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# Physico-chemical Parameters and Species' Distribution Patterns of Extremophilic Bacteria in Kitagata and Ihimbo Hot Springs in South Western Uganda

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**Abstract**

Extremophiles are organisms that live in extreme environmental conditions of pressure, temperature, salinity that are considered uninhabitable in comparison to the physico-chemical characteristics of the normal environment of human cells. Extremophiles include thermophiles, barophiles, acidophiles and alkaliphiles. Extensive studies of extremophiles ecology, physiology, and molecular biology have yielded valuable information about life processes with a number of important industrial applications. The study sought to profile the thermophilic bacteria present in the less studied two hot springs of Kitagata and Ihimbo. Water samples were collected in a sterile thermo flask and taken to the laboratory, and then the colonial and biochemical tests were done and in comparison to the Bergey's Manual of Determinative Bacteriology nine species were identified. The most common were *Bacillus spp, Klebsiella spp, Escherichia coli, and Enterobacter spp*. Identification of these thermophile bacteria has become a key step in mapping potential sources of the sought thermo-stable enzymes and also empowered future microbial ecology research and bio-discovery of thermo-stable enzymes that are important in the biotechnological breakthrough.

***Key words:*** Extremophiles; Thermopiles; Hot springs.

## 1. Introduction

Extremophiles are organisms that thrive in habitats which are intolerably hostile or even lethal for other life-forms[1]. Examples extremophiles organisms include thermophiles, psychrophiles, alkaliphiles, and acidophiles[2]. These habitats such as hot springs, saline and/or alkaline lakes, deserts and the ocean beds found in nature are too harsh for life to exist since they possess extreme combined values of physico-chemical parameters such as pressure, temperature, pH, salinity, oxidative stress, radiation, chemicals (oxygen, $H_2S$, $CH_4$) and metals (Fe, Cu, Mo, Zn, Cd, Pb [3].

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The terrestrial hot springs that exist on earth represent hot spots for unusual forms of life, genes, and metabolites since they present extreme hot niches whose pristine quality is maintained over long a period of time[4]. The thermophile bacteria thrive at high temperatures, above $45^{o}C$ which are present in hot springs.

The stability and activity of the thermophiles at hot conditions have made them useful alternatives to the easily altered mesophilic molecules. This is due to the membrane lipids containing more saturated and straight chain fatty acids than do mesophilic, which grow typically between 15°C and 40°C [5]. This allows thermophiles to grow at higher temperatures by providing the right degree of fluidity needed for membrane function. The presence of chaperones which refold denatured proteins increase the stability of thermophilic proteins [6]. Also, thermophilic proteins appear to be smaller and in some cases more basic, which may also result in increased stability importance of thermophile [7]

These thermophile bacteria have attracted several studies due their uniqueness and importance in biotechnological breakthroughs. Since they have proven to be sources of thermo-stable enzymes such as protease and amylases used in the food, detergent and pharmaceuticals industries, as well the DNA polymerase for polymerase chain reaction due to their ability to do work under such conditions that would denature enzymes taken from “normal” bacteria[8][9]. Also some bacteria though to be mesophilic have strains that have been found in hot springs such as *Escherichia coli*, *Enterobacter spp*, *Klebsiella spp, Citrobacter spp* and *Leclercia spp* which are the occasional pathogens[4].

In Uganda, the hot springs are found within the left lift valley region and areas along nearby. These include Karungu, Bubale, Kagamba, Kisiizi, Minera, Rubabo, Birara, Rubaare, Ihimbo, Kitagata Kabuga, Kabenge, Ndugutu, Rwimi, Rwagimba, Sempaya, Panyimur, Amuru, Amuru hot springs[10][11]. This study based on two hot springs of Kitagata and Ihimbo which are among the chain of hot springs located along outside the geothermal areas of the rift valley in Western Uganda. Kitagata hot spring is (0°40$^{1}$42.00’’S, 30°09$^{1}$38.0’’E) on an elevation of 1495m within Kitagata town Council, Sheema district on the Ishaka - Kagamba road about 16 km from Ishaka town. It lies besides a small river Ngaromwenda and surrounded with papyrus swamps. Ihimbo hot spring is (0°58$^{1}$41.00’’S, 29°17$^{1}$26.0’’E) on an elevation of 1028m in Bwambara subcounty, Rujumbura county, Rukungiri district. The hot spring is situated downhill in Ihimbo forest at a distance of 1.5 km from the Rukungiri - Kihihi road via Bugangari, Bwambara and Nyamirama and 2 km from River Ntungwa. It is close to the presumed major boundary steep rift fault, at the escarpment front of the western Rift Valley[12].

