EVALUATING THE REVERSAL POTENTIAL OF CHITOSAN MICROSPHERE IN DIET INDUCED TYPE II DIABETES

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DEDICATION SHEET

This Thesis is heartily dedicated to our respectful parents and family without their constant support this thesis would not be possible. They always inspire us in learning and doing everything possible to walk in the path of our success. "No matter how far we come, our parents are always with us".

NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
NAAC ACCREDITED 'A+' GRADE

CERTIFICATE

This is to certify that the thesis entitled **"Evaluating the reversal potential of chitosan microsphere in diet induced Type II diabetes"** submitted to the Institute of Science, Nirma University in partial fulfillment of the requirement for the award of the degree of M.Sc. (Biotechnology/Microbiology), is a record research work carried out by **Harshita Keshwani (20MBT015), Lakshita Sharma (20MBT025), Riya Sharma (20MBT041), Arti Varma (20MMB035)** under the guidance of **Dr. Sriram Seshadri**. No part of this thesis has been submitted for any other degree or diploma.

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The above Dissertation project was carried out in a group by **Harshita Keshwani, Lakshita Sharma, Riya Sharma, Arti Varma** under my guidance.

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"Thankfulness is the beginning of gratitude. Gratitude is the completion of thankfulness. Thankfulness may consist merely of words. Gratitude is shown in acts."

-Henri Frederic Amiel

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Abbreviations

- ***AGIs: Alpha-Glcucosidase Inhibitor***
- ***AUC: Area Under Curve***
- ***CM: Chitosan Microsphere***
- ***CD14: Cluster of Differentiation14***
- ***CRP: C-Reactive Protein***
- ***CVD: Cardio Vascular Diseases***
- ***DAMP: Damage Associated Molecular Pattern***
- ***GABA: Gamma Amino Butyric Acid***
- ***GIT: Gastro Intestinal Tract***
- ***HDL: High Density Lipoprotein***
- ***HSFD: High Sugar Fat Diet***
- ***IKK: Inhibitor of kappa B Kinase***
- ***IL: Interlukin***
- ***IR: Insulin Resistance***
- ***IGT: Impaired Glucose Tolerance***
- ***JNK: C-Jun N-Terminal Kinase***
- ***LDL: Low Density Lipoprotein***
- ***LPS: Lipopolysaccharides***
- ***LTAs: Lipoteichoic Acids***
- ***NF-kB: Nuclear Factor kappa B***
- ***OGTT: Oral Glucose Tolerance Test***
- ***PAMPs: Pathogen Associated Molecular Patterns***
- ***PBS: Phosphate Buffer Saline***
- ***PRR: Pathogen Recognition Receptor***
- ***SCFA: Short Chain Fatty Acid***
- ***SGOT: Serum Glutamic Oxaloacetic Transaminase***
- ***SGPT: Serum Glutamic Pyruvic Transaminase***
- ***T2D: Type II Diabetes***
- ***TG: Triglycerides***
- ***TLRs: Toll Like Receptors***

- ***UPR: Unfolded Protein Response***
- ***WTAs: Wallteichoic Acids***

ABSTRACT

Type 2 diabetes (T2D) is a multi-dysfunctional condition involving metabolic and immunological disorders, which is now well reported to be connected with altered gut microbiota. One of the major cause of T2Dis lifestyle change including High Sugar Fat diet and alteration in gut microflora causing dysbiosis. Dysbiosis of the gut microbiota leads to an increase in the proportion of gram-negative, consequently increased LPS production in plasma also altered short-chain fatty acids, impaired insulin signaling cascades via a variety of biochemical pathways and ultimately leading to diabetes. There are several pharmacological medications available that are useful for treatment, such as Metformin, Sulfonylureas, and biguanides which are used as treatment strategy but it has side effects and long term impact. So to overcome these disadvantages Chitosan is used ,a natural polymer having multiple unique properties such as mucoadhesive, hypocholesterolemic, hypolipidemic, and prebiotic.It has been reported that it modifies the gut microbiota and reduces inflammation, preventing insulin resistance.

