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Effect of sound on "*Salmonella Typhimurium*" and "*Escherichia Coil O157:H7*"

De Silva L. M. M*, Swarnamali A. D. D, Marasinghe B. N. A, Rathnayake R. M. S. U. K, Aponsu G. M. L. P, Hettiarachchi C. N

Lecturer, Department of Physical Sciences and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka
Undergraduate Student, Department of Physical Sciences and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka
Undergraduate Student, Department of Food Science and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka
Professor, Dean of the Faculty, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka
Professor, Head of the Department, Department of Physical Sciences and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka
Instructor, Center for Computer Studies, Sabaragamuwa University of Sri Lanka, P.O. Box 02, Belihuloya 70140, Sri Lanka

ABSTRACT:
Purpose: The most popular way of destroying bacteria in a mediumis chemical treating method whereas which may destroy the useful bacteria as well. Therefore, there should have a method to destroy a targeted bacterium. This research have been performed to identify the most appropriate sound frequency or frequency band to destroy "Salmonella Typhimurium" and "Escherichia Coil O157:H7 (E-coil)".
Research Method: Samples of both bacteria were treated with discrete frequency sound signals in different ranges such as infra, audible and ultra, then relative colony count of treated samples was measured relative to the control samples of both bacteria.
Findings: For E-coil,varying relative colony forming efficiency was observed for infrasoundtreatment and comparatively high relative colony forming efficiency was observed for audible sound treatments whereas low colony forming efficiency was observed for ultrasound treatment. For Salmonella Typhimurium, varying relative colony forming efficiency was observed for infrasound treatment and low relative colony forming efficiency was observed for audible sound treatments whereas very low colony forming efficiency was observed for ultrasound treatment. This concludes that high bacteria distortion is possible at ultrasound treatment for both bacteria.
Research Limitations: Discrete sound frequency signals were used instead of continues frequency signals to represent each frequency range.
Originality/ Value: Ultrasound treatment is possible to destroy both "Salmonella Typhimurium" and "Escherichia Coil O157:H7" bacteria.

KEYWORDS: Audiblesound, Escherichia Coil O157:H7, Infrasound, Salmonella Typhimurium, Ultrasound

I. INTRODUCTION

In this developing world, scientists are trying find effective and ecofriend methods to destroying bacteria which are in different media such as water, soil, foods… etc. The most popular and cost effective method is using chemicals to destroy bacteria which are in above mentioned media. Another popular method is drying media to make them bacteria free. Especially, "Salmonella Typhimurium" and "Escherichia Coil O157:H7" bacteria can be, abundantly, found in polluted water bodies such as polluted lakes and rivers. Frequently, these water bodies are used to fulfill the urban water requirement where main water purification method is chemical treatment method. These methods are mainly used since they are cost and time effective.

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Even though above mentioned methods are time effective and cost effective, these methods may destroy useful microorganisms and used chemical compounds may be harmful for human beings since they are consumed directly or non-directly. Therefore, having a method which destroys targeted set of bacteria in a media is very useful.

In this research, we are investigating the effect of sound frequencies of all three rangeson "Salmonella Typhimurium" and "Escherichia Coil O157:H7" bacteria through a sound treatment method. A similar research (L. M. M. De Silva *et al.*, 2018) reported that *Staphylococcus Aureus*bacterium have shown 33% and 50% of relative colony forming efficiency for acoustic sound and ultrasound treatment respectively.

II. MATERIALS& METHODS

Pure samples of "Salmonella Typhimurium" and "Escherichia Coil O157:H7" were obtained from Medical Research Institute (MRI) and all the steps of the research were carried out in the pathology laboratory of the Faculty Agricultural Sciences, Sabragamuwa University of Sri Lanka. Both bacteria were treated at the same time to fix the laboratory conditions such as temperature and the relative humidity.

This research was carried out under 8 sub steps as mentioned below. Each step was carried out for both "Salmonella Typhimurium" and "Escherichia Coil O157:H7" bacteria. The most important factor to be noticed here is that the amounts of both bacteria used for each step is same as mentioned in the followings steps.

A. Subcluturing the Pure Samples

A 3.25g of nutrient broth was dissolved in 250ml of distilled water in a 250ml volumetric flask and it was tightly capped with a cotton wool plug and then it was covered tightly with an aluminum foil. After that the nutrient broth was autoclaved for 20 minutes at 121 °C, at 15 psi pressure (15 atm). Then it was taken out and it was allowed to cool down to the room temperature. Then an inoculation loop, pure sample, sprit lamp and the autoclaved nutrient broth were taken into the laminar floor and the inoculation loop was heated until red hot. Finally, the broth was inoculated and then incubated for 2 days.

