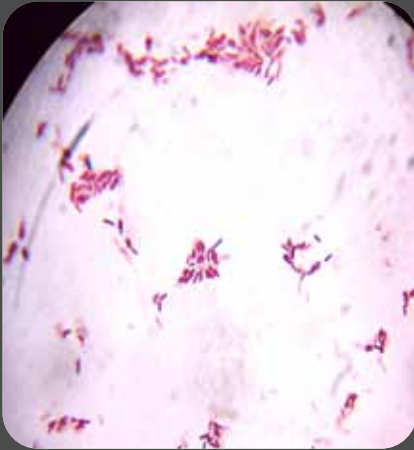


STUN PROJECT



MICROBIOLOGICAL ANALYSIS OF URINE, STRUVITE AND STRUVITE-EFFLUENT FOR THE ORGANISMS:

- ❖ *Enterococcus* spp
- ❖ *E coli*



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Aim of the study:

A) Isolation and enumeration of bacteria (*Enterococcus* spp and *E-coli*) from spiked and unspiked:-

- ❖ Urine samples (stored urine)
- ❖ Effluent samples
- ❖ Struvite

B) Isolation and enumeration of the same two bacteria from spiked and unspiked struvite at different temperatures and relative humidity to compare the pathogen inactivation.

Materials and methods

Sample collection

Sample were collected in sterilized bottles and brought to the lab and kept in refrigerator and processed for the experiment on the second day of arrival.

Procedure:

Isolation and enumeration of bacteria from stored urine and effluent samples.



Method used: Membrane filtration method :

➤ Diluted sample (2ml of sample with 98ml of sterile distilled water to make a total volume of 100ml) was filtered through a sterile membrane filter kept in a special filter apparatus contained in a suction flask.



➤ After filtration, the membrane containing bacteria was aseptically transferred keeping its upper side upwards on to a sterile plate of selective agar medium like Bile aesculin azide agar for *Enterococcus* spp and MacConkey agar for *E. coli*.

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➤ The plates were then incubated at 37°C for 24hrs and the colonies **which** developed after incubation were counted and the average of the duplicate microbial counts were determined and results were expressed per 100ml of sample by applying the formula:

No. of bacteria per 100 ml of sample = Colony count / volume of sample used * 100

ISOLATION AND ENUMERATION OF BACTERIA FROM SPIKED INPUT (stored urine) AND EFFLUENT

Method used : Calibrated loop-direct streak method.

In this method, a calibrated bacteriological loop to deliver a known volume of liquid is used to streak agar plates of selective media.

After incubation, number of colonies present on the plate was determined. Calculation was then made for the bacteria per ml of the urine sample by applying the formula:

Bacteria per ml of urine = Number of colonies developed x Factor that converts known volume of loop to 1 ml



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Spiking of urine

➤ 1000 ml (1L) of stored urine was filled in a sterilized beaker and placed on a magnetic stirrer at ~200 rpm. (Bastian, 2009).

➤ Urine was spiked with 2ml of 0.5 McFarland standard suspension of 18 hrs old bacterial culture of *Enterococcus* sp and *E coli*.

Note 2: (A 0.5 McFarland standard solution consists approx. cell density of 1×10^8 CFU/ml).



Plate showing the growth of *Enterococcus* sp



Plate showing the growth of *E coli*

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PRODUCTION OF SPIKED STRUVITE/EFFLUENT (same protocol was followed as described by Mr. Loic and Mr. Bastian)

- After spiking of urine sample with bacteria (as mentioned above), Magnesium chloride (3.04gm per 1000ml of sample) was added and stirring was done for 15 mins.
- The mixture was then filtered using sterilized nylon fabric in the filter unit. Weight of the nylon fabric and struvite was noted and nylon filter+struvite was placed carefully in the desiccator filled with saturated salt solution (NaCl) and stored at wished temperature.
- In this experiment, three temperatures were used i.e. 4°C, 23.9°C (±0.1°C) and 33°C.