As Chitosan have many biological properties which helps in restoration of microflora altered in T2D. It also works for restoring the microbial population of gram positive bacteria, thus helps in reduction of inflammation and insulin resistance. Therefore, the hypothesis for this study was that on administration of the chitosan microsphere the gut microbiota dysbiosis which occurs in type II diabetes could be restored.

Objectives-1.To prepare the chitosan microsphere and its characterization.

2.To understand the alterations in gut microbiota and diabetic characteristics following administration of chitosan microsphere in diet induced diabetic rats.

the purpose of this study was to “Evaluating the Reversal potential of chitosan Microsphere in the diet-induced Type II Diabetes”. Male Wistar rats(IS/PHD/27/2020/032) were divided into four groups Normal Control (NC) with a normal diet, Diabetic Control (DC) with a high sugar fat diet (HSFD), treatment group DC with chitosan (40mg/kg), and DC with metformin (100mg/kg). After 12 weeks of study, a decrease in glucose level, triglycerides, and LDLc were observed in the treatment group as compared to the DC group. This indicated a decrease in insulin resistance. There was an increase in the proportion of butyrate than other SCFA in

treatment groups as compared to the DC group. It indicated an increase in the proportion of gram-positive bacteria that causes the activation of TLR-2 and other signaling pathways leading to a reduction in inflammation. These results suggest that chitosan could prevent the progression of T2D through restoring the gut microbiota dysbiosis and its mediated inflammation.

INTRODUCTION AND REVIEW OF LITERATURE

I. GUT MICROBIOTA:-

Gut microbiota can be referred as organ within an organ which is a domicile of trillions of microorganisms resides within human intestine. Researchers have shown an adult human body contains as many bacterial cells as human cells. Numerous of researches have shown that gutmicrobiota contributes towards human health and diseases in a direct or indirect manner. During infancy , gut microbiota develops in Gastrointestinal tract(GI) (AI Bander et al., 2020). Microbial community residing in GI tract largely comprises prokaryotic domain (bacteria) and to lesser extent fungi, archaea and parasites. More than 90% of bacteria residing in gut belongs to four major phyla- Bacteroidetes , Firmicutes , Actinobacteria and Proteobacteria from these Firmicutes and Bacteroidetes are dominant one (Figure) (Nishida et al.,2021). It has been shown that the GI tract of an adult human being harbors trillions of microbial cells in both its stomach's acidic environment as wells as in basic environment of intestine (Patterson et al ., 2016). The composition of microbiota living in particular environment of GI tract may alter due to various factors and life events of host .Literature have shown that greater the diversity of microbial composition better will be the health outcomes (AI Bander et al., 2020).

Gut microbiota influences various functions of host such as nutrient metabolism , natural defence of host against infection and immune response. Various approaches and methods have been used to study gut microbiota and its relationship with host to construct the map showing its affect both beneficial and detrimental on host health (Patterson et al ., 2016).

It has now been completely understood from the researches and studies done in past years that host-microbiome interaction together co-metabolize certain kind of dietary components that produces large arrays of signaling molecules which has beneficial impacts on host health (Shanahan et al., 2009).Pharmabiotics referred as bioactive metabolites produced by microbes residing in gut such as SCFA's , conjugated fatty acids , neuroactive metabolites such as GABA ,these metabolites have certain kind of benefits to host health . thus host-microbial interaction is quite important for optimum health of host (Patterson et al.,2014).