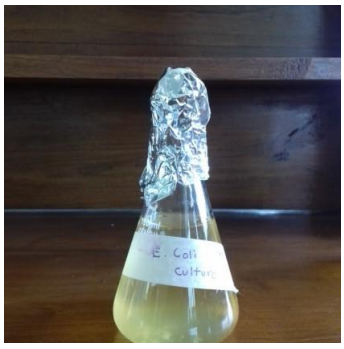

Figure 01: Sub cultured Salmonella

B. Preparation of Dilution Series

Initially 12 test tubes were sterilized at 160°C for 2 hours. The laminar floor was turned on and UV treated for 30 minutes. Meanwhile 500ml of distilled water was autoclaved at 121°C & at 15 psi pressure for 20 minutes and it was allowed to cool down to the room temperature. After that the sub cultured sample was taken from the incubator. Then each test tube was filled with 27 ml of autoclaved distill water and 3ml of subcultured sample was added into the first test tube and it was mixed well. After that 3ml from the first test tube was measured by a pipette and poured into the second test tube and mixed well. This procedure was continued until the 12^{th} test tube and the dilution series was made. Finally, all the test tubes were labeled accordingly up to $10^{-12}$and all of them were capped with a cotton wool plug and covered with an aluminum foil. This dilution series was stored in the refrigerator for further usage.

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C. Determining the Correct Dilution Factor for the Experiment

15 petri plates and 5 pipettes of 1ml were sterilized at 160°C for 2 hours. Meanwhile 7g of nutrient agar and 2.5g of agar was measured and mixed with 250ml distilled water in a 250ml volumetric flask. Then it was tightly capped and covered with an aluminum foil and it was autoclavedat 121°C & at 15 psi pressure for 20 minutes and then cooled to the room temperature. Then the laminar floor was UV treated for 30 minutes and it was sterilized with 70% alcohol solution. After that, it was poured to each and every petri plate and allowed to further cool down. Then from 10^{-8} (units are needed) dilution series to 10^{-12} dilution series, 3 petri plates (replicates) was cultured by spread plate method for each dilution factor. After the culturing, each petri plate was sealed by using para films and incubated for 2 days. At the end of the 2 days incubation period, the colonies in each petri plate were counted and the observations were recorded.

D. Ultra Sound Treatment

Initially 5 beakers of 100 ml, 1 pipette of 5 ml were washed well and sterilized at 160°C for 2 hours. Meanwhile the apparatus as shown in belowfigure 02 was set up inside the laminar floor and UV treated for 30 minutes. After that 5 ml from 10^{-8} dilution was pipetted to each 100ml beaker and placed in the respective treating unit and treated for 3 hours with 20 kHz, 40 kHz, 60 kHz, 80 kHz sound waves separately. A control sample was maintained. The temperature, humidity and light intensity during the experiment was recorded by Pasco temperature sensor, humidity sensor and light sensor respectively.

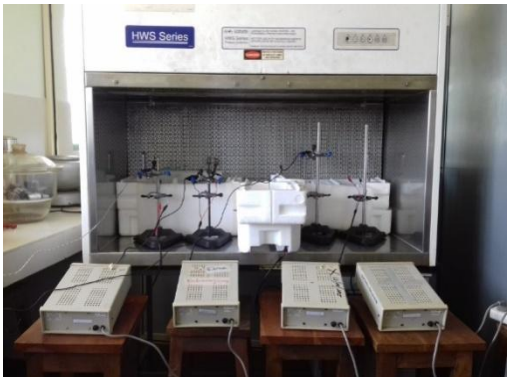

Figure 02: Experimental Structure

E. Acoustic Range Sound Wave Treatment

Initially 5 beakers of 100ml, 1 pipette of 5 ml were washed well and sterilized at 160°C for 2 hours. Meanwhile the apparatus was set up inside the laminar floor and UV treated for 30 minutes. After that 5 ml from 10^{-8} dilution was pipetted to each 100 ml beaker and placed in the respective treating unit and treated for 3 hours with 100 Hz, 1 kHz, 5 kHz, 15 kHz sound waves separately. The temperature, humidity and light intensity during the experiment was recorded by Pasco temperature sensor, humidity sensor and light sensor respectively.

F. Infrasound Range Sound Wave Treatment

Initially 5 beakers of 100 ml, 1 pipette of 5 ml were well washed and sterilized at 160°C for 2 hours. Meanwhile the apparatus was set up inside the laminar floor and UV treated for 30 minutes. After that 5 ml from 10^{-8} dilution was pipetted to each 100 ml beaker and placed in the respective treating unit and treated for 3 hours with 5 Hz, 10 Hz, 15

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Hz, 20 Hz sound waves separately. The temperature, humidity and light intensity during the experiment was recorded by Pasco temperature sensor, humidity sensor and light sensor respectively.

G. Culturing the Sound Treated Bacteria

15 petri plates, spreader and 5 pipettes of 1 ml were sterilized for 2 hours at 160°C. Meanwhile 7 g of nutrient agar and 2.5 g of agar was measured and mixed with 250 ml distilled water in a 250 ml volumetric flask. Then it was tightly capped and covered with an aluminum foil and it was autoclaved at 121°C & at 15 psi pressure for 20 minutes and cooled to the room temperature. Then the laminar floor was UV treated for 30 minutes sterilized with 70% alcohol solution. After that autoclaved culture media was poured to each and every petri plate and further cooled down. Then 3 replicates for each sound wave were cultured by inoculating 0.2 ml from the each treated sample for each plate by spread plate method and each plate was sealed by para films and incubated for 48 hours.Finally, colonies were counted by a colony counter.