For the temperature of 4°C the desiccator was placed in the refrigerator, for the temperature of 33°C the desiccator was placed in incubator and the temperature 23.9°C was the room temperature

- The effluent so obtained was used for the isolation and enumeration of bacteria using the same calibrated loop-direct streak method and identification of bacteria was done using specific tests.

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Isolation, enumeration and identification of bacteria from spiked struvite:

(Enumeration was done on day 0, day 2 and day 3 at different temperatures and relative humidity and results obtained were compared)

Method used: Serial dilution agar plating

Procedure:

➤ 5mg of struvite was weighed and dissolved in 10ml of citrate buffer of pH 6 by vortexing at high speed.

➤ Serial dilution was performed till the required dilution was achieved.

➤ Then from appropriate dilutions 1ml of suspension was pipette

to the center of appropriately labelled bile aesculine azide agar plate (for *Enterococcus* spp) and MacConkey agar plate (for *E coli*).

➤ The suspension was then spread over the entire surface of agar plate using a sterile bent glass rod and incubated at 37°C for 24 hours in an inverted position.

➤ After proper incubation number of colonies on the plates were counted and the no of bacteria per gram of struvite was calculated using the formula

No of cells per gram of struvite = No of colonies x dilution factor / weight of struvite



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Isolation, enumeration bacteria from unspiked struvite.

Struvite was produced as described above. The so produced struvite was then dried in the incubator at 37°C for 2 days at relative humidity of 88%.

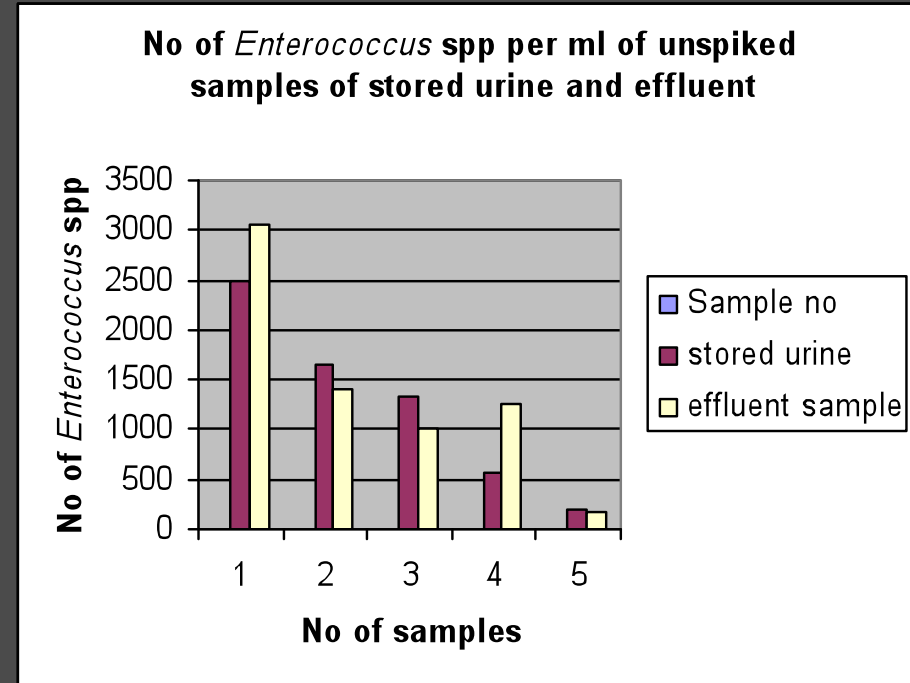
➤ Then after it was removed from the incubator and placed in a petriplate, dried in the room temperature until it was dried completely and then proceeded for the isolation and enumeration of bacteria by serial dilution method.

