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Comparative Study On Intestinal Flora Of The Type Ii Diabetes Mellitus Patients

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ABSTRACT

Background: To study the Intestinal flora of the regularly medicated Type II Diabetes Mellitus patients. To analyse the comparative study of Type II Diabetes Mellitus patients with the normal Intestinal flora. To study the Drug sensitivity of the intestinal flora to anti-diabetic drugs. Isolation of the intestinal flora using culture media.

Methods: The micro-organisms are cultured using culture media like DCA (Deoxycholate citrate agar), XLD (Xylose lysine Deoxycholate) and MacConkey agar. Biochemical tests like Indole test, TSI, urease utilization, Citrate utilization are used to determine the species of the organisms. Study takes place in Central laboratory of A.C.S. Medical College & Hospital. A Comparative study with the period of the study Six (6) months and Sample size is 30 Faeces samples with the **Inclusion criteria:** Faecal samples of the diabetic patients under regular antidiabetic medication, **Exclusion criteria:** Faecal samples of non-medicated diabetic patients and normal human.

Results: In total of 30 Patients, 15 diabetic patients and 15 normal patients' sample were taken and processed. In those diabetic patients has less E.colidominance and has Klebsiella Dominance. E.coli was sensitive to anti diabetic drug like metformin.

Conclusion: This study is done to find the changes in the regularly medicated patients and how it is changed. The intestinal flora is sensitive to the drugs and regular medication can degrade the intestine and high doses can cause permanent degradation in the intestinal flora. The regular medication can inflammable to intestine and can regenerate by the indigenous system of the medicine and the intestinal environment is maintained by the natural medication.

Keywords: *Intestinal flora, diabetes, anti-diabetic drugs, dominance, E.coli, Klebsiella.*

1. INTRODUCTION

Gut microbiota, also known as gut flora or gut microbiome, is a community of microorganisms that lives in the digestive tracts of animals. This includes bacteria, archaea, fungi, viruses, parasites, and other organisms.

These contain common phyla of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. It helps in energy harvesting, digestion, and immune defense.

It maintains the integrity of the mucosal layer of the intestine by butyryl synthesis. It also synthesizes vitamins like vitamin K, riboflavin, biotin, nicotinic acid, pantothenic acid, pyridoxine, and thiamine.

The blood glucose level should be maintained in a narrow range. The low level of blood glucose is known as hypoglycemia and is caused by various reasons. This leads to the condition called diabetes mellitus.

Diabetes mellitus is of two types. Type I Diabetes Mellitus is also called juvenile diabetes and insulin-dependent diabetes mellitus. Type II diabetes mellitus is also called adult-onset diabetes and non-insulin diabetes mellitus. This Type II Diabetes Mellitus is caused when the insulin goes to the resistant stage and does not respond to the low level of blood glucose. This causes the risk of atherosclerosis. This was maintained by some drug administration. Drugs like Metformin and Glipicide, etc., are used orally for the control of blood glucose daily.

During the last decade, the role of intestinal flora has drawn much attention across the world. Understanding the interplay of intestinal flora and diabetes would provide new insight into developing therapeutics for diabetes.

Because the drugs can affect the flora system, which is present in our intestine naturally. Species like E. coli, Helicobacter pylori, Lactobacillus acidophilus, Clostridium perfringens, Brewer's yeast, etc. are affected, and the proportion of species is also affected. Thus, the changes are occurring in the digestive system and lead to gastrointestinal disturbances, diarrhoea, and constipation.

The microbes in the intestine are vulnerable to the normal oral drugs, which are used on occasions. Those drugs can affect the flora present in the intestine. This changes the microbe's foe for a while. For this small dosage and time period, the floral changes may occur. Likewise, drug administration for a long period may change the intestinal flora drastically. Due to the exposure to drugs for a long period, the microbes may have changed physiologically, morphologically, and genetically.

These changes can be found through culturing and isolation of microbes from the microbes. The microbiota in the normal human intestine is compared with the Type II Diabetes Mellitus patients with regular medications. This comparative study will state the changes in the intestinal flora environment due to the regular medication and exposure of microbes to drugs.

And the drug sensitivity of microbes of regularly medicated Type II Diabetes Mellitus. The drugs used for the sensitivity tests are Metformin and Glipicide. These sensitivity results will show the microbes sensitivity to the antidiabetic drugs.

In this study, the microbiota of the Type II Diabetes Mellitus with regular medication is compared with the normal human microbiota. The drastic change in the environment is observed with E. coli degradation and causes degradation of mucosal integrity.

