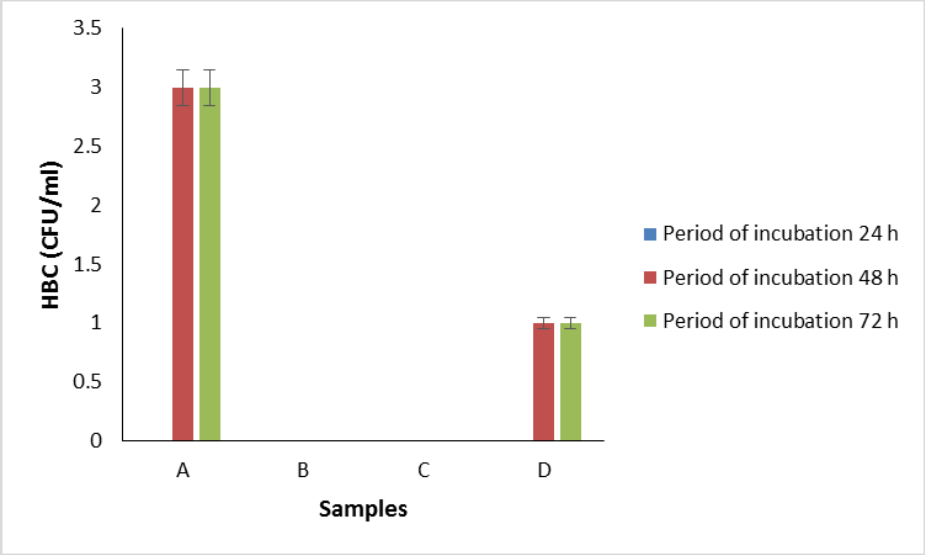

Figure 5 Comparison of HBC at 37 °C on nutrient agar

3.1.6. Growth on yeast extract agar at 37 ℃ with varied incubation periods

As presented in figure 6, after 24 h incubation period on yeast extract agar, samples A, B, C and D had 0 CFU/ml HBC each. However, after 48 h period of incubation, samples A, and B gave a count of 3 CFU/ml and 3 CFU/ml respectively while samples C. and D gave a bacterial growth yield of 2 CFU/ml and 11 CFU/ml respectively on yeast extract agar. After 72 h, the growth yields recorded for samples A, B, C, and D were also 3 CFU/ml, 3 CFU/ml, 2 CFU/ml and 11 CFU/ml respectively as figure 6 showed.