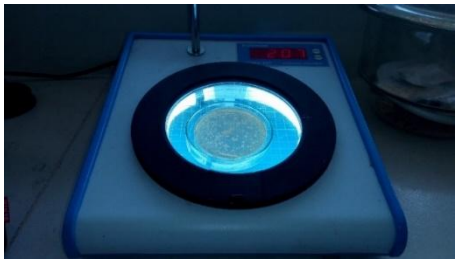

Figure 03: Counting colonies

H. Interpreting the Results

The method of results interpreting in this research is the calculation of percentage increment or decrement of colonies relative to the control sample after the sound treatment. This method have been used in a previous research (Shaobin et al., 2010) as the "relative colony forming efficiency". According to Shaobin et al., 2010, the relative colony forming efficiency has been calculated by the following equation.

$$\text{relative colony forming efficiency} = \frac{\overline{N_i}}{\overline{N_c}} \times 100\%$$

III. RESULTS & DISCUSSION

A. Selecting the Dilution Factor

The following table indicates the average colony count of five different dilution factors for three replicates of both bacteria

Table 01: Dilution factor with average colony count

Dilution Factor	Colony Count			Average Colony Count	
	Replicate Number	For *Salmonella Typhimurium*	For *Escherichia Coil O157:H7*	For *Salmonella Typhimurium*	For *Escherichia Coil O157:H7*
	1	66	60		

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10^{-8}	2	87	87	81	89
	3	90	90		
10^{-9}	1	43	43	39	37
	2	37	37		
	3	38	36		
10^{-10}	1	36	35	39	40
	2	44	44		
	3	37	uncountable		
10^{-11}	1	28	27	32	35
	2	33	33		
	3	36	36		
10^{-12}	1	15	15	16	14
	2	21	41		
	3	13	13		

According to a previous literature (Bree and Dotterrer1916), the number of colonies allowable on satisfactory agar plate have been determined as 30 to 300. 30 have been selected as the lowest possible value since 30 is the lowest possible value for a proper statistical analysis.

300 has been selected as the highest possible number of colony count. Petri plates which having colony count more than 300 were categorized as too numerous to count (TNTC) plates. When the colony count is more than 300, the colonies cannot be distinguished from one another by a clear separating margin. And also crowded colonies can interfere the growth of neighboring colonies and then the results may not bereliable. However, another research (Tomasiewicz et al., 1980) reports that the most appropriate range of colony count is 25 – 250. By considering all those literature, 10^{-8} was selected as the most appropriate dilution factor for this research purpose. Here,for both bacteria, 10^{-8} was selected as the most appropriate dilution factor since it has the appropriate mean value of colony count compared to the mean colony count for other dilution factors as shown in the above table.

Ultrasound is a major area that so many researchers have been done in recent decade. Ultrasound induced biological effects and also biophysical mechanisms have been thoroughly investigated. Ultrasound is the sound waves with the frequency of 20 kHz or beyond (Brondum et al., 1998; Butz and Tauscher, 2002). The most applicable generation of ultrasound is carried out using the electrostrictive transformer principle.

This is based on the elastic deformation of ferroelectric materials within a high frequency electrical field and is caused by the mutual attraction of the molecules polarized in the field (Raichel, 2000). For polarisation of molecules a high-frequency alternating current will be transmitted via two electrodes to the ferroelectrical material. Then, after conversion into mechanical oscillation, the sound waves will be transmitted to an amplifier, to the sound radiating sonotrode and finally to the treatment medium. In general use, ultrasound equipment uses frequencies from 20 kHz to 10 MHz. Power ultrasound which has the ability of cavitation, is in the range of20 to 100 kHz. Cavitation is the main principle that is used to microorganism inactivation (Piyasena et al; 2003).

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Pasteurization, ultra high temperatures are some of conventional methods that thermal energy is used for the inactivation of microorganisms. Because of the thermal energy essential thermal liable nutrients can be destroyed while occurring so many undesirable flavors. But in a ultrasound process cavitation caused for the killing of microorganisms which based on the pressure changes created by the ultrasonic waves.
During the ultrasound treatment, longitudinal sound waves are created and when it meets the liquid medium alternating compression and expansion areas are created (Sala et al; 1995). Due to this pressure change, the cavitation occurs and gas bubbles are formed in the liquid medium. During the expansion cycle, the bubble surface area becomes larger due to increasing the diffusion of the gas. With the time there is a point where the provided ultrasonic energy is not sufficient to retain the vapour phase in the bubble. Because of that a rapid condensation occurs and then condensed molecules collide with each other creating shock waves. Shock waves create regions where having very high temperature &high pressure. Pressure changes results from these implosions act as the main bactericidal effect of ultrasound. Zones where having high temperature can kill some bacteria. However, since these are limited to a very little area, the high temperatures do not effect for large areas (Piyasena et al; 2003). This is the principle of bactericidal effect of ultrasound even shown in this study.

To determine the effect of sound on *Salmonella Typhimurium &Escherichia coli,* the colony count at each and every sound wave was compared with the colony count of the control sample.