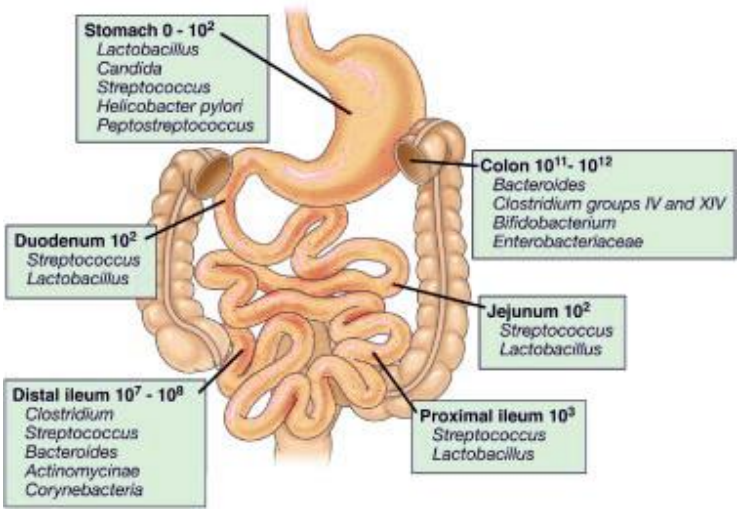


Figure:-1.Microbiota composition of GI tract (Sartor., 2008).

Literature published in recent years have linked gut microbiota to every single disease starting from GI diseases , diabetes , obesity , cancer and even to neurological diseases such as Autism , Parkinson's disease and anxiety (Patterson et al ., 2016). Recently, various advancements have been done in gene sequencing techniques and bioinformatics tools which has enabled researchers to have insight knowledge of composition of gut microbiota and its significant effect on host physiology and health (Nishida et al.,2021).

It has been shown that Diet plays pivotal role in shaping the composition of gut microbiota. Recent studies have shown that different dietary intake in obese and overweight humans have significant effects on the gut microfloral composition. Researchers have found that individuals consuming unhealthy diet such as high sugar intake and less vegetables , fruits and water have high inflammatory profile as compared to individuals consuming healthy diet (Kong et al., 2014). Healthy dietary intake is directly related to rich microbial diversity of gut and shows the part of diet in shaping gut microbial composition. There is also a direct or indirect link between gut microflora and T2D, it has been evaluated that about 80% of T2D patients are obese and overweighed and this one of the factor contributes to higher risk of T2D. recent studies focuses on link between T2D and gut microflora, it has been observed that T2D patients shows endotoxemia(Kallio et al., 2015). Various researches have shown that gut microflora contributes to endotoxemia related inflammatory response along with insulin resistance in T2D. In T2D

patients microbial endotoxin plays key role in insulin resistance , it has been observed that in T2D patients there is altered gut microbial profile (Patterson et al.,2014).

II. GUT MICROFLORA DYSBIOSIS AND TYPE II DIABETES-

The gut microbiota used to be called as the microflora of the gut and combined with dysbiosis, it is known as disbalance in the microflora of the gut. Various organisms such as eukaryotes, archaea and bacteria present in the gut are known as the gut microbiota.

Gut microbiota is a complex bacterial community that regionally colonizes the gastrointestinal tract. In human, the gut microbiota provides protection to the host by competing for space and nutrition against pathogens and facilitates the digestion of food, and take energy from vitamin synthesis and indigestible food such as carbohydrate and also immune cells development.

They also maintain the intestinal permeability and intestinal integrity of the epithelial lining of the gut. The gut microbiota mainly consists of four phyla(families) –Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. Some other species are Fusobacteria, Verruromicrobia, Cyanobacteria occurs in gut but less in abundance. In normal conditions, the greatest portion of the gut microbiota is covered by the bacteria Firmicutes(64%), Bacteroidetes(23%), Proteobacteria(8%) and Actinobacteria(3%) (Cunningham et al.,2021).