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RESULTS:

Enumeration of bacteria (*Enterococcus* spp from unspiked samples of stored urine and effluent)

Sample no	Stored urine	Effluent
1	2500	3050
2	1650	1400
3	1325	1000
4	575	1250
5	200	175



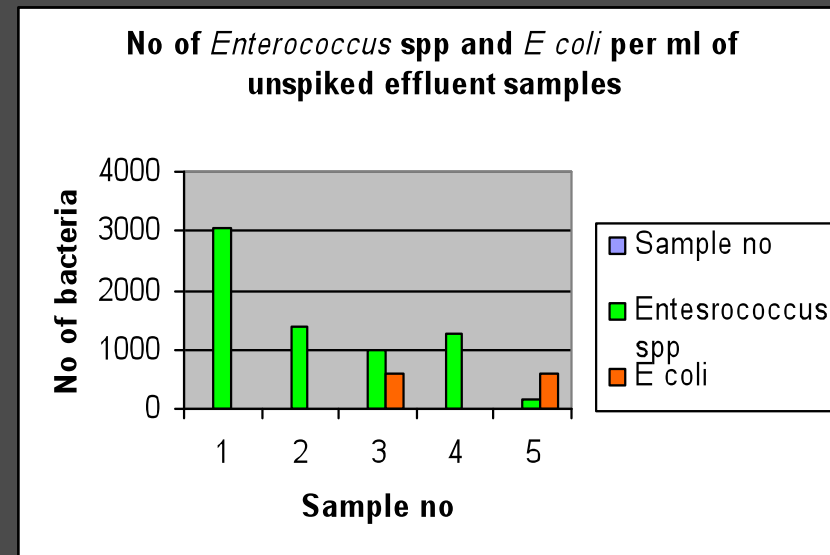
No of *Enterococcus* spp per ml of unspiked samples of stored urine and effluent

Graph 1: Graph showing the no of *Enterococcus* spp per ml of unspiked samples of stored urine and effluent.

Enumeration of bacteria (*E coli* from unspiked samples of stored urine and effluent)

Sample no	Effluent	Stored urine
1	0	0
2	0	0
3	600	0
4	0	0
5	575	0

No. of *E coli* per ml of unspiked urine and effluent samples



Graph 2: Graph showing the no of *Enterococcus* spp and *E coli* per ml of unspiked urine samples

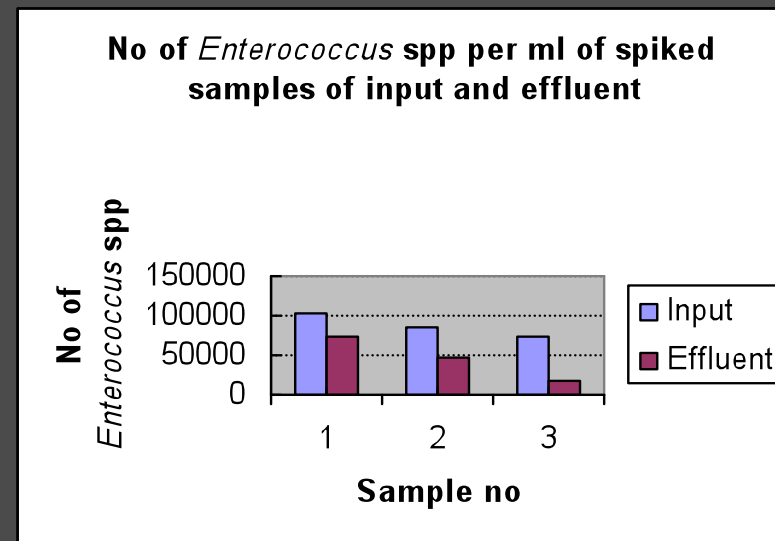
Pathogen enumeration from spiked input (stored urine) and effluent samples.