B. Results for Salmonella Typhimurium in Infrasound Range

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency& average increment or decrement percentage of colony forming efficiency at 5Hz, 10Hz, 15Hz & 20Hz frequencies

Table 02: Frequency with average colony count & colony forming efficiency at infrasound

Frequency (Hz)	Colony Count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	548	566	563	559		
5	452	468	465	461	82.9	-17.1
10	650	621	642	637	114.0	14.0
15	654	638	631	641	114.7	14.7
20	542	539	529	536	96.0	-4.0

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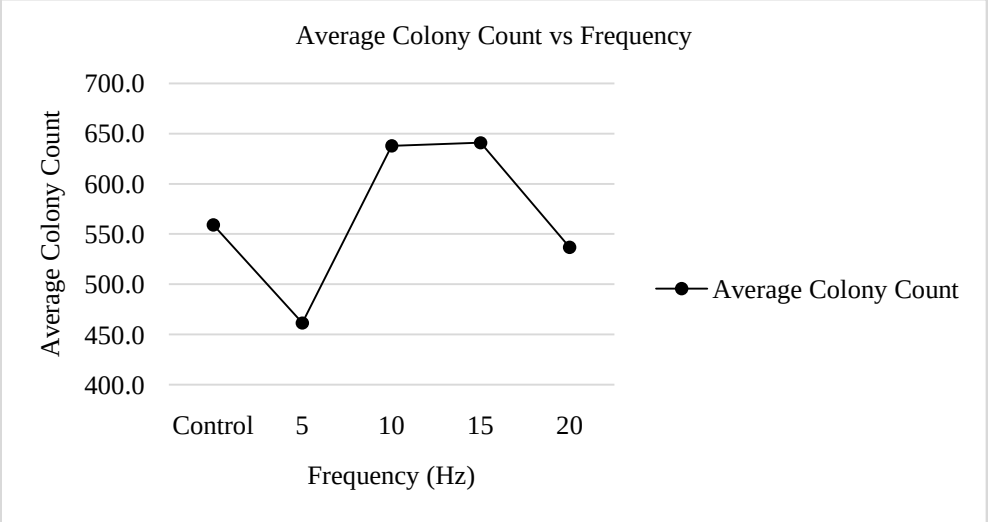

Figure 04: Average colony count variation with the infra sound frequencies

The above graph clearly shows that both increment and decrement of average colony count of replicates of the bacteria are possible compared to the control sample. At 5Hz & 20 Hz, average colony count havebeen decreased whereas at 10Hz & 15Hz, average colony count have been increased compared to the control sample.

C. Results for Salmonella Typhimurium inAudiblesound Range

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency & average increment or decrement percentage of colony forming efficiency at 100Hz, 1000Hz,5000 Hz and 15000 Hz frequencies.

Table 03: Frequency with average colony count & colony forming efficiency at audible sound

Frequency (Hz)	Colony count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	496	530	542	522		
100	480	450	441	457	87.4	-12.6
1000	427	430	432	429	82.2	-17.8
5000	188	186	179	184	35.3	-64.7
15000	151	146	142	146	28.0	-72.0

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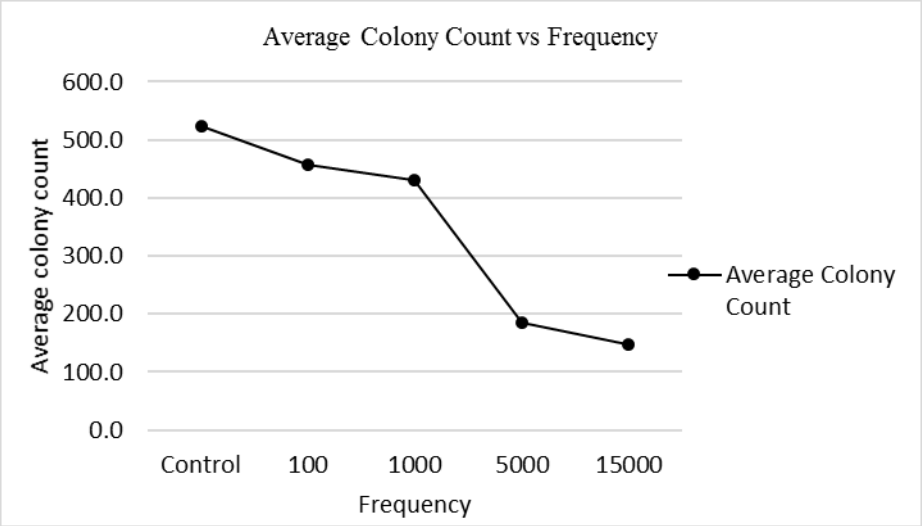

Figure 05: Average colony count variation with the audible sound frequencies

The above graph clearly shows that there is a decrement of average colony count at all the selected frequencies compared to the control sample. At 100Hz & 1000Hz, the decrement is moderately low whereas at 5000Hz & 15000Hz, the decrement is quite high. Especially, this decrement is vast when the frequency of the treating sound in the "KHz" range. And also it is clearly shown by the graph that this decrement takes place gradually when the frequency of the treating sound is increased.

D. Results for Salmonella Typhimurium in Ultrasound Range

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency & average increment or decrement percentage of colony forming efficiency at 20000Hz, 60000Hz, 40000Hz & 80000Hz frequencies.