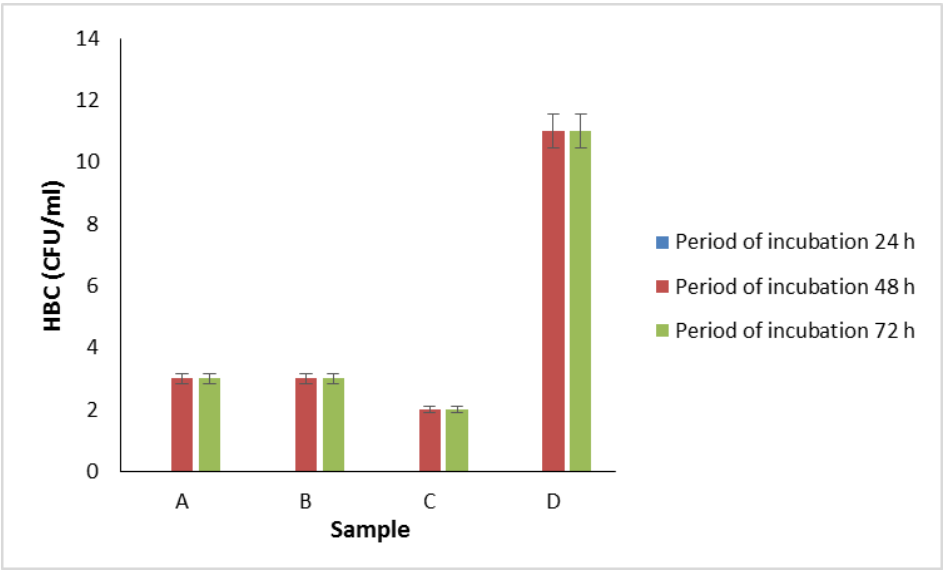

Figure 6 Comparison of HBC at 37 °C on yeast extract agar

3.1.7. Total coliform count (TCC) at 37 ℃ after 24 h

According to the result in figure 7 below, at 37 °C after 24 hours, sample A had no total coliform count, sample B had the highest total coliform count of 12 CFU/100 ml, sample C had 8 CFU/100 ml while sample D had 6 CFU/100 ml. Total coliform count at 37 °C on MacConkey for the four samples after 24 h incubation period ranged from 0 to 14 CFU/100 ml while on EMB, sample A had the highest total coliform count of 19 CFU/100 ml followed by sample B which had 7 CFU/100 ml while samples C and D had 4 CFU/100 ml respectively (Figure 7). However, there was no faecal coliform observed in all the sachet water samples at 44 °C on all the media used.

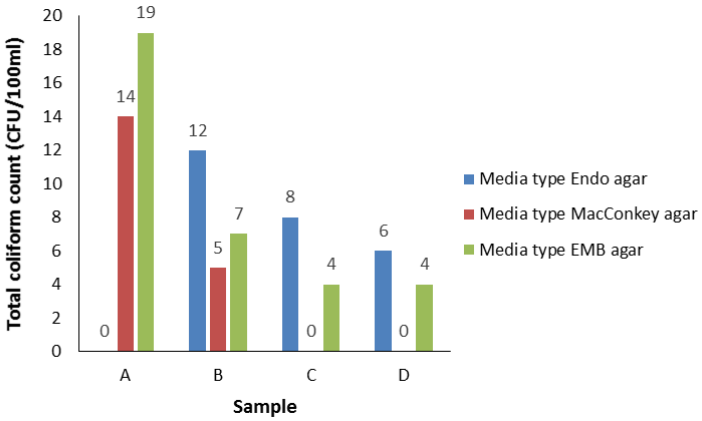

Figure 7 Comparison of the total coliform count of the samples at 37 °C after 24 h on different media

Table 1 Bacterial isolates from the samples

Serial number	Sample code	Probable bacterial isolate
1	A	*Enterobacter spp.*
		Staphylococcus aureus
		Bacilluss sphericus
2	B	*Bacillus cereus*
		Micrococcus roseus
3	C	*Staphylococcus epidermidis*
		Pseudomonas aeruginosa
4	D	*Bacillus sphericus*
		Staphylococcus aereus
		Klebsiella pneumonia

3.2. Physicochemical analysis

3.2.1. Physical qualities of the water

According to the result obtained from physical analysis of the sachet water samples, samples A, B and C had 26.6 °C temperatures while sample D had 26.4 °C. Sample A had the lowest pH value of 3.43, sample B had 6.03, sample C had 6.41 while sample D had 6.47. The conductivity value of sample A was 155.56 µs/cm, sample B had a conductivity value of 51.6 µs/cm, while that of samples C and D were 51.1 µs/cm and 97.6 µs/cm respectively. Turbidity for sample A was 0.443 NTU which was the highest, sample B had 0.352 NTU, sample C had 0.371 NTU while sample D had 0.395 NTU. The sachet water samples had a total dissolved solids ranging from 93.36 mg/l to 30.66 mg/l which were obtained from samples A and C respectively. Sample A and B had biological oxygen demand value of 7.44 mg/l, sample C had 7.41 mg/l while sample D had 7.42 mg/l (Table 2).

Table 2 Physical qualities of the water samples

Parameter	Unit	Sample A	Sample B	Sample C	Sample D
Temperature	°C	26.6	26.6	26.6	26.4
pH		7.43	6.03	6.41	6.47
Conductivity	µs/cm	155.6	51.6	51.1	97.6
Total dissolved solids	mg/l	93.36	30.96	30.66	58.56
Turbidity	NTU	0.443	0.352	0.371	0.395
BOD	mg/l	7.44	7.46	7.41	7.42

PPT = Parts per trillion, µs/cm= Microsemen per centimeter, NTU = Nephlometric turbidity unit, mg/l = Milligram per litre, BOD = Biological Oxygen Demand

3.2.2. Chemical qualities

Based on the result obtained from the chemical analysis of sachet water samples, sample A and D had the highest total hardness value of 34.2 mg/l each while sample B and C had the total hardness of 17.1 mg/l each. Although samples A and D had nitrite values of 0.006 mg/l each, sample B had a value of 0.007 mg/l while sample C had 0.003 mg/l nitrite value. Sample A had a nitrate value of 9.4 mg/l sample B had 4.9 mg/l, sample C had 6.1 mg/l while sample D had 5.0 mg/l nitrate value. The sulphide –N value for sample A was 9 mg/l, samples B and C had 3 mg/l while sample D had 5 mg/l (Table 3).