2. METHODS

Material:

Inoculation Loop, Petri-dish, Incubator, Faecal sample container, Test tube, Glass slides, Microscope, Bunsen Burner, Drug sensitivity discs, Micropipette, Pipette tips, Cotton plugs, Microscope with 100 x oil immersion, Cedar wood oil.

Culture:

The micro-organisms are cultured using culture media like DCA (Deoxycholate citrate agar), XLD (Xylose lysine Deoxycholate) and MacConkey agar.

Bio-chemical test: [IMVIC]

Biochemical tests like Indole test, TSI, urease utilization, Citrate utilization are used to determine the species of the organisms.

Methodology:

Site of the study : Central laboratory of A.C.S. Medical College & Hospital.

Type of study : Cross sectional Period of the study: Six (6) months

Sample size : 30 Faeces samples

Inclusion criteria : Faecal samples of the diabetic patients under regular antidiabetic medication.

Exclusion criteria : Faecal samples of non-medicated diabetic patients and normal human

Intervention:

Sample Collection:

Label the container with name, date of birth, and the date. Instruct the patient to place something in the toilet to catch the poo, such as a potty or an empty plastic food container, or spread clean newspaper or plastic wrap over the rim of the toilet. Make sure the poo doesn't touch the inside of the toilet. Use the spoon or spatula that comes with the container to put the required amount of poo into the container, then screw the lid shut. Aim to fill around a third of the container—that's about the size of a walnut. Tip the remaining pee into the toilet. Put anything you used to collect it in a plastic bag, tie it up, and put it in the bin. Wash your hands thoroughly with soap and warm running water.

Growth media:

DCA agar

It is particularly useful for the isolation of organisms that cause bacillary dysentery, Salmonella strains that cause food poisoning, and Salmonella para typhi.

Media was prepared using DCA agar Hi-Media with the dilution of 70.52 gm in 1000 ml of sterile water for 30 plates. Mix well in the distilled water and autoclave for 1 hour at 121 ℃, then pour in the petri dishes and dry in the drying oven to set.

It is not so selective for Salmonella Typhi. This growth medium is inhibitory to most gut bacteria, in particular species of the genus Proteus, although these species do survive on DCA agar. It is therefore essential that suspected pathogens must be sub-cultured on a less inhibitory medium prior to identification. Salmonella spp. appear to be yellow or colourless colonies, often with a dark centre. As there are many bacteria that also look like Salmonella on DCA, it is widely recommended that more selective agars be used for the identification of Salmonella, namely xylose lysine deoxycholate (XLD) agar. This growth medium is heat-sensitive and should be poured and cooled as soon as possible after addition of the deoxycholate; otherwise, it tends to become very soft and difficult to handle. It has a pH of approximately 7.3 and, when poured and cooled, appears light to dark pink in colour.

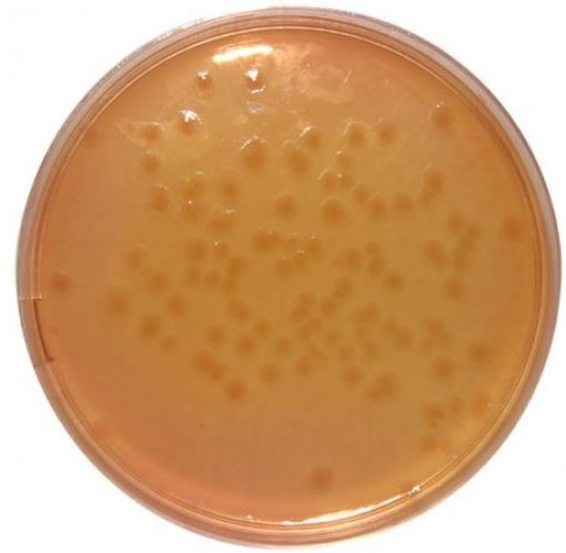


Figure 1: DCA medium with yellow colonies

XLD Agar:

Xylose Lysine Deoxycholate agar (XLD agar) is a selective growth medium used in the isolation of Salmonella and Shigella species from clinical samples and from food.

Media prepared using XLD agar Hi-Media withdrew 56.68 grams in 1000 ml of sterile water for 30 plates. Mix well in the distilled water and autoclave for 1 hour at 121°C. Then poured into the petri dishes and dried in the drying oven to set.