Table 04: Frequency with average colony count & colony forming efficiency at ultrasound

Frequency (Hz)	Colony count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	550	556	562	556		
20000	148	142	146	145	26.1	-73.9
40000	138	141	145	141	25.4	-74.6
60000	76	95	83	84	15.2	-84.8
80000	44	38	50	44	7.9	-92.1

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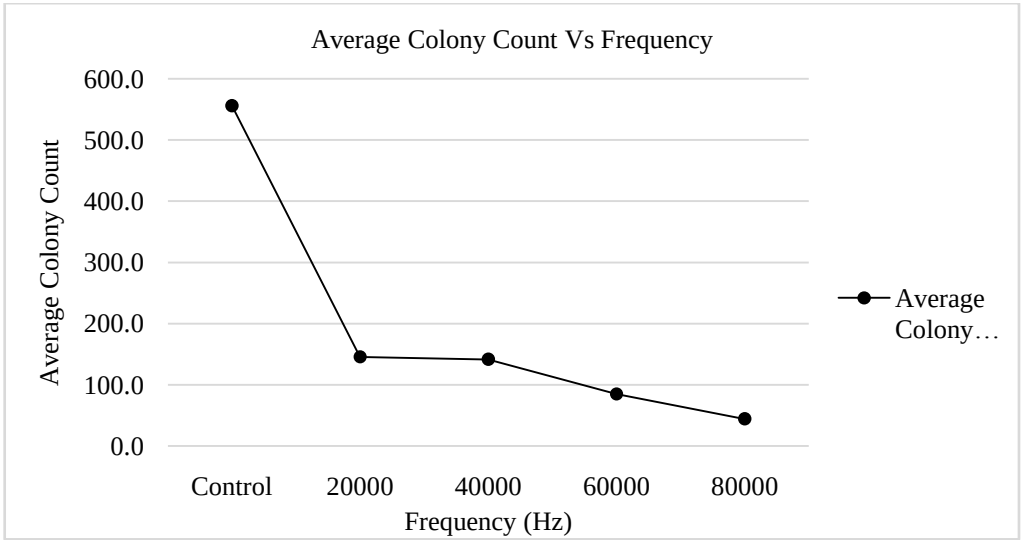

Figure 06: Average colony count variation with the ultra sound frequencies

The above graph also clearly shows that there is a dramatical decrement of average colony count at all the selected frequencies compared to the control sample. The most important factor at this range is that it shows almost 70% above decrement at all the selected frequencies in the Ultrasound range.
Compared to the infra & audible range, in this range decrement of the number of colonies after the sound treatment is very high. Therefore, this range can be considered as the most effective range among the selected ranges.

E. Results for Escherichia Coli in Infrasound Range

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency & average increment or decrement percentage of colony forming efficiency at 5Hz, 10Hz, 15Hz & 20 Hz frequencies.

Table 05: Frequency with average colony count & colony forming efficiency at infrasound

Frequency (Hz)	Colony Count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	76	23	73	57		
5	31 (1L.C.)	49 (1L.C.)	56 (1L.C.)	46	80.0	-10.0
10	176 (1L.C.)	100 (1L.C.)	22 (1L.C.)	100	175.4	75.4
15	10 (1L.C.)	9 (1L.C.)	12 (1L.C.)	11	19.2	-80.8
20	10 (1L.C.)	16 (1L.C.)	41 (1L.C.)	23	40.3	-59.7

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According to the results of this study, one large colony (L.C) was observed in all samples for all frequencies. This large colony was also considered as a single colony for count assuming that the large colony also have been formed by a single microorganism

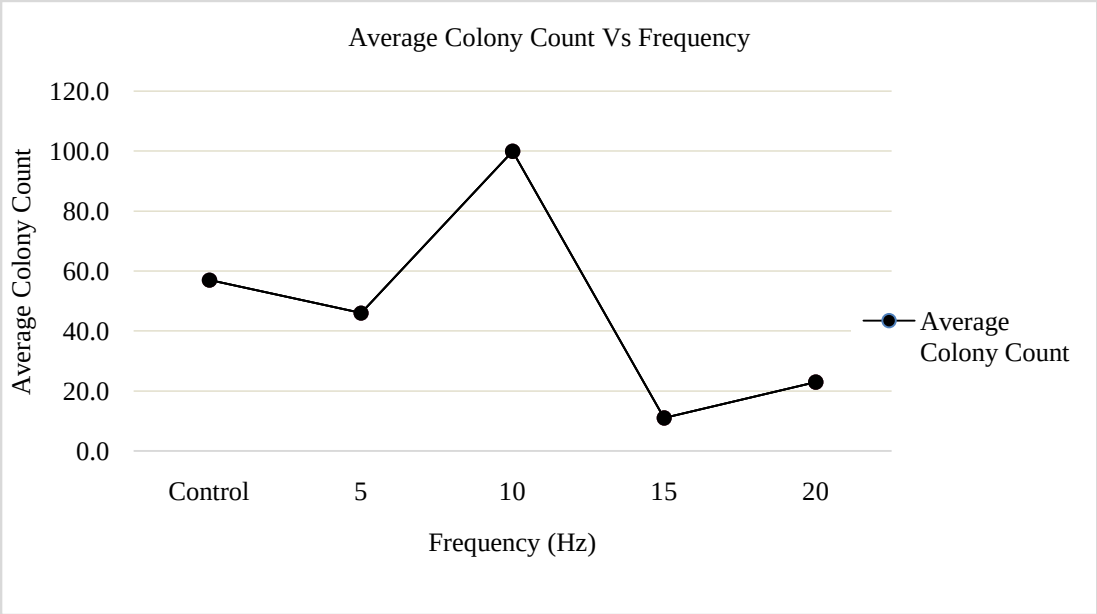

Figure 07: Average colony count variation with the infra sound frequencies

As in the case of Salmonella, the above graph clearly shows that both increment and decrement of average colony count of replicates of the bacteria are possible compared to the control sample. At 10 Hz, the average colony count has been increased whereas at 5 Hz, 15 Hz & 20 Hz the average colony count have been decreased compared to the control sample. However, at 15 Hz, the average number of colonies have decreased dramatically.