Several studies on hot springs have been done worldwide specifically on thermophilic bacteria for example in Yellowstone national park USA [1] Orissa, India [13] however , in Africa with in the left rift valley where hot springs are prominent, studies have been mainly Ethiopia[14]. In Uganda few studies have been in the geothermal region within the rift valley region such as Kwatwe- Kikorongo [15, 12]whereas the hot springs scattered along outside this region in which the two hot springs of this study (Kitagata and Ihimbo hot springs)[11][10], fewer studies have been made to explore the thermophiles inhabiting them, thus this study aimed at profiling and identifying the thermophile bacteria in these less studied hot springs of Kitagata and Ihimbo has become a key step in mapping potential sources of the sought thermo-stable enzymes and also assessing the health status due to prevalence of these bacteria.

## 2. Methods

### *2.1 Sampling sites*

In Kitagata hot springs, the water oozes from underground on one site and then flows along down in the pools of the spring. Five sampling sites were selected purposively guided by the morphology, flow regimes to locate the points. In the main hot spring; the sampling points were mapped according to the physical divisions by rocks that formed the pools and the flow of water as K1, K2, K3, K4 and K5 from the adjacent small spring approximately 30M apart (Plate 1). In Ihimbo hot springs, water oozes from the sand, flows into a small stream where in the middle of the stream it forms a pool and then flows out into a small stream. Four sampling points were selected purposively guided by the morphology and flow regimes as H1, H2, H3 and H4 from an adjacent spring at point water oozes from the underground (Plate 2).


**Figure 1:** Kitagata hot spring showing the sampling sites (K1, K2, K3, K4, and K5).


**Figure 2:** Ihimbo hot spring showing the sampling sites (H1, H2, H3 and H4**).**

### *2.2 Collecting water samples for thermophilic bacteria*

Water sample were collected from Kitagata and Ihimbo hot spring in a sterile thermo flasks[16] and brought into micro-biology laboratory at Mbarara University of Sciences and Technology on same day for analysis to reduce the proliferation of mesophilic bacteria. Temperature was measured by using mercury bulb thermometer, electro conductivity and pH using ACH multi-meter handheld probe, model HQ40D in situ. Each water sample collected, 1ml was incubated for 24 hours on the nutrient agar using the pour plate method[17]. The total bacteria count was determined using total plate counting method adopted[18] and expressed as colony forming units (CFUs). Colonies obtained on nutrient agar, were isolated and further purified on nutrient agar using streak plate method as recommended by Rath and Subramanyam[19].

### *2.3 Identification of thermophile bacteria*

Isolated colonies were identified on the basis of routine morphological and biochemical tests[20]. The bacterial colonies were differentiated on the colonial characteristics of size, elevation, pigment, surface, colour, edge and shape as observed on nutrient agar and on microscopic observation of isolated colonies for gram staining to distinguish the Gram-positive and Gram-negative then were sub-cultured onto a prepared plate containing blood agar and on MacConkey agar respectively using spread plate method and streak plate method as recommended by Rath and Subramanyam [19]. The isolates were finally identified as *Bacillus spp Entrobacter spp, Staphylococcus spp, Klebsiella spp, Psuedomonas spp, Streptococcus spp* and *Escherichia coli* by analyzing the producing strain of biochemical characteristics that included indole production test, citrate utilization test, oxidase test, catalase test, starch hydrolysis, and carbohydrate fermentation test in comparison to characteristics given in the Bergey's Manual of Determinative Bacteriology[21]. The biological characteristics considered to fully and exhaustively identify the partially observed genera were catalase, oxidase, citrate, Indole, motility, coagulase and spore staining tests. This was adapted as recommended by the Berg's manual

## *3.* Results

From this study, nine thermophilic bacteria were identified using the traditional methods of morphological, microscopic observation and biochemical characterization (Table 1&2) and comparison with the characteristics given in Bergy's manual. These tests indicated that existing bacteria belonged to the *Bacillus subtullis, Klebsella species*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Escherichia coli, Enterobacter agglomerans and Streptococcus faecalis;* though full taxonomic description recommended to be done with scanning electron microscopy and comparison of the base sequences of the ribosomal RNA.

### *3.1 Morphological characteristics and gram staining test for thermophile bacteria in Kitagata and Ihimbo hot springs*

Morphological and microscopic observation indicated that most occurring bacteria belonged to the *Bacillus spp, Klebsiella spp, Escherichia coli, Psuedomonas spp* and *Enterobacter spp* shown in Table 1 below. Morphologically, these bacteria colonies showed some variation in the colour, margin, shape, and texture of the colonies. Also basing on Gram staining, the isolates were found to be Gram-positive and Gram-negative and microscopic observation on the Gram-positive revealed mostly spore- forming, rod-shaped bacterium arranged in chain and Gram-negative were only rod-shaped bacterium arranged in chain. Growth occurred on nutrient agar, blood agar while on MacConkey agar only the Gram-negative grew. Furthermore, the different thermophile bacterial species exhibited different characteristics when they were grown on two kinds of culture; MacConkey argar and blood argar. Colonies appeared finely wrinkled and adherent to the agar surface. Most exhibited alpha- or beta-haemolytic activity on 5% RBCs but also others were non-haemolytic.