Note: Urine was spiked with 2ml of bacterial solution of 0.5 McFarland standard . (A 0.5 McFarland standard solution consists approx. cell density of 1×10^8 CFU/ml)

Number of *Enterococcus* spp per ml of Input and Effluent

<i>Input</i>	<i>Effluent</i>
<i>103125</i>	<i>72250</i>
<i>86000</i>	<i>47375</i>
<i>73125</i>	<i>17625</i>

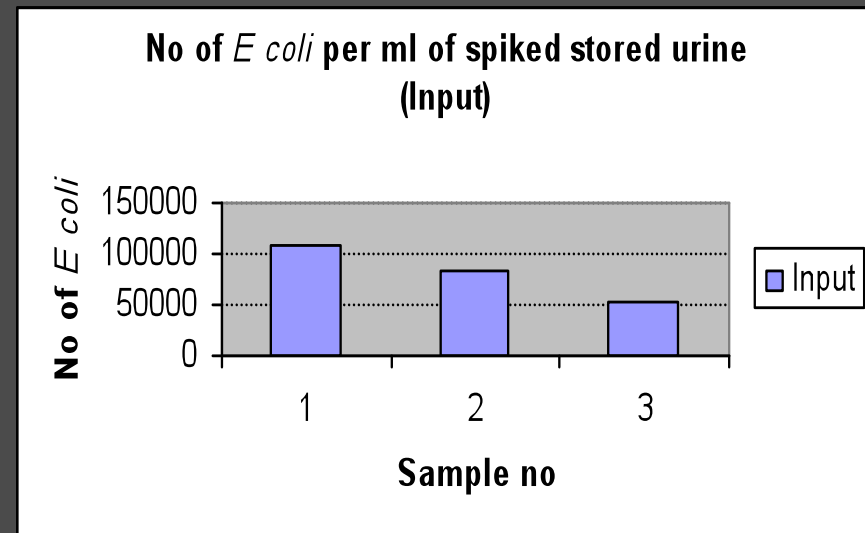
Correlation between no of bacteria in Input and Effluent was calculated which gave a positive value of 0.991157. Thus there is a positive correlation that is when the no of bacteria increases in input, the no of bacteria also increases in effluent



Graph 3: Graph showing the no .of *Enterococcus* spp per ml of spiked samples of input and effluent.

Number of *E coli* per ml of Input and Effluent

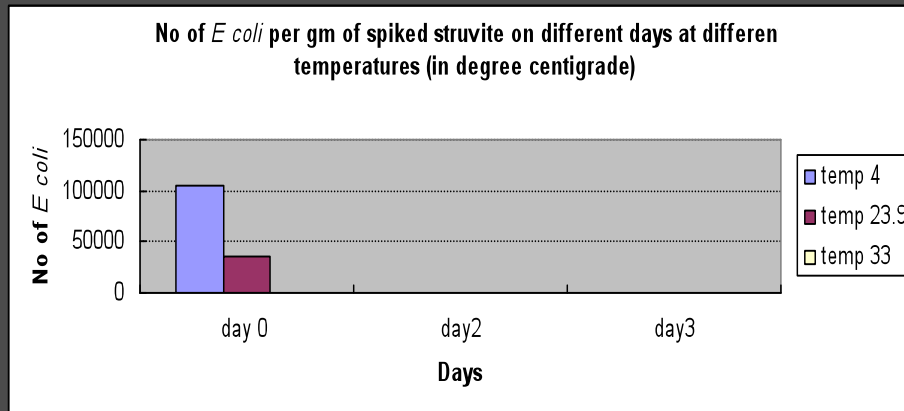
<i>Input</i>	<i>Effluent</i>
109000	0
84500	0
53500	0



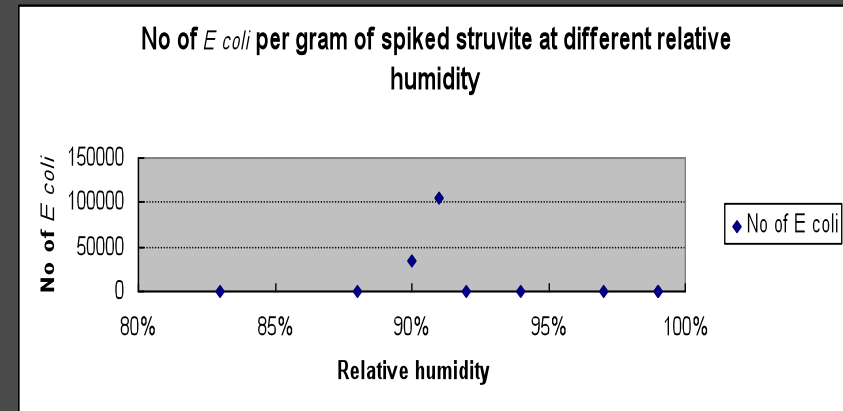
Graph 4: Graph showing the no of *E coli* per ml of spiked input