The agar was developed by Welton Taylor in 1965. It has a pH of approximately 7.4, leaving it with a bright pink or red appearance due to the indicator phenol red. Sugar fermentation lowers the pH, and the phenol red indicator registers this by changing to yellow. Most gut bacteria, including Salmonella, can ferment the sugar xylose to produce acid; Shigella colonies cannot do this and therefore remain red. After exhausting the xylose supply, Salmonella colonies will decarboxylate lysine, increasing the pH once again to alkaline and mimicking the red Shigella colonies. Salmonellae metabolize thiosulfate to produce hydrogen sulphide, which leads to the formation of colonies with black centres and allows them to be differentiated from the similarly coloured Shigella colonies.

Figure 2: XLD medium with pink colonies

MacConkey Agar:

MacConkey agar is a selective and differential culture medium for bacteria.

Media was prepared by using MacConkey agar HiMedia with the dilution of 49.53 grams in 1000 ml of sterile water for 30 plates. Mix well in the distilled water and autoclave for 1 hour at 121°C, then pour in the petri dishes and dry in the drying oven to set.

It is designed to selectively isolate Gram-negative and enteric (normally found in the intestinal tract) bacteria and differentiate them based on lactose fermentation. Lactose fermenters turn red or pink on MacConkey agar, and non-fermenters do not change colour. The media inhibits the growth of Gram-positive organisms with crystal violet and bile salts, allowing for the selection and isolation of gram-negative bacteria. The media detects lactose fermentation by enteric bacteria with the pH indicator neutral red.

Figure 3: MacConkey Agar with pink and white colonies

Culture through streaking in media:

In this method, the inoculation of the sample in the culture media is done. Firstly, label the petri dishes. The inoculation loop is heated to red hot and cooled, then the sample is inoculated in the media using the inoculation loop. An oval in the 1/4th corner of the petri dish is drawn in the inoculation loop, then the loop is heated again and cooled. This inoculum is known as the mother inoculum, and from this mother the lines are drawn parallel to each other; this is called the primary inoculum. From these other parallel lines drawn as secondary inoculum in the same way, tertiary is done. Lastly, the tail is drawn with no intersection.

Bacterial Cell Determination:

1. Label a clean glass slide using a red wax marker. Note that it is important to recognize the side of the glass slide that you put your bacterial sample on.

2. Add a small drop of saline to the slide. This can be done by placing a drop of saline onto your inoculation loop and then transferring it to the slide. If you use the saline dropper directly on the slide, do not release a full drop.

3. With an inoculation loop or needle, pick up a small number of bacteria. Mix it well with the saline and spread the mixture over a wider area of the slide. Be careful not to have the two smears run into each other.

4. Air dry the bacterial specimen on the slide (slide warmers may also be used).

5. When slides are completely air-dry, heat fix the bacterial specimen by passing the slide slowly over the flame twice (your instructor will demonstrate this).

- Heat fixing kills cells and adheres them to the slide.
- Cells will be rinsed off the slides if they are not fixed properly.
- Be careful not to overheat the slides in this procedure.
- After heat-fixing is complete, you are ready to simply or gram stain your slide.

Gram Staining:

Gram staining differentiates bacteria by the chemical and physical properties of their cell walls. Gram-positive cells have a thick layer of peptidoglycan in the cell wall that retains the primary stain, crystal violet. Gram-negative cells have a thinner peptidoglycan layer that allows the crystal violet to wash out on addition of ethanol. They are stained pink or red by the counterstain, commonly safranin or fuchsine. Lugol's iodine solution is always added after the addition of crystal violet to strengthen the bonds of the stain with the cell membrane.

Procedure for Staining:

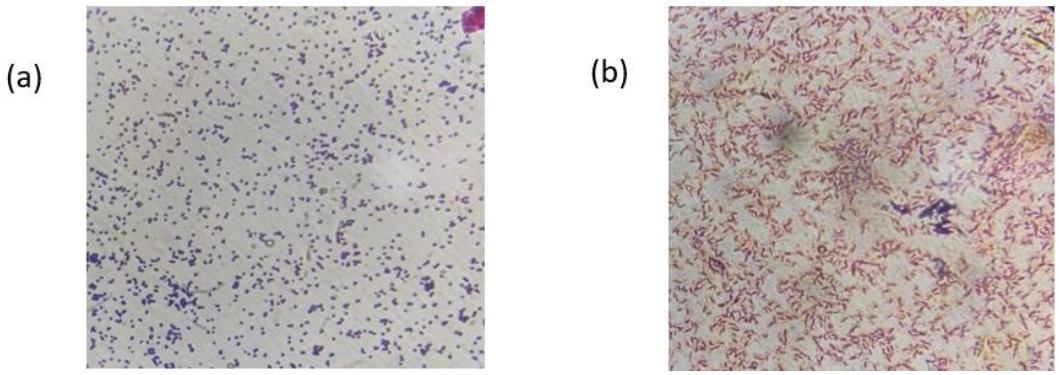
1. Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture. Heat fixation kills some bacteria but is mostly used to affix the bacteria to the slide so that they do not rinse out during the staining procedure.