**Table 1:** Morphological characteristics and gram staining test for thermophile bacteria in Kitagata and Ihimbo hot springs**.**

| Species | Growth on | | Colonial characteristics | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MacConkey Argar | Blood argar | Size | Texture | Surface | Elevation | Adges | Colour | Gram stain |
| *Bacillus subtullis* | NG | H | Large | Rough | Dry | Raised | Dentated | White-grey | †ve Bacilli |
| *Klebsiella spp* | NLF | NH | Large | Mucoid | Moist | Raised | Entire | white- grey | –ve Bacilli |
| *Psuedomonas spp* | NLF | H | Large | Rough | Dry | Flat | Dentated | Creamy | –ve Bacilli |
| *Staphylococcus spp* | NG | H | Medium | Smooth | Moist | Raised | Entire | White-grey | †ve Cocci |
| *E coli* | LF | NH | Medium | Smooth | Moist | Raised | Entire | Pink | –ve Bacilli |
| *Entrobacter spp* | LF | NH | Medium | Smooth | Moist | Raised | Entire | White- grey | –ve Bacilli |
| *Entrobacter spp* | NLF | NH | Medium | Smooth | Moist | Raised | Entire | White-grey | –ve Bacilli |
| *Streptococcus spp* | NG | H | Small | Smooth | moist | Raised | Entire | White | †ve Cocci |

***NG= no growth*LF = lactose fermenter *NLF = non lactose fermenter *H = haemolytic *NH = non haemolytic**

### *3.2 Biochemical characteristics of thermophiles in Kitagata and Ihimbo hot springs*

Thermophillic bacteria species isolated from the two hot springs of Kitagata and Ihimbo were further subjected to biochemical tests such as catalase, oxidase, citrate, indole, motility, spore staining, coagulase and capsule staining test. These tests were carried out and the results were observed as either positive or negative (Table .2) and in comparison with the characteristics given in Bergy's manual were identified up to the species.

**Table 2:** Biochemical characteristics of the isolates from Kitagata and Ihimbo hot springs.

| Catalase | Oxidase | Citrate | Indole | Motility | spore staining | coagulase | cupsule Staining | Organism |
|---|---|---|---|---|---|---|---|---|
| + | + | – | – | + | + | | | *Bacillus subtullis* |
| + | – | + | + | + | – | | | *Entrobacter cloacae* |
| + | + | – | – | – | – | + | | *Staphylococcus aureus* |
| + | – | + | – | – | – | | + | *Klebsiella spp* |
| + | – | – | – | + | – | – | | *Entrobacter agglomerans* |
| + | + | – | – | + | – | | | *Psuedomonas aeruginosa* |
| + | + | + | + | + | – | – | | *E coli* |
| + | – | – | – | + | – | | | *Entrobacter aerogenes* |
| – | – | + | – | – | – | | | *Streptococcus faecalis* |

+ Positive to test – Negative

### *3.3 Spatial distribution of thermophile bacteria in the Kitagata and Ihimbo hot springs*

Results indicated that in Ihimbo hot springs, only eight of the nine isolated thermophile bacteria species were identified (Table 3). Only one species of thermophile bacteria, *Entrobacter agglomerans* was not found in any of the four points sampled. On the other hand, *Bacillus subtullis* was the most dorminant and was found in all the four sampling points in Ihimbo hotsprings while *Streptococcus faecalis* was only found at on sampling point in Ihimbo, where water is the hottest, H1. For Kitagata hot springs, results indicated that only six of the nine species of the thermophile bacteria species were found to be present. Only two species were absent in Kitagata hot springs. These were *Streptococcus faecalis* and *Psuedomonas aeruginosa.*

Furthermore, results showed that *Bacillus subtullis* was found almost at all the sampling sites in both hot springs of Ihimbo and Kitagata. *Klebsiella spp* and *Escherichia coli* were also the next prominent in all sites though not found at the points where water was oozing from underground with the highest temperatures (68°C) ie H1, H4, and K1. *Enterobacter species* were found in some sites in both hot springs however *Psuedomonas aeruginosa* was only in Ihimbo hot spring. *Entrobacter agglomerans* and *Streptococcus faecalis* were only found once in all the samples. The most common identified species were of *Bacillus*, *Klebsiella* species. The genus *Bacillus* was isolated from all samples collected in the study.