When microbiota of the gut get disbalanced , influences human health and that disruption increases the pro-inflammatory conditions and various disorders including obesity, type II diabetes mellitus, inflammatory bowel disease and cancer.For the normal functioning of the intestine of the host, microflora of the gut plays a major role in maintaining the intestinal permeability and intestinal barriers of the host by allowing nutrients and other components to pass through the membrane of the gut and allowing the toxic substances from leaving the intestinal barriers and transfers to the other parts of the body.(Zhang et al.,2021)

Dysbiosis or alterations of the stable gut microflora hinder the intestinal permeability due to which increase the absorption of lipopolysaccharide(LPS) that is present in the gram negative bacterial cell wall causes the activation Toll-like receptor (TLR-4) this induces the activation of pro-inflammatory cytokines or inflammation in the gut that leads to increase in intestinal

permeability and the intestinal barriers get damaged. Due to the activation of these receptors, insulin receptors by decreasing it's phosphorylation and signaling impairment was observed (Caricilli et al.,2013)

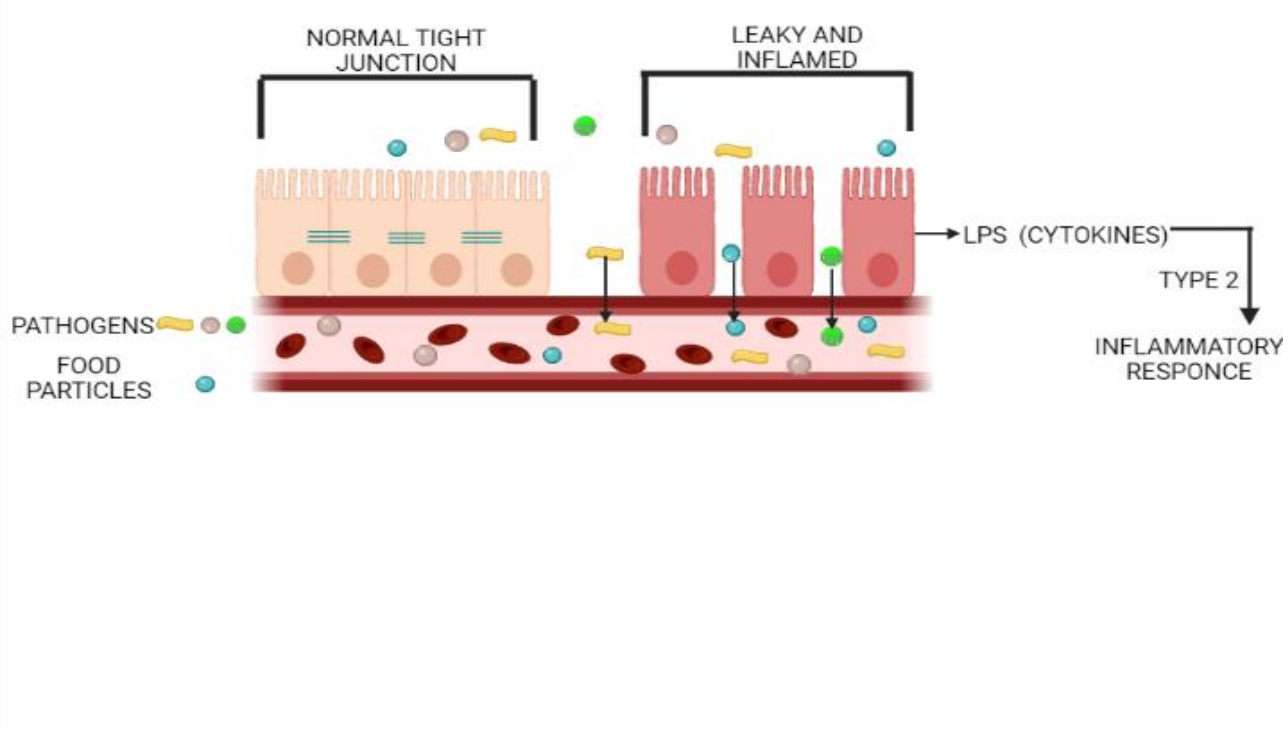


Figure:-2.Disruption of intestinal homeostasis.

Disruption of intestinal homeostasis (dysbiosis) is one of the major leading cause of obesity, insulin resistance, diabetes, and cardiovascular diseases.