2. The addition of iodine, which binds to crystal violet and traps it in the cell

3. Rapid decolorization with ethanol or acetone

4. Counter staining with safranin. Carbol fuchsin is sometimes substituted for safranin since it more intensely stains anaerobic bacteria, but it is less commonly used as a counterstain.

Summary of Gram stain			
Application of	**Reagent**	**Cell color**	
		Gram-positive	**Gram-negative**
Primary dye	crystal violet	purple	purple
mordant	iodine	purple	purple
Decolorizer	alcohol/acetone	purple	colorless
Counterstain	safranin/carbolfuchsin	purple	Pink or red



(a)gram positive cocci **(b) gram negative bacilli**

Figure 4: Microscopic view of gram-stained slide

Biochemical tests:

Oxidase test:

The oxidase test is used to determine whether an organism possesses the cytochrome oxidase enzyme. The test is used as an aid for the differentiation of Neisseria, Moraxella, Campylobacter, and Pasteurella species (oxidase positive). It is also used to differentiate pseudomonads from related species.

1. Strip of Whatman's No. 1 filter paper is soaked in a freshly prepared 1% solution of tetra-methyl-p-phenylene-diamine dihydrochloride.
2. After draining for about 30 seconds, the strips are freeze-dried and stored in a dark bottle tightly sealed with a screw cap.
3. For use, a strip is removed, laid in a petri dish, and moistened with distilled water.
4. The colony to be tested is picked up with a platinum loop and smeared over the moist area.
5. A positive reaction is indicated by an intense deep-purple hue appearing within 5-10 seconds, a "delayed positive" reaction by coloration in 10-60 seconds, and a negative reaction by absence of coloration or by coloration later than 60 seconds.

Indole test:

The indole test determines the ability of an organism to produce indole from the. Degradation of the amino acid tryptophan. Tryptophan is hydrolysed by tryptophanase to produce three possible end products—one of which is indole; the others are pyruvate and ammonium ion, as shown by the following reaction.

The peptone water with the emulsified isolated colony is incubated for 24 hrs, and Kovac's reagent is added to find the colour change.

Urease test:

The urease test is used to differentiate urease-positive organisms (e.g., Proteus) from other organisms. It can also be used to detect the presence of *Helicobacter pylori*. This test can be used for differentiation between the yeasts Candida albicans and Cryptococcus neoformans.

Prepared by using urea agar base (Christensen) of HiMedia with the dilution of 24.01 in 950 ml of distilled water for 30 test tubes. Mix well in the distilled water and autoclave for 1 hour at 121°C. Then it was poured into a test tube for 1/4 and dried in the slant position in the drying oven to set. Then the stroke method is used for inoculation and incubated for 24 hrs to identify the microorganisms.

Citrate test:

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen.

Prepared by using citrate agar (Christensen) of HiMedia with the dilution of 24.80 in 1000 ml of distilled water for 30 test tubes. Mix well in the distilled water and autoclave for 1 hour at 121°C, then pour in a test tube for 1/4 and dry in the slant position in the drying oven to set. Then the stroke method is used for inoculation and incubated for 24 hrs to identify the microorganisms.

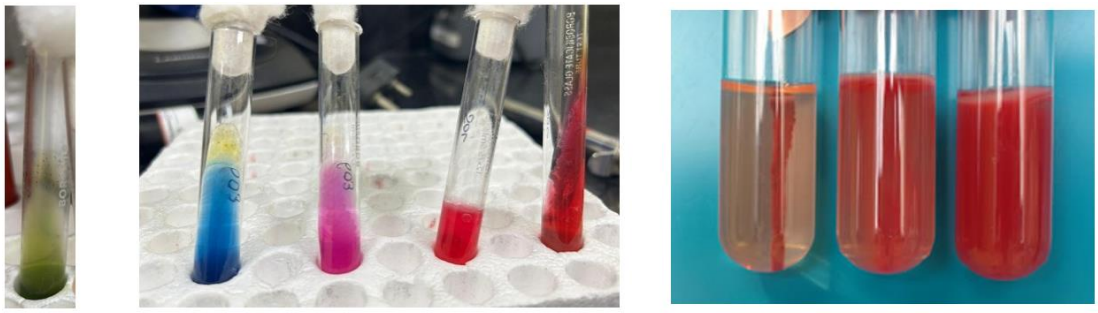
TSI (triple sugar iron) test:

Prepared by using triple sugar iron agar of HiMedia with the dilution of 64.32in 1000 ml of distilled water for 30 test tubes. Mix well in the distilled water and autoclave for 1 hour at 121°C, then pour in a test tube for 1/4 and dry in the slant position in the drying oven to set. Then the stroke method is used for inoculation and incubated for 24 hrs to identify the microorganisms.

Mannitol Motility Medium:

Mannitol motility medium is a bacterial growth medium used to detect the ability of bacteria to ferment mannitol and produce nitrogen gas and to indicate the motility of the organism.

Prepared by using Mannitol motility test medium of HiMedia with the dilution of 26.04 in 1000 ml of distilled water for 30 test tubes. Mix well in the distilled water and autoclave for 1 hour at 121°C, then pour in a test tube for 1/4 and dry in the drying oven to set. Then the stroke method is used for inoculation and incubated for 24 hrs to identify the microorganisms.



(a) (b) (c)

(a)Negative **(b) Positive** **(c) MMM test**

Figure 5: Biochemical Tests Citrate utilization, Urease utilization, Mannitol motility medium, Triple sugar iron

3. RESULT

In total of 30 Patients, 15 diabetic patients and 15 normal patients' sample were taken and processed. In those diabetic patients has less E.colidominance and has Klebsiella Dominance. E.coli was sensitive to anti diabetic drug like metformin.

In normal patients, E. coli had established dominance more than the others, and they were contributing more in the gut environment. In type 2 diabetic patients, the Klebsiella genus has the dominance, especially K. pneumoniae and K. oxytoca. S. typhi presence was due to the past history of the enteric fever.

Table- 1 - shows that the number of intestinal organisms present in diabetic patients varies significantly compared to non-diabetic individuals.

Diabetic patients	
Species	Count
K. pneumonia	6
E. coli	3
S. para typhi B	1
K. oxytoca	5

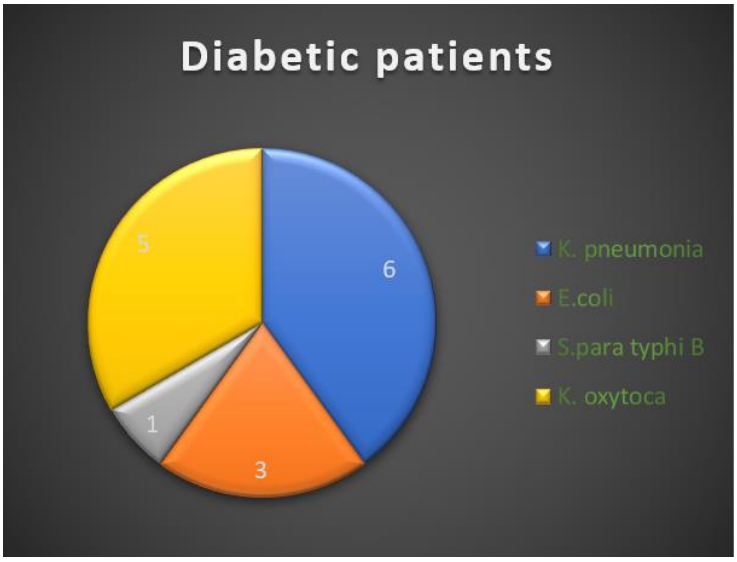


Chart 1

Table 2- shows that significant numbers of E.coli present in the samples collected from normal individuals.