**Table 3 :**The thermophilic bacteria distribution in Kitagata and Ihimbo hot springs**.**

| Species | **Distribution of thermophile bacteria in the two hot springs** | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **IHIMBO HOT SPRINGS** | | | | | | | | **KITAGATA HOT SPRINGS** | | | | | | | | | |
| | **DRY** | | | | **WET** | | | | **DRY** | | | | | **WET** | | | | |
| | **H1** | **H2** | **H3** | **H4** | **H1** | **H2** | **H3** | **H4** | **K1** | **K2** | **K3** | **K4** | **K5** | **K1** | **K2** | **K3** | **K4** | **K5** |
| *Bacillus subtullis* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | _ | _ | + |
| *Klebsiella spp* | _ | _ | + | _ | _ | + | + | _ | _ | + | + | + | + | _ | + | + | _ | + |
| *Enterobacter cloacae* | _ | + | _ | _ | _ | _ | + | _ | _ | + | + | + | + | _ | _ | + | _ | _ |
| *Entrobacter aerogenes* | + | _ | _ | _ | _ | _ | + | _ | _ | _ | _ | _ | _ | _ | + | _ | + | _ |
| *Staphylococcus aureus* | _ | + | + | _ | _ | _ | _ | _ | _ | _ | + | + | _ | _ | _ | _ | _ | _ |
| *Escherichia coli* | _ | + | + | _ | _ | + | + | _ | _ | _ | + | + | + | _ | _ | + | + | + |
| *Psuedomonas aeruginosa* | _ | _ | + | _ | _ | _ | + | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ |
| *Entrobacter agglomerans* | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | + | _ | _ | _ | _ | _ |
| *Streptococcus faecalis* | + | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ | _ |

+ Present _ Absent

Considering the total thermophilic bacterial counts, the mean total colony counts of thermophilic bacteria was higher for Kitagata hot springs (1080±241.65) compared to that of Ihimbo hot springs which was 674.27±168.57 (Table 4). As indicated in Table 4.3, there is no significant variation between total bacterial colony counts obtained from Kitagata and Ihimbo hot springs (P = 0.367). However, results showed that Kitagata hot springs had more total colony counts of thermophilic bacteria (n = 20) whereas Ihimbo had fewer total colony of thermophilic bacteria (n = 16).

**Table 4:** Comparison of total bacterial counts between Kitagata and Ihimbo hot springs**.**

| Hot spring | N | Mean SE | Standard deviation | P value |
|---|---|---|---|---|
| Kitagata | 20 | 1080 ± 241.65 | 846.5 | 0.367 |
| Ihimbo | 16 | 674.27 ± 168.57 | 168.57 | |

### *3.4 Selected physico-chemical parameters of water of Kitagata and Ihimbo hot springs*

The selected physico-chemical parameters for this study included temperature, pH and electrical conductivity. From the results shown in table 4.5, the measured values varied considerably along the sites sampled. The mean water temperature levels were particularly high at the H1 and H4 for Ihimbo hot springs (64.25 ± 1.03 and 68.25 ± 0.85 respectively) and K1 for Kitagata hot springs (65.25 ± 0.48). These were the sites where hot water emerged from the hot springs. Temperature decreased along its flow.

The mean pH values for water samples obtained from Kitagata hot springs raged from 8.84 ± 0.07 to 8.57 ± 0.18 whereas for Ihimbo hot springs, from 9.77 ± 0.16 to 9.38 ± 0.39. Observation also indicated that the mean pH values of water at all sampling points in Ihimbo hot springs were higher than those observed from all sampling points of Kitagata hot springs (Table 5). In both Kitagata and Ihimbo hot springs, highest mean pH values were obtained from sampling points where temperature was higher than the rest of the sampling points.

Generally, the mean values of Electrical conductivity were found to be higher for all considered sampling points in Kitagata hot springs compared to those in Ihimbo hot springs (Table 5). As noted from Table 5, lower electrical conductivity was observed at sites K1 and K5 for Kitagata hot springs and sampling point H1 and H4 for Ihimbo hot springs. These are the sites with higher temperatures than the rest in the two hot springs. The sites in each hot spring had a similar pattern in EC value falling in the range of (1063- 1324) as shown in Table 5 below.

**Table 5:** Selected physico-chemical characteristic of water samples**.**

| Hot spring ID | Collection Site | Mean ± SE of physico-chemical parameters | | |
|---|---|---|---|---|
| | | Temp (˚C) | pH | Electrical conductivity |
| Kitagata | K1 | 65.25 ± 0.48 | 8.68 ± 0.13 | 1293.50 ± 7.82 |
| | K2 | 56.00 ± 1.00 | 8.65 ± 0.13 | 1303.00 ± 8.34 |
| | K3 | 46.00 ± 0.71 | 8.63 ± 0.12 | 1307.25 ± 6.60 |
| | K4 | 40.00 ± 0.71 | 8.57 ± 0.18 | 1315.50 ± 7.89 |
| | K5 | 47.25 ± 0.25 | 8.84 ± 0.07 | 1286.00 ± 31.82 |
| Ihimbo | H1 | 64.25 ± 1.03 | 9.77 ± 0.16 | 1066.25 ± 16.64 |
| | H2 | 45.50 ± 0.50 | 9.75 ± 0.19 | 1069.25 ± 16.60 |
| | H3 | 38.00 ± 1.15 | 9.54 ± 0.27 | 1075.50 ± 14.43 |
| | H4 | 68.25 ± 0.85 | 9.38 ± 0.39 | 1064.25 ± 20.77 |