Table 3 Chemical qualities of the water samples

Parameter	Sample A	Sample B	Sample C	Sample D
Total hardness (mg/l)	34.2	17.1	17.1	34.2
Nitrate (mg/l)	0.006	0.007	0.003	0.006
Nitrite (mg/l)	9.4	4.9	6.1	5.0
Chloride (mg/l)	45.52	17.07	17.07	28.45
Sulfide (mg/l)	9	3	3	5
Salinity $(^{o}/_{oo})$	0.08	0.03	0.03	0.05

3.2.3. Heavy metals in the water

From the result obtained from heavy metal analysis of the sachet water samples, sample A had a nickel value of 0.059 mg/l, sample B had 0.047 mg/l while samples C and D had 0.049 mg/l. Sample A had cobalt value of 0.026 mg/l, sample B had no cobalt, sample C had 0.021 mg/l while sample D had 0.014 mg/l. Chromium value for sample A was 0.018 mg/l, sample B had 0.025 mg/l, sample C had 0.011 mg/l while sample D had 0.023 mg/l. Iron value for samples A and D were 0.04 mg/l each, sample B showed no iron content while sample C had 0.01 mg/l iron level. Sample A also had zinc value of 0.14 mg/l, samples B and C had 0.17 mg/l each while sample D had 0.08 mg/l as presented in table 4 below.

Table 4 Heavy metal content of the water samples

Parameter	Sample A	Sample B	Sample C	Sample D
Nickel (mg/l)	0.059	0.047	0.049	0.049
Cobalt (mg/l)	0.026	0.000	0.021	0.014
Chromium (mg/l)	0.08	0.03	0.011	0.023
Iron (mg/l)	0.04	0.00	0.01	0.04
Zinc (mg/l)	0.14	0.17	0.17	0.18

The total heterotrophic count showed a significant difference (variations) with incubation temperature, time and media type used. This is based on the result of a two way ANOVA analysis in which the Fcal (5.0) was greater than the Fcrit (3.1) at 95% confidence level. Although no microbial growth was recorded after 24 hours in all the media used and at all temperatures using the serial dilution, a direct plating method of the samples on the different media used yielded some bacteria after 24 hours at 28 ^{o}C. This suggests that the microbial load in the water samples was too few that the dilution process resulted in the absence of microbial growth on all the media and at all temperatures.

The result of the heterotrophic plate count showed that all the water samples were safe for drinking since the count were within the EPA standard of 1.0×10^{2} CFU/ml as stated in USEPA [10]. The highest heterotrophic plate counts were obtained on yeast extract agar when compared to counts obtained from nutrient agar plates, with the counts increasing as the period of incubation increases at 28 ^{o}C. Although heterotrophic bacterial counts were also high on YEA at 4 ^{o}C and 37 ^{o}C compared to counts on nutrient agar plates, the counts were constant as the incubation period increased from 24 h to 72 h. The total coliform count by membrane filtration revealed that Eosin methylene blue was the best medium for recovery of coliforms from drinking water followed by Endo agar and then MacConkey agar at 37 ^{o}C after 24 h of incubation.

Table 5 World health organization (WHO) standard for drinking water

Parameter	**WHO standard**
Temperature	25
pH	6.5-9.5
Hardness	200
Conductivity	1000 (μs/cm)
Total dissolved solids	600 mg/l
Chloride	250 mg/l
Sulphate	250 mg/l
Nitrate	50 mg/l as NO_3^-
Nitrite	3 mg/l as NO_2^-
Total coliform organisms/100 ml	0
Faecal coliform/100 ml	0
Iron	0.3 mg/l
Zinc	0.05 mg/l
Chromium	0.07 mg/l
Cobalt	-
Nickel	-
Copper	1.0 mg/l

WHO guideline for drinking water (2004)

From the water analysis, all the water samples were free from faecal coliform. This is in agreement with the World Health Organization [11] standard of zero faecal coliform per 100 ml in drinking water. However, all the water samples had total coliform counts and according to Osuinda and Enuezia [12], coliform count values indicate that the water samples were contaminated. Isa *et al*. [13] also stated that the occurrence of total and faecal coliforms in high concentration makes the water unfit for human consumption based on WHO recommendation, although the standard organization of Nigeria [14] gave a coliform guideline of 10 CFU/100 ml making all the sachet water samples analysed to be fit for human consumption. There was a significant difference in the total coliform counts obtained in all the media types within the samples as Fcal > Fcrit (Fcal = 5.3, Fcrit = 3.1) in a two way ANOVA analysis.