Normal patients	
Spices	Count
E. Coli	15
K. Pneumonia	0
S. Para typhi b	0
K. Oxytoca	0

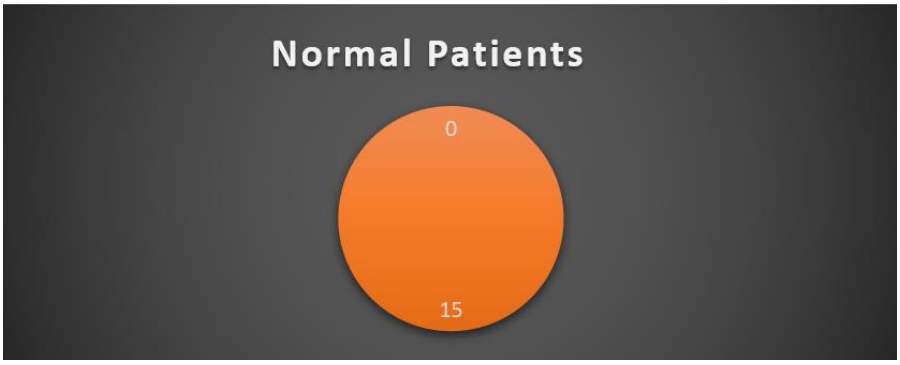


Chart -2: Normal Patients

Table 3: Distribution of intestinal flora of the diabetic and non-diabetic patients.

SPECIES	**E. coli**	**K. pneumonia**	**S. typhi B**	**K. oxytoca**
Non diabetic	15	0	0	0
diabetic	2	7	1	6

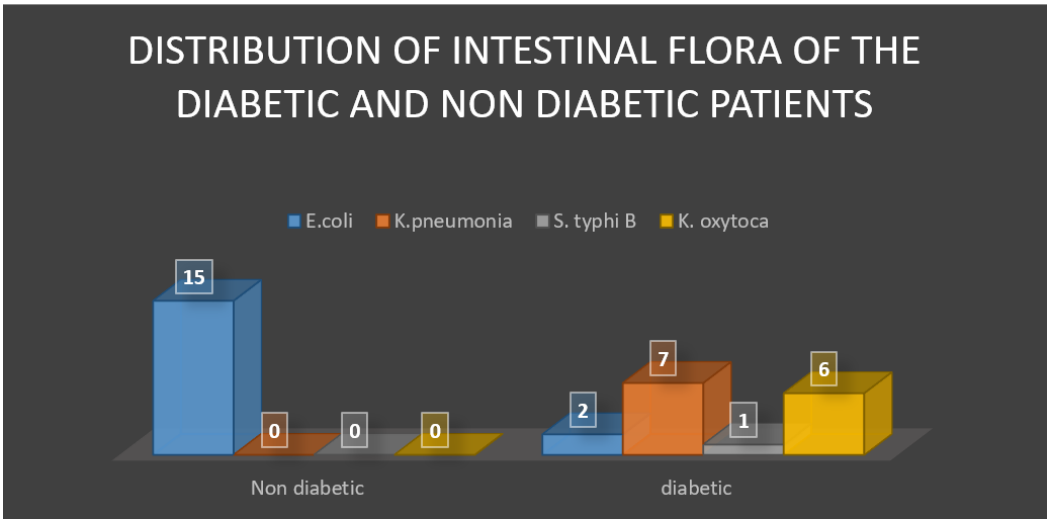


Chart 3

4. DISCUSSION

E. coli is present in the gut naturally and helps in the maintenance of the integrity of the mucosal layer of the intestine. It releases the chemical called butyrate, which plays the major role in the maintenance of mucus integrity in the intestine.

Diabetic patients, E. coli disintegration has appeared due to the oral intake of anti-diabetic drugs like metformin and glipizide.

E. coli is sensitive to metformin and causes dysbiosis of the intestinal flora.

This causes the dominance of Klebsiella pneumonia and oxytoca. Then the butyrate-producing rate becomes slow, and the degradation of the integrity of the mucosal layer in the intestine. occurs. E. coli produces 70% of the gross amount of the butyrate. After the degradation of the mucus layer weakens it, it is easily inflammable and infectable. This causes the patient to become immunosuppressed gradually.

This leads to a lack of nutrients and leakage of nutrients, improper digestion, chronic septicaemia, etc.

5. CONCLUSIONS

This study is done to find the changes in the regularly medicated patients and how it changed. The intestinal flora are sensitive to the drugs, and regular medication can degrade the intestine, and high doses can cause permanent degradation in the intestinal flora. The regular medication can be inflammatory to the intestine and can be regenerated by the indigenous system of the medicine, and the intestinal environment is maintained by the natural medication.

6. LIMITATIONS

The study only concentrates on orally medicated type II diabetes mellitus patients. The organisms are isolated when there is dominance in the entire media. No considerations of the other colonies than the dominant colonies. The pathogenic bacterial organisms are excluded during the isolation (entire sample).

The drugs are diluted using sterile distilled water at the lowest level. The filter papers are used after sterilizing as drug discs.

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