F. Results forEscherichia Coli in Audiblesound Range

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency & average increment or decrement percentage of colony forming efficiency at 100Hz, 1000Hz, 5000Hz & 15000Hz frequencies.

Table 06: Frequency with average colony count & colony forming efficiency at audible sound

Frequency (Hz)	Colony count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	44	47	126	72		
100	61	30	66	52	72.2	-27.8
1000	241	246	270	252	350.0	250.0
5000	320	57	115	164	227.7	127.7
15000	61	30	66	52	72.2	-17.8

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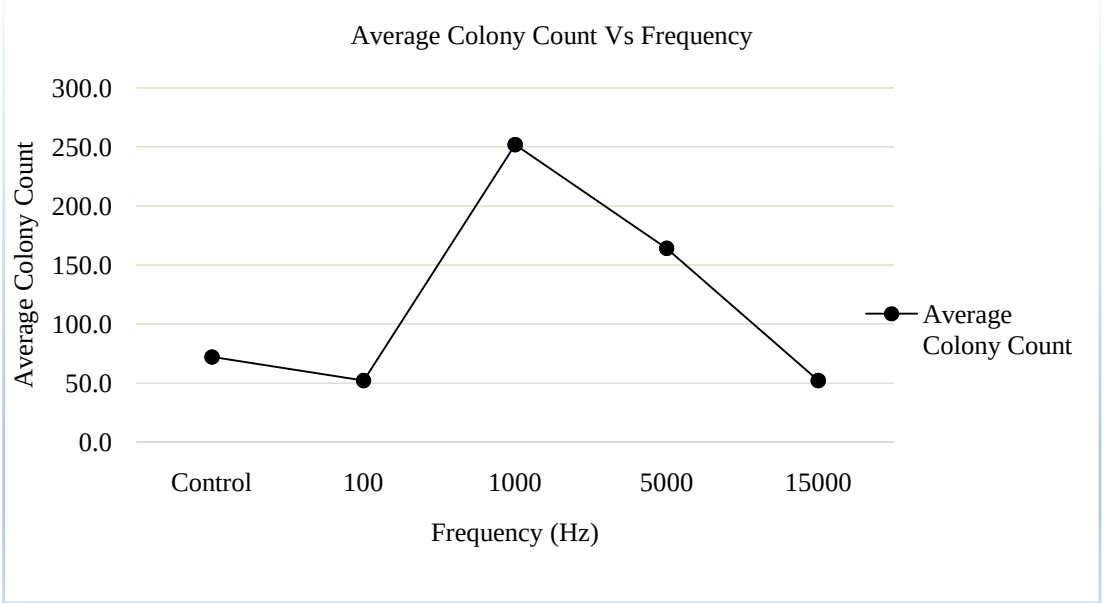

Figure 08: Average colony count variation with the audible sound frequencies

The above graph clearly shows that both increment and decrement of average colony count of replicates of the bacteria are possible compared to the control sample. According to the graph, it can be predicted that this range is highly favorable for the growth of bacteria. At 1000 Hz, the maximum increment have been reported compared to the control sample.

A similar research (Shaobin et al., 2010) reports that the relative colony forming efficiency of E. coli is 141.6%, 130.0% and 131.1% after stimulation of 22 hours by sound wave with the frequency of 1000, 5000 and 10,000 Hz, separately, which were significantly higher than that of the control (100%). By this study, it clearly shows that 1000Hz is the most promising wave to stimulate growth of E.coli.

G. Results for Escherichia Coli in UltrasoundRange

The following table indicates the colony count of three replicates, average colony count, colony forming efficiency & average increment or decrement percentage of colony forming efficiency at 20000Hz, 60000Hz, 40000Hz & 80000Hz frequencies.

Table 07: Frequency with average colony count & colony forming efficiency at ultrasound

Frequency (Hz)	Colony Count (Replicates)			Average Colony Count	Colony forming efficiency (%)	Average increment or decrement percentage (%)
Control	42	45	136	44		
20000	(L.C)	(L.C)	(L.C)			

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40000	38	39	203	38	86.3	-13.7
60000	(L.C)	(L.C)	(L.C)			
80000	35	38	127	36	81.8	-19.2

At 20 KHz and 60 KHz large clouds were formed for all three replicates. Therefore, those frequencies were neglected for the data interpretation. Third replicates of 40 KHz and 80 KHz were also deviated from the 1st and 2nd replicates, therefore, colony count of 3rd replicates of these two frequencies (40 KHz and 80 KHz) have not been taken into account when the average colony count is calculated.