### *3.5 Relationship between physico-chemical parameters and total thermophilic bacteria counts*

With the Eye-ball method of interpretation of correlation coefficients, results in Kitagata hot springs, showed that total bacteria counts had a strong negative correlation with temperature (r= -0.6529) and the correlations were significant (P = 0.0018) and the linear regression explaining about 42.63% of the relationship and also the correlations were moderate positive (r=0.4187, P= 0.0662) and very weak positive (r= 0.1573, P= 0.5077) relationship with conductivity and pH respectively and were not significant. On the other hand, Ihimbo hot springs' total bacteria counts had a significant very strong negative correlation (r= -0.8638) with temperature, and the correlations were significant (P= 0.0001) and a linear regression analysis explaining 74.62% of the relationship and also very weak positive (r=0.1835, P= 0.4962) and (r= 0.0571, P= 0.8335) relationships with electrical conductivity and pH respectively and were not significant.

**Table 6:** Relationship between total bacterial count and physico-chemical parameters.

| | | **Correlations** | **Regressions** | | | | |
|---|---|---|---|---|---|---|---|
| **Hot spring** | | **Total count** | ***α*** | ***B*** | ***R²*** | ***β(control)*** | ***P*** |
| Kitagata (n=20) | Temperature | -0.6529 | 4785.14 | -77.38 | 0.4263 | -72.39 | **0.0018** |
| | PH | 0.1573 | -5061.88 | 681.36 | 0.0247 | 609.63 | 0.5077 |
| | EC | 0.4187 | -18760.73 | 15.07 | 0.1753 | 10.49 | 0.0662 |
| Ihimbo (n =16) | Temperature | -0.8638 | 2958.80 | -44.36 | 0.7462 | -44.22 | **0.0001** |
| | PH | 0. 0571 | -172.01 | 76.55 | 0.0033 | -35.59 | 0.8335 |
| | EC | 0.1835 | -3630.73 | 3.91 | 0.0337 | 3.43 | 0.4962 |

For both Kitagata and Ihimbo hot springs, results indicated that the total thermophile bacteria counts for all the points sampled in each of the two hot springs increased with a decrease in temperature of the water (Fig 1 and Fig. 2). Generally, Kitagata hot springs had a higher number of total thermophile bacteria counts (Fig. 1) compared to Ihimbo hot springs (Fig.2).