Gut microbiota affects gut permeability, insulin sensitivity, metabolism of glucose and modulates inflammation and homeostatis in the host. Elevation of pro-inflammatory cytokines, inflammatory proteins and chemokines is directly associated with type 2 diabetes.

Lipopolysaccharide layer of gram negative bacteria induces metabolic endotoxemia and inflammation which mainly leads to cause type 2 diabtetes. This inflammation that is caused by the change in the microbiota of gut activates the lipopolysaccharide and CD14/ toll-like receptor(TLR)4.

The elevation of plasma lipopolysachharide concentration in host induced by HSFD(high sugar fat diet) is refer to as metabolic endotoxemia. CD14 has a major role in innate immunity, when LPS layer binds to the complex of CD-14 and TLR-4 at the surface of innate immune cells activates the inflammation reaction.(Gurung et al.,2020)

There are various pro-inflammatory stimuli are present such as LPS, fatty acids, lipids, and chemokines activates IKKβ kinase intracellularly. The activation of IKKβ stimulates NF-**K**b(transcription factor) and then the expression of other inflammatory mediators will also increase that can cause insulin resistance.

Type 2 diabetes is associated with the several parameters of innate immunity. It has been hypothesized that this disease is immune-dependent in a way that the immune cells produce different levels of pro-inflammatory cytokines, which has a bad effect on type 2 diabetes.

IL-6, IL-18 and TNF-alpha are the major innate inflammatory cytokines and there elevated serum levels are associated with type 2 diabetes. There is a cross-linked relationship with between type 2 diabetes and innate immunity. CRP that is C-reactive protein is positively associated with the mechanism of insulin resistance(IR), insulin concentration in plasma, body weight and triglycerides level and negatively associated with the HDL concentration.(Sepehri et al.,2016)

The innate immune system has PRRS (Pathogen recognition receptors). PRR recognizes the cell surface of microbes known as PAMPS (Pathogen associated molecular patterns) and DAMPS(damaged associated molecular patterns). DAMPS are endogenous molecules that are produced by the cells during inflammation or infection.

Here, PRR including TLR and NLRS (Toll-like receptors) and (Nod like receptors) respectively. Both TLR and NLR activates the NF- **K**B leads to induce the pro inflammatory cytokines expression.

TLRS are basically innate immune cell receptors, insulin resistance mechanism of TLRS involves activation of TLR ligands. TLR 2 ligand interaction has several inflammatory effects leads to progression of type 2 diabetes.

It has been reported that in type 2 diabetes, inflammation is one of the major cause of pancreatic B cells dysfunction.

So the crosstalk of NLRs and TLRs leads to activate NF- **K**B (Nuclear factor kappa –light-chain enhancer of activated B cells) which induces inflammation cause pancreatic B cells to get insulin resistant and the major pathogenesis of T2D (Prajapati et al.,2014).