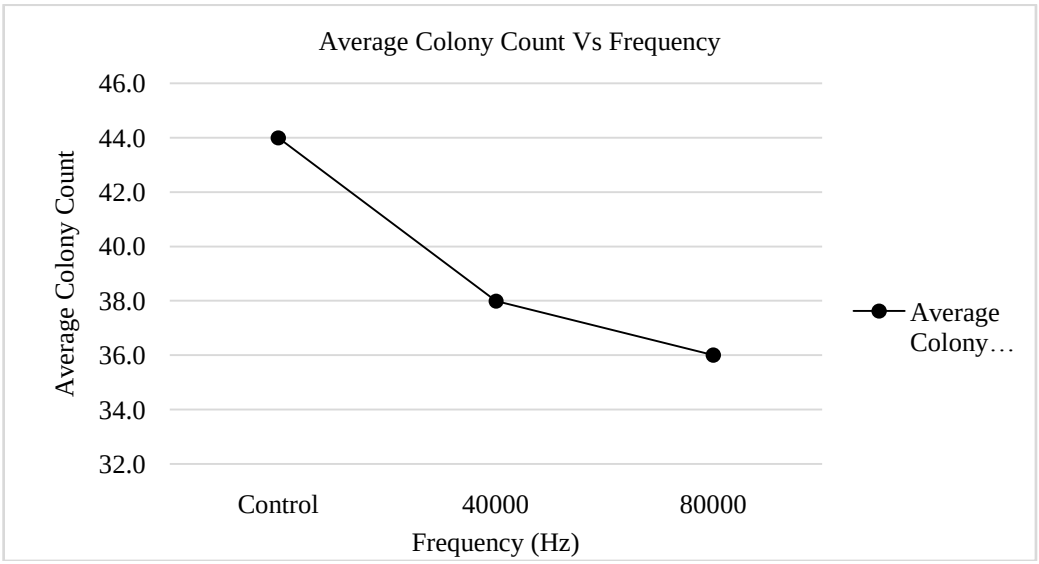

Figure 09: Average colony count variation with the infra sound frequencies

The above graph clearly shows that there is a dramatical decrement of average colony count at both frequencies compared to the control sample. However, the percentage decrement of colony count is low compared to the control sample

H. Results of Laboratory Conditions at the Research Duration

Three laboratory conditions such as Relative Humidity, Temperature and Light Intensity were recorded throughout the research. The following figures indicate the variation of these conditions with the time at each sound treatment.

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At Infrared Sound Treatment

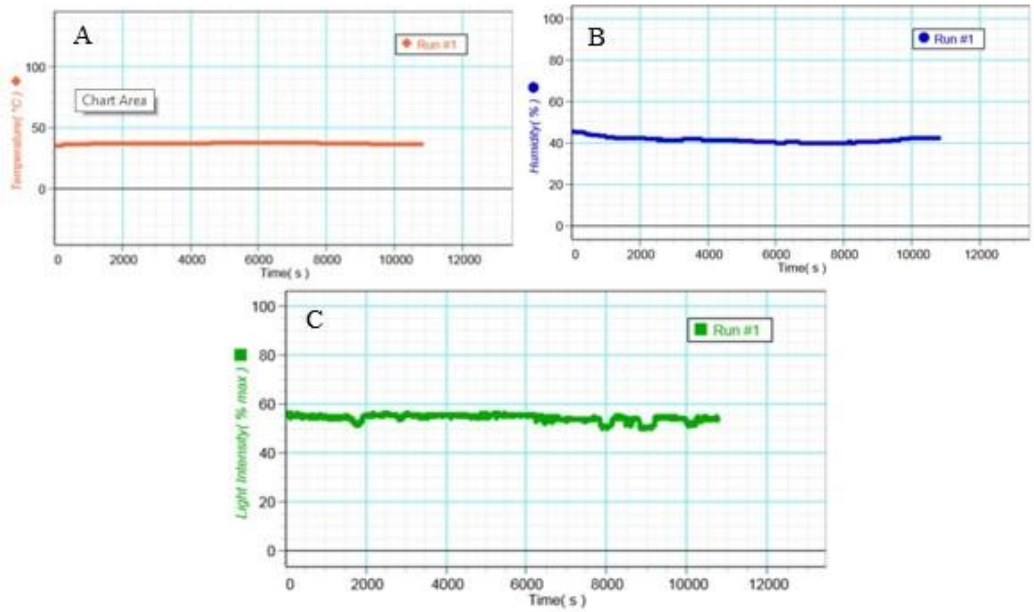

Figure 10: The variation of Temperature (A), Humidity (B) and Light intensity (C) with time