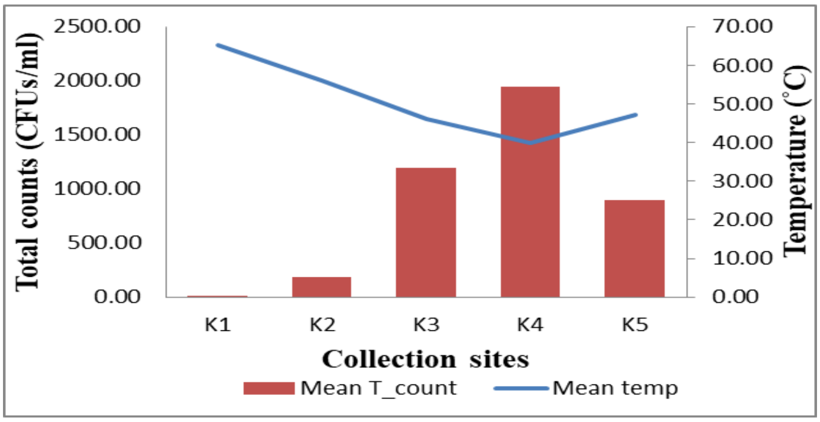


**Figure 3:** Relationship between temperature and total thermophile bacteria count in Kitagata hot spring.

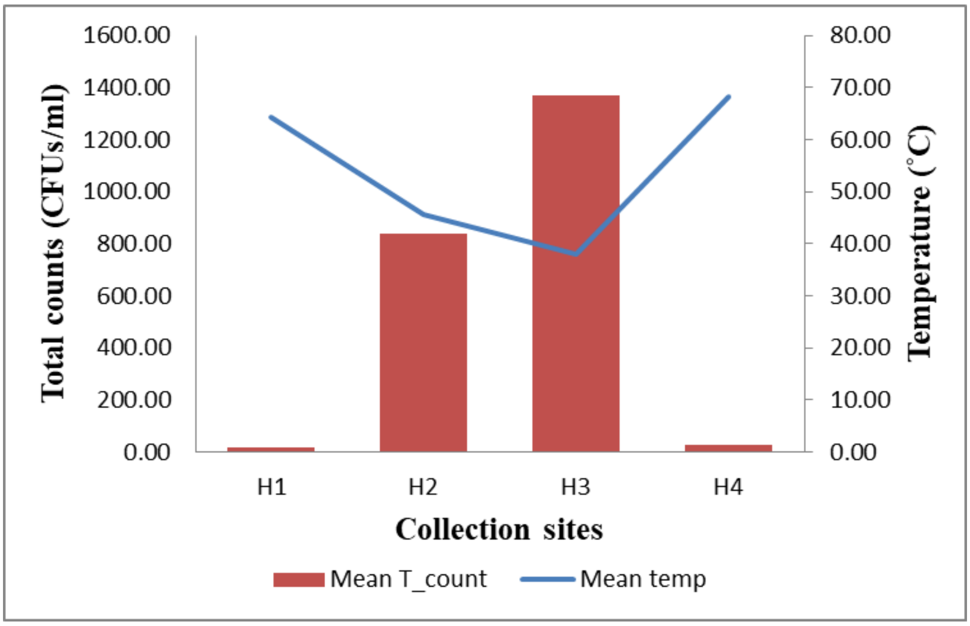


**Figure 4:** Relationship between temperature and total bacteria count Ihimbo hot spring**.**

## 5. Discussion

### *5.1 Morphological characteristics and gram staining test for thermophile bacteria in Kitagata and Ihimbo hot springs*

Nine species of thermophilic bacteria were identified. The species identified were; *Bacillus subtullis, Klebsella species, Enterobacter cloacae, Pseudomonas aeruginosa, Enterobacter aerogenes, Escherichia coli, Enterobacter agglomerans and Streptococcus faecalis.* This finding is consistent with the findings of several studies which all point out that all these above listed species of bacteria are thermophiles [22]. The use of colony characteristics as a basis for identification of these thermophiles from the two hot springs put into consideration several aspects of the colony that included; size, edge, colour, texture, surface and elevation. In addition, the samples were allowed to grow on two types of nutrient agar, MacConkey and blood agar as was described in Bergy's manual. Gram staining was also employed to further identify the species and 3 genera of thermophilic bacteria obtained from the two hot springs showed a positive test with gram staining (Table 1) which showed the same characteristics of no growth when cultured on MacConkey agar and haemolysis when cultured on blood agar. These included; *Bacillus subtullis, Staphylococcus spp and Streptococcus.* These findings were consistent with findings in the study[23] which documented that these genera are thermophilic, gram positive and exhibit the same characteristics as those observed by this study when cultured on the two high nutrient agar; MacConkey and Blood agar. However, others showed a negative gram test on analysis (Table 1) and exhibited different characteristics of growth on MacConkey and blood agar where they were cultured. From the observation *Klebsella spp, Pseudomonas spp* and *Enterobacter spp* did not ferment lactose when cultured on MacConkey agar though only *Pseudomonas spp* showed haemolysis of blood agar culture. These findings are consistent with the findings of Rothschild and Manicinelli,[2] who documented these thermophiles with similar culture characteristics on the two high nutrient agars. However, *E.coli* and *Enterobacter spp* were found to ferment lactose when cultured on MacConkey agar and at the same time could not haemolyse blood agar. The characteristics showed by these two thermophiles are consistent with the findings of Takano [24] where the same genera of thermophiles fermented lactose when cultured on MacConkey agar and could not show haemolysis of blood agar. Several studies have documented that the ability to ferment lactose and haemolyse blood agar is attributed to an intrinsic factor, the ability to possess enzymes suited for fermentation of lactose and haemolysis of blood components [5], [8], [23], [25]. This therefore implies that these thermophiles possess the enzymes required to carry out such.

### *5.2 Biochemical characteristics of thermophiles in Kitagata and Ihimbo hot springs*

Nine thermophilic bacterial species from Kitagata and Ihimbo hot springs were identified using these biochemical tests and in comparison with the characteristics given in Bergy's manual. All tested positive for catalase except *Streptococcus faecalis*. The positive catalase thermophiles are able to secrete catalase enzyme. The enzyme, catalase is produced by bacteria that respire using oxygen, and protects them from the toxic by-products of oxygen metabolism. All catalase positive bacteria identified in the study (Table 2) are therefore considered as strict aerobes and facultative anaerobes, although they have the ability to respire using oxygen as a terminal electron acceptor. On the other hand, Steptococcocus faecalis gave a negative test for catalase enzyme suggesting that it is an anaerobe that only relies on fermentation and does not respire using oxygen as a terminal electron acceptor. This finding is consistent with the findings of the Virtual Interactive Bacterial Laboratory, Michigan State University, 2021.

Considering the citrate test, four thermophile bacteria species from the hot springs of Kitagata and Ihimbo gave a positive test for Citrate biological test. These were; *Enterobacter cloacae*, *Kleibsella spp*, *Escherichia coli* and *Streptococcus faecalis*. In this test, citrate agar was used to test an organism's ability to utilize citrate as a source of energy. Bacteria that can grow on this medium produce an enzyme, citrate-permease which converts citrate to pyruvate that enters the organism's metabolic cycle for energy production [26]. The identification of E. coli as citrate positive thermophile bacteria is in consistent with the findings of Goh *and his colleagues*[27] whose findings identified and classified as a negative citrate test thermophile bacteria. Only five species of thermophile bacteria obtained from Kitagata and Ihimbo hot springs were observed to show no growth and no colour change as the slant remained green.