Results of the enumeration of bacteria (*Enterococcus* spp and *E coli*) per gram of spiked struvite)

	Temperature in degree centigrade	Relative humidity	No of <i>Enterococcus</i> spp	No of <i>E coli</i>
Day 0	4	91%	78000	105200
Day 2	4	88%	69200	0
Day 3	4	83%	120000	0
Day 0	23.9	90%	92000	35200
Day 2	23.9	92%	72000	0
Day 3	23.9	94%	104000	0
Day 0	33	97%	72000	400
Day 2	33	99%	67200	0
Day 3	33	99%	44000	0



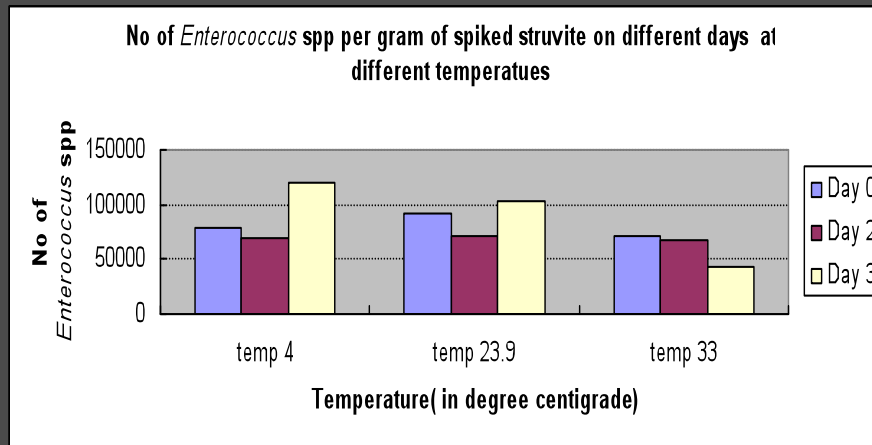
Graph 5: Graph showing number of *E coli* per gram of spiked struvite on different days at different temperatures



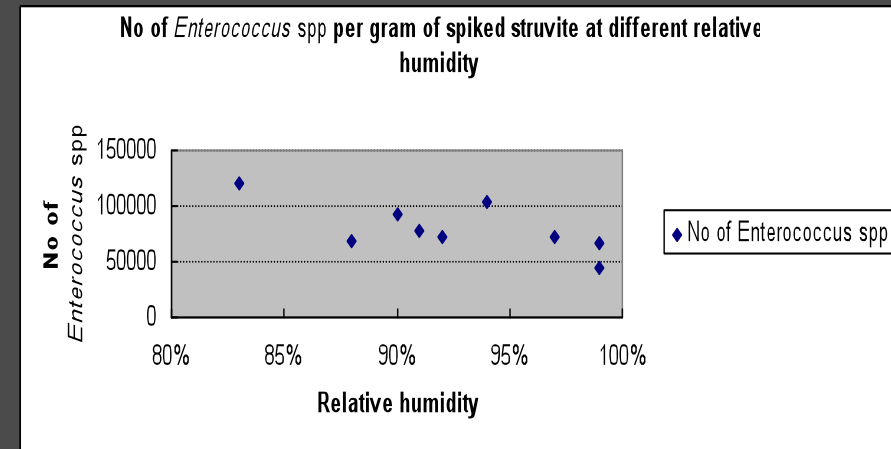
Graph 6: Graph showing number of *E coli* at different relative humidity

When correlation was calculated between growth and temperature for *E coli*, a negative value of -0.43349 was obtained. That is when temperature increases, growth of bacteria decreases. Similarly when correlation was calculated between growth and relative humidity for *E coli*, a negative value of -0.16657 was obtained. That is when relative humidity increases, growth decreases.