At Audible Sound Treatment

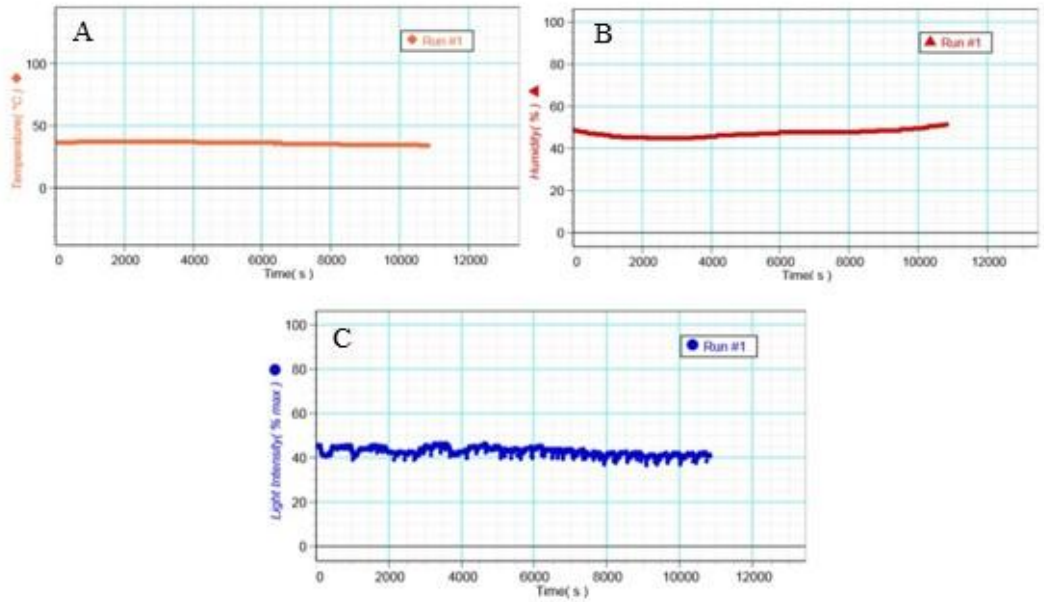

Figure 11: The variation of Temperature (A), Humidity (B) and Light intensity (C) with time

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At Ultra Sound Treatment

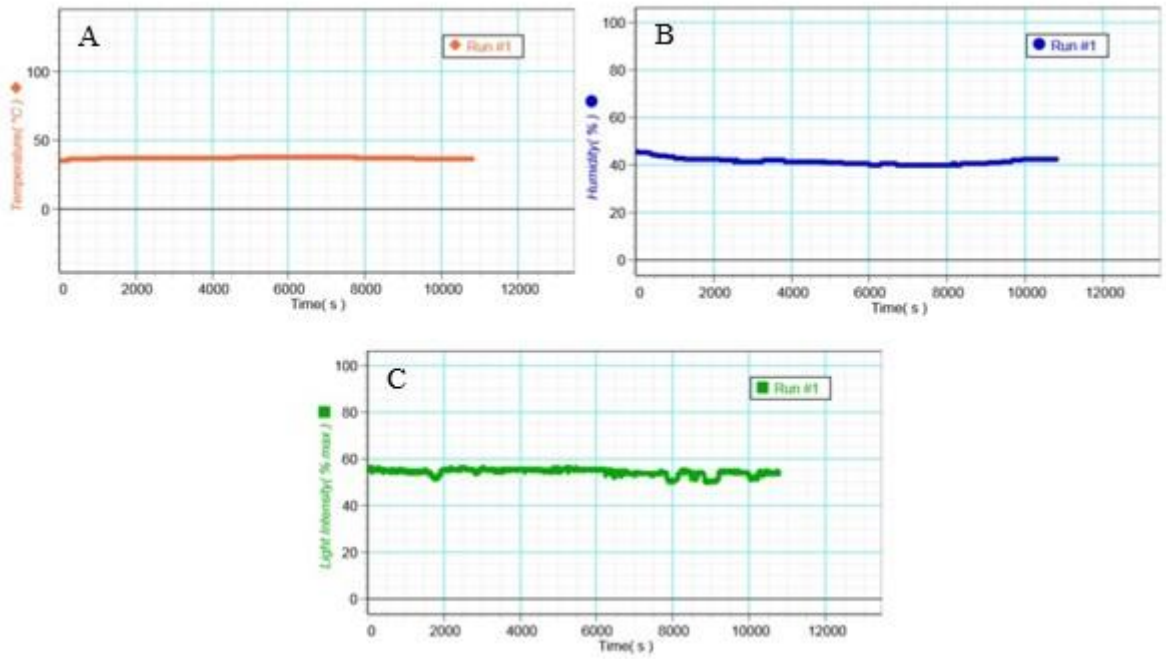

Figure 12: The variation of Temperature (A), Humidity (B) and Light intensity (C) with time

IV. CONCLUSION

According to the research findings of *Salmonella* bacterium, infrasound range acts as both growth promoter anddemoter at different frequencies in this range. However, both audible sound range and ultra sound range act as growth promoter to this *Salmonella* bacterium. Ultra sound range demotes *Salmonella* bacterium rather than audible range since research findings show dramatical colony decrement in Ultra sound range.

According to the research findings of *Escherichia coli* bacterium, as in the case of*Salmonella* bacterium, infrasound range acts as both growth promoter and demoter at different frequencies in this range with quite high variations compared to the control. However, middle region of the audible range acts as a bacteria growth promoter while upper and lower region of this ranges act as a growth demoter to this bacterium. At the middle region of the audible range, the growth promoting ability is very high compared to the control sample (it is about 250% times higher than the control sample). As in the case of *Salmonella* bacterium, Ultra Sound range acts as a growth demoter to *Escherichia coli* bacterium as well whereas percentage demoting ability of *Escherichia coli* bacterium is lower than that of to the *Salmonella* bacterium in this Ultra Sound range.

As the final conclusion, it can be stated that both positive and negative sound effects are available on both *Escherichia coli* bacterium and *Salmonella* bacterium at different sound ranges.

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