Thermophile bacteria obtained from Kitagata and Ihimbo hot springs that tested positive for cleaving Indole from tryptophan included: *Enterobacter cloacae* and *E. coli.* These bacteria have been documented to have tryptophan operon which is a regulator for tryptophan biosynthesis from other amino acids. Therefore bacteria have substrate tryptophanase and can use tryptophan as an energy source. The presence of *Enterobacter cloacae* as a positive indole thermophilic bacterial species is consistent with the findings of Cihan[28] who suggested that most of the enterobacterial species show negative indole test except *Enterobacter cloacae.* More studies by[29, 30]showed that 96% of *E.coli* is indole positive except coli from other indole-negative enteric bacteria.

Following culturing on blood and MacConkey agar (Table 1), the swarming patterns as a type of motility was further analysed under a light microscope. Results further showed that six thermophile bacteria species obtained from Ihimbo and Kitagata hot springs showed swarming motile patterns. These included *Bacillus subtullis*, *Enterobacter cloacae*, *Enterobacter agglomerans, Pseudomonas aerogenes* and *Escheichia coli*. These bacteria species have been shown to show positive motile test in other studies [31, 32]. Motility as a test is closely associated with cell structure, mainly the presence of flagella and different types of flagella arrangements. These are found in gram negative bacteria since most of the gram positive bacteria are atrichous (without flagella) implying that they are non-motile. However, most of the gram-positive, catalase negative cocci are generally motile. This explains the reason as to why *Bacillus subtullis*, gram positive bacteria that is catalase positive exhibited motion unlike the other gram positive bacteria that have been documented to show a negative motile test. *Enterobacter cloacae*, *Enterobacter agglomerans, Pseudomonas aerogenes* and *Escheichia coli* are all gram negative bacteria that are capable of exhibiting swarming patterns of motion which are consistent with the findings of[33]. On the other hand, *Staphylococcus aureus*, *Kleibsiella spp* and *Streptococcus faecalis* gave a negative test for motility. All these are atrichous and have been documented by several studies to give a negative motile test [33, 34].

The study observed that only one thermophile bacteria species gave a positive test for the coagulase test. This was *Staphylococcus aureus*. Bacteria of the genus *Staphylococus* have been traditionally grouped into two groups on the basis of their ability to clot blood plasma (the coagulase test) and several studies have shown that the coagulase-positive staphylococci constitute the species, *Staphylococci aureus*. This explains why it was the only species that gave the positive coagulase test.

ssSimilarly, *Klebsiella spp* were observed to be the only thermophile bacterial species obtained from Kitagata and Ihimbo hot springs that showed a positive capsule staining test. The findings of this study are inconsistent with many studies which have shown that *Klebsiella spp* is a non-spore forming with an optimal growth temperature of 37˚C. There is therefore need to further isolate and come to the species level.

However, some of the identified species such as *Klebsiela ssp, Streptococcus faecalis and E coli* have been identified as the common intestinal microorganisms and occasional pathogens [35]. The bacteria are basically used as indicators of faecal contamination of water since they are considered as inhabitants of intestinal tracts of homeothermic animals [36] thus of concern on sanitary quality of water .

***5.3 Spatial and species distribution of thermophile bacteria in Kitagata and Ihimbo hot springs***

The total number of thermophile bacteria species obtained from both Kitagata and Ihimbo hot springs was found to be nine species from the study (Table 2). However, there was interspecific variation in the distribution of thermophilic bacteria obtained from the both Kitagata and Ihimbo hot springs. Results indicated that Kitagata had only six thermophile bacterial species while Ihimbo had eight species. The slight variation in the number of thermophilic bacteria in the two hot springs can be attributed to their location and exposure to outside contamination forces. Similar studies suggested that microbial abundance of the hot springs is mainly influenced by mainly external sources [7, 22, 32, 35].

The study observed that the only thermophile bacteria that was observed to be absent in Ihimbo hot springs was

*Entrobacter agglomerans.* It was observed during the study that generally, Ihimbo hot spring had hotter water than Kitagata hot springs. The temperature optimal range of *Entrobacter agglomerans* has been found not to vary a lot and has been shown to thrive well in relatively warmer waters. This further explains why it was not found in any of the hottest points sampled but rather in only one site in Kitagata hot springs that corresponds to the relatively warm areas. Results showed that *Bacillus subtullis* and *Klebsiella* species were the commonest in two hot springs. The presence of *Bacillus subtullis* in all sampled locations could be due to the ability of this genus to grow at high temperatures and their resistance to harsh environmental conditions [29]. In addition to its adaptation for hot surroundings[9]. Strains of *Bacillus* have been documented to be dominant in studies carried out by different researchers cross the world [29, 37] as it has been found to be a great source of enzyme amylase and protease which are useful in the food industry. In addition, results showed that there was no *Streptococcus faecalis* and *Pseudomanas aeruginosa* in Kitagata. Kitagata was observed to have waters with a relatively low alkaline pH which might explain the reason as to why these high alkaline demanding thermophiles might be absent in Kitagata hot springs and present in Ihimbo hot springs where waters were observed to have a higher pH that favours their establishment.