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Graph 7: Graph showing no of *Enterococcus* spp per gram of spiked struvite on different days at different temperatures.



Graph 8: Graph showing no of *Enterococcus* spp at different relative humidity

Correlation: When correlation was calculated between growth and temperature for *Enterococcus* spp, a negative value of -0.46142 was obtained. That is when temperature increases, growth of bacteria decreases.




Similarly when correlation was calculated between growth and relative humidity for *E coli*, a negative value of -0.70345 was obtained. That is when relative humidity increases, growth decreases.

Result for unspiked struvite sample

No *E coli* or *Enterococcus* spp was isolated.

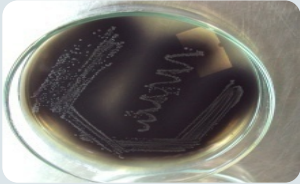
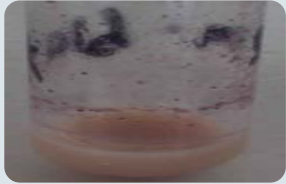


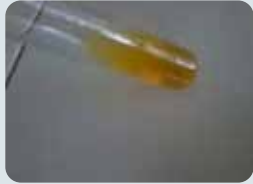
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Test performed and its results

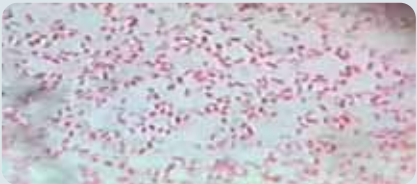


Organism	Gram staining reaction	Growth medium
<i>Enterococcus</i> spp	Gram positive cocci, occurring in pairs or short chains. 	On bile aesculin azide agar. The bacteria formed dark black or brown colonies of 1mm diameter. On blood agar:some were non hemolytic and some showed alpha hemolysis  

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Biochemical tests performed

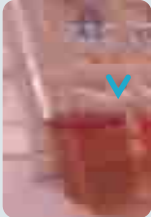



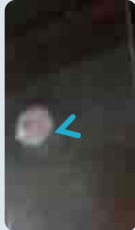

Aesculin hydrolysis test	Litmus milk reduction test	Catalase test	Indole production test	TSIA test
Positive	Positive	Negative	Negative	Acid/Acid
				

Test for *E coli*

Organism	Gram staining reaction	Growth medium
<i>E coli</i>	Gram negative rods 	On MacConkey agar: The bacteria formed smooth pink colonies of 1mm diameter. On blood agar showed hemolytic reaction  

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Biochemical tests

Organism	Indole production test	Methyl red test	Voges proskauer test	Citrate utilization test	Catalase test	TSIA test
<i>E coli</i>	Positive 	Positive 	Negative 	Negative 	Positive 	Acid/Acid, gas + 

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SUMMARY OF THE FINDINGS

- With **unspiked** input(urine) and effluent samples in case of *Enterococcus* spp, there was considerable inactivation of the organism in the effluent as compared to the input (stored urine) sample.
- In case of *E coli*, out of five samples , three samples showed complete absence of the organism in the effluent sample.
- Also with Spiked input and effluent samples in case of *Enterococcus* spp, there was considerable inactivation of the organism in the effluent sample as compared with the input sample.
- Whereas there was cent percent inactivation of *E coli* in the effluent sample
- In case of spiked struvite number of both the organisms decreased with increase in temperature and relative humidity. Infact complete absence of *E coli* was found at relative humidity of 83%, 88%, 92%, 94% and 99%.

CONCLUSION:

E coli has greater inactivation rate as compared to *Enterococcus* spp in the stored urine and effluent.

THANK YOU