All the bacteria isolated from the samples were majorly members of the *Enterobacteriaceae* family including *Klebsiella pneumonia, Enterobacter sp*. etc. Other species presents in the sachet water samples were *Bacillus species, Staphylococcus sp., Micrococcus roseus* and *Pseudomonas aeruginosa.*

The result from this study that incubation period, temperature and media types affect the amount of bacteria recovered from drinking water corroborates with the report by WHO [2] that there were several factors that affect the genera of bacteria in drinking water and their level of presence during HPC; and as reported by Mmuoegbulam *et al*. [15]. These factors include the type of medium used to grow the bacteria, the temperature of incubation used, how long the plates were incubated, where the water sample was collected, the time of the year and the age of the sample. It is also important to know that the concentrations and types of bacteria that are recovered at the same sampling location can vary over time [2]

Although, some of the heterotrophic bacteria recovered from the water samples in this study were opportunistic pathogens, numerous studies have indicated that heterotrophic bacteria isolated from water using HPC methods which were opportunistic pathogens possess very few virulence factors [16], with two large epidemiological studies showing no association between the ingestion of drinking water containing heterotrophic bacteria and the incidence of gastroenteritis in participants [16]. However, in a study by Mmuoegbulam *et al*. [5,6], a lot of opportunistic pathogens were isolated both from river and tap water samples and it was reported that *Klebsiella variicola* and *Enterobacter* species which were isolated from a river in Nigeria were enterotoxigenic and had some histolopathological effects [5].

Physical parameters such as the taste, odour and colour were in conformity with the World Health Organization Standard as all the water samples were odourless, colourless and tasteless.

It has been reported that most sachet water producing companies generally get their water from underground water sources or bore holes which then undergo treatments to achieve its aesthetic properties. However, natural underground water is usually free from contamination because of the purification properties of soil when compared with surface water which is exposed to contamination through anthropogenic activities [17]. Water sources can also be contaminated due to improper construction, shallowness, proximity to toilet facilities, sewage, refuse dump site and human activity around the bore holes but when this bore holes or underground water undergo appropriate treatment it becomes potable and are considered safe for drinking. Based on the result of the mean physicochemical quality of the samples using a two way ANOVA, there was no significant difference ($P < 0.05$) in the physical and chemical qualities observed within the samples, Fcal =0.35, Fcrit = 4.3 (Fcal < Fcrit). All the samples had their pH towards neutrality which is in line with the WHO guideline of 6.5 – 9.5. However, WHO [18] emphasized on the importance of determining the pH of water as a guide to the contact time and the level of free residual chlorine to be added at the end of the contact time for an effective chlorination.

4. Conclusion and recommendation

From the results obtained, it can be concluded that all the sachet drinking water samples analyzed were microbiologically safe for drinking as the water samples had no faecal coliform. However, although the coliform counts were within the SON regulatory standard of 10 CFU/ ml, the high coliform counts above the WHO standard of 0 coliform count/100 ml revealed that there is need for improvement in the treatment processes employed by all the sachet water companies. Based on the result in this study, both media type, incubation temperature and length of incubation all play important role in the recovery of bacteria present in drinking water. Moreover, the physicochemical analysis result obtained revealed that the water samples meet the world health organization standard for drinking water.

We therefore recommend that routine investigations on the quality of water produced by potable water companies should be carried out by appropriate agencies in Nigeria. Underground water sources such as bore hole water should be dug in areas far from possible contamination and the level of ground drilled should meet the approved standard. Moreover, pipes should be checked regularly so as to detect damaged pipes or holes in pipes and the need for replacement with new pipes to avoid contamination. Routine water assessment including physical, chemical and bacteriological examinations should be carried out to ascertain the level of contamination with the aim of eliminating the contamination. Variations in terms of media, temperature and length of incubation should be employed during water analysis before effective conclusions are made about the quality of portable drinking water supplies.

Compliance with ethical standards

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Disclosure of conflict of interest

There were no conflicts of interest/ competing interests.

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