There was no significant difference in the average total bacteria count (CFUs) and species richness between Kitagata and Ihimbo hot springs due to similar geochemical environments possessed in the springs. However, on comparing average mean of total bacteria counts (CFUs) of Kitagata (1080.70±241.65) were higher than Ihimbo (874.27±168.57). This could be attributed to easy access of users due to their difference in geographical locations of the two that have lead kitagata hot spring be polluted thus causing higher bacteria counts.

### *5.4 Selected physico-chemical parameters of Kitagata and Ihimbo hot springs*

The slight variation in the selected physical chemical parameters considered in the study (temperature, pH and Electrical conductivity) (Table 5) is as a result of internal factors such as the type of the rock beneath the earth's surface and external factors such as anthropogenic activities around the hot springs[14]. The mean temperature for water at all points where water oozed from in Ihimbo hot springs was considered in the study was higher (Mean: 64.25±1.03 and 68.25±0.85 ) for H1and H4 respectively compared to Kitagata hot springs (Mean: 65.25±0.48) for point K1. The variation might be due to the internal factor, mainly the heating of the rock underneath the earth's surface by the earth's core.

The study observed that Ihimbo hot spring was found to have a higher pH (Mean: 9.77±0.16 to 9.38±0.39) compared to Kitagata hot springs (Mean: 8.84±0.07 to 8.57±0.18) (Table 4.5). pH is influenced by several factors which range from the nature of the rocks underneath and the activities of macro and microorganisms in a water body [37] to external factors such as anthropogenic activities like farming especially crop growing on the hill sides and trading centre that is in close proximity with Kitagata hot spring in which running water from these fields carry organic and inorganic substances that may have been the cause of its lower Ph. in comparison to Ihimbo hot spring that is surrounded by a natural forest where there is no much human influence.

Generally, the mean values of Electrical conductivity were found to be higher for all sites in Kitagata hot springs than Ihimbo hot springs (Table 4.5). Similar studies in Ethiopia and Kenya have shown that conductivity is an indicator of anthropogenic activities around the hot springs [3, 14]. The slight higher electrical conductivity for Kitagata hot springs might be due to the fact that several forms of pollution mainly by run offs since Kitagata is located in the valley and near the road and also floods from the nearby stream Ngaromwenda compared to Ihimbo hot springs which is located inside the pristine Ihimbo forest a slightly raised ground [38].

### *5.5 Relationship between physico-chemical parameters and total thermophilic bacteria counts in Kitagata and Ihimbo hot springs*

Physico-chemical parameters have been considered to exert primary control on microbial community assemblages [39]. Total Bacterial counts (CFUs) declined with increasing temperature, suggesting that temperature plays a significant role in shaping the distribution of microbial taxa. Similar patterns have been observed in a variety of terrestrial thermal springs on different continents, including Great Boiling Spring [40] and Yellowstone National Park springs [41] in North America, and Iceland springs [42] in Europe. As observed from Table 6, results showed that in both Kitagata and Ihimbo hot springs, there is a strong negative correlation between temperature and total observed thermophile bacteria counts ($r = -0.6529$ and -0.8638 for Kitagata and Ihimbo hot springs respectively). This observed difference is attributed to the fact that as temperature decreases from the points where it oozes out in the hot springs to the sites far away from the point of discharge, more thermophile bacterial species thrive. This is consistent with similar studies by Getachew [4] who stated that not all thermophiles will inhibit the hottest areas but will have a tolerable temperature range they inhabit.

The pH and electoral conductivity (EC) had a weak positive correlation with no significant relationship with thermophile bacteria. The pH of water was alkaline in nature and varied from 8.57 to 8.84 and 9.38 to 9.77 in Kitagata and Ihimbo hot springs respectively. This high pH value may be due to low water level and high salts concentrations approach's it towards alkalinity[43]. Electrical conductivity was found to be good indicator of the water quality[44]. In this study, electrical conductivity varied from 1286.00 to 1315.50 μS/ cm and 1066.25 to 1084.25 μS/ cm in the hot springs of Kitagata and Ihimbo respectively. The EC increases from point where water come from the underground as it flows along the other points. This might be due to high anthropogenic activities.

## 6. Conclusions

This study has concluded that both Kitagata and Ihimbo hot springs were characterised by several thermophilic bacterial species. Nine species of thermophilic bacteria were identified; of these, six and eight thermophilic bacterial species were found in Kitagata and Ihimbo hot springs respectively. Furthermore, the study found out that each of the selected physico-chemical parameters had an influence on the total bacteria counts and distribution of the obtained thermophilic bacterial species in both Ihimbo and Kitagata hot springs. The influence of each physico-chemical parameters on thermophile bacteria distribution and total bacteria counts in the two hot springs of Kitagata and Ihimbo varied.

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