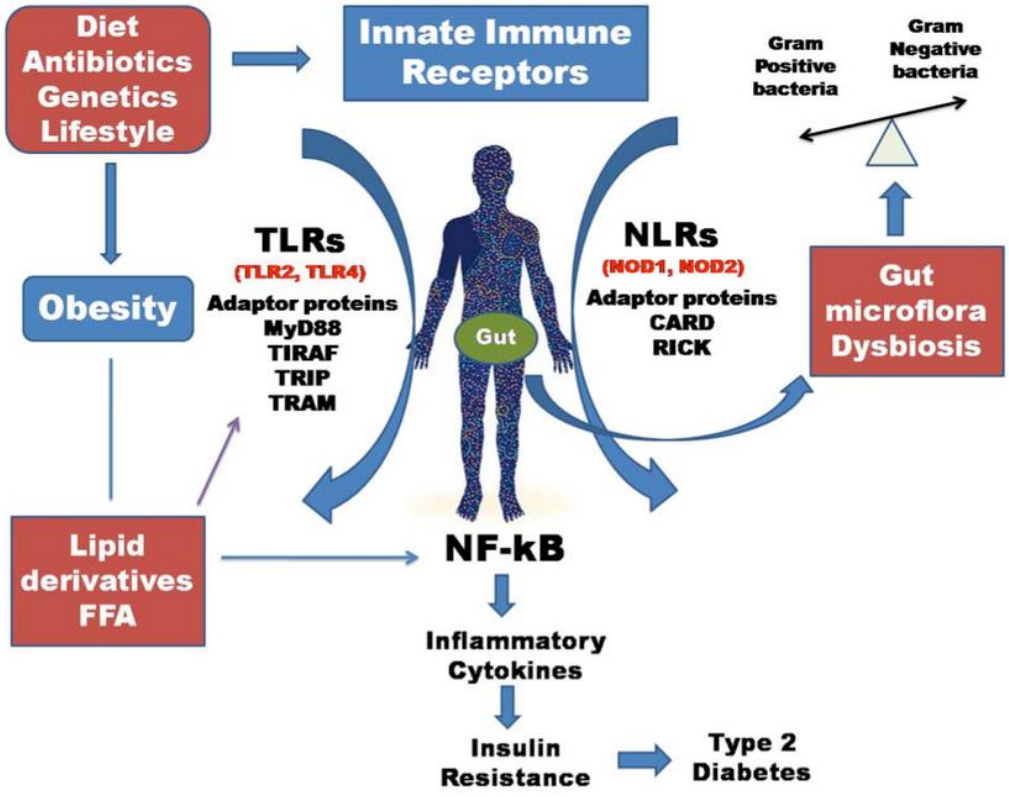


Figure:-3.The NLR and TLR cross talk in pathogenesis of T2D (Prajapati et al.,2014).

III. TREATMENT STRATEGIES-

Various non-insulin anti-diabetic drugs are used for the treatment of Type II Diabetes such as-

A. **Biguanides**-this is one of the major class of anti-diabetic drug , metformin is one of them, it is most commonly used drug used in treatment of T2D (Holman, 2007).It has been proved to be effective in lowering down blood glucose level, increases sensitivity towards insulin and reduces hypoglycemic risk (Hundal et al., 2003). it is the only agent which reduces mortality rates in T2D patients. It shows its antihyperglycemic effects through suppression of AMPK-dependent and independent pathways. Metformin is more

effective when given orally rather than intravenously. it has been suggested that metformin should be avoided for the patients with renal(both chronic or acute) insufficiency(Ripsin et al. ,2009).

B. **Sulfonylureas-** this is a line of drugs used for treatment of patients with T2D who are not highly obese. This type of drugs directly acts on islet βcells by closing ATP – sensitive potassium ion channels leads to stimulation of insulin secretion(Ashcroft et al.,2013). they are dependent on presence of β cells in sufficient amount for their function .their effect can be aggravated through interactions with various drugs like aspirin , phenylbutazone. Along with its beneficial effect , one of its side effect is weight gain.

C. **Thiazolidinediones (TZDs) –** this is a class of drugs which includes rosiglitazone ,troglitazone and pioglitazone. These are PPAR-γ ligands which controls the hepatic insulin sensitivity when administrated (Hevener et al. , 2007). Their action in regulation of hyperglycemia is more lasting than metformin and sulfonylureas. Their efficiency increases when used in combination of other anti-diabetic drugs. When used with insulin it reduces insulin dosage improves T2D glycemic control . It also exhibits various side effects such as higher risk of bladder cancer ,weight gain and edema. Rosiglitazone and troglitazone not in use anymore because of their higher risk of myocardial infarction(Mamtani et al., 2012).

D. **α-Glucosidase inhibitors (AGIs)-**AGIs includes drugs such as acarbose , voglibose and miglitose. These are widely used in postprandial hyperglycemia .they inhibits α-glucosidase enzyme present in intestinal mucosa works for conversion of complex polysaccharides to monosaccharides , leads to carbohydrate absorption decrement. Acarbose reduces the risk of myocardial infarction which is common in T2D. side effects of these drugs are abdominal bloating , flatulence and diarrhea (Wu et al., 2014).

E. **Incretin-based therapies**- Incretins are hormones which stimulates insulin secretion and suppresses postprandial glucagon secretion .these are secreted by intestinal endocrine cells along with GIP and GLP-1. This is one of the best treatment measure for T2D because of its higher efficiency, weight loss and lower risk of hypoglycemia (Wu et al., 2014).

F. **GLP-1 receptor agonists-** This class of drugs includes exenatide and liraglutide. They target GLP-1 receptors, reduces HbA1c level from 0.8% to 1.5% .they stimulates insulin

secretion , reduces glucagon secretion and lowers the hypoglycemic risk. This class of agents can be used for treatment of T2D in both elder as well as younger patients(Bourdel et al.,2011).

other than anti-diabetic drugs new therapeutic strategies have also been developed for the treatment of T2D which are more effective and have less adverse effects , such as:-

A. SGLT2 inhibitors-Sodium glucose co-transporter type 2 (SGLT2) inhibitors are one of the glucose – lowering agents. These agents prevents reabsorption of glucose back in circulation filtered in kidney and increases secretion of glucose in urine leads to lowering down of glucose concentration in blood. These inhibitor are also effective in reducing HbA1c level, fasting blood glucose , body weight and hyperglycaemia (Bays et al., 2013).

B. **Stem cell educator therapy-** Many researches have suggested that patients with T2D shows chronic metabolic inflammation and various immune dysfunctions .different researches have shown that may be macrophages are the key players whose contribution leads to such chronic inflammations and insulin resistance of cells in patients . this therapy have been developed to reverse the immune dysfunction and to make the cells insulin sensitive .the procedure in this therapy includes – collection of blood from patient ,purifying lymphocytes from blood , co-culturing of those cells with adherent cord blood-derived multi-potent stem cells (CB-SCs) *in vitro* and again administrating educated lymphocytes in patients (Zhao et al ., 2012). Phase I and Phase II studies have suggested that the therapy is safe to use and it have shown remarkable efficiency , increases insulin sensitivity in early stage T2D patients (Wu et al., 2014).

C. Antioxidant therapy- It is one of the newly developed strategy for the treatment of T2D patients. It is beneficial in reducing the risk factors related to T2D and its complications(Ceriello et al ., 2009) . Various types of antioxidants like vitamins , supplements , plant-derived antioxidants and drugs having antioxidant properties are used in treatment of oxidative stress in patients with T2D. β- carotene , vitamin-C&E are few antioxidants which are effective in reducing oxidative stress and have low complications. For example- vitamin C reduces HbA1c level , fasting blood glucose and improves insulin action in patients' body(Rahimi et al ., 2005) . plants with substances having antioxidant properties like monoterpenes, cinnamic acids, flavonoid. These antioxidants

are proven to be highly effective in T2D treatment with lower side effects (Wu et al., 2014).

These anti-diabetic drugs and therapies have several disadvantages such as the microbiota becomes resistant towards it , various side effects on host body and they does not provide targeted delivery. So to overcome such disadvantages we need to formulate such compound which is more effective, provide targeted drug delivery .So one such compound is Chitosan which is used in restoration of gut microbiota due to its various biological properties.

IV.INTRODUCTION TO CHITOSAN-

Chitosan is cationic natural biodegradable polysaccharide. It contained two monosaccharide subunits, D-glucosamine and N-acetyl-D-glucosamine connected together by β-(1-4) glycosidic bonds. Chitosan is mainly found from the chitin that is distributed in nature and it also originate from cells of crustaceans, exoskeleton of arthropods, crabs and cell wall of fungi. It is derived from chitin through N- deacetylation. Chitosan was first found by Roguet in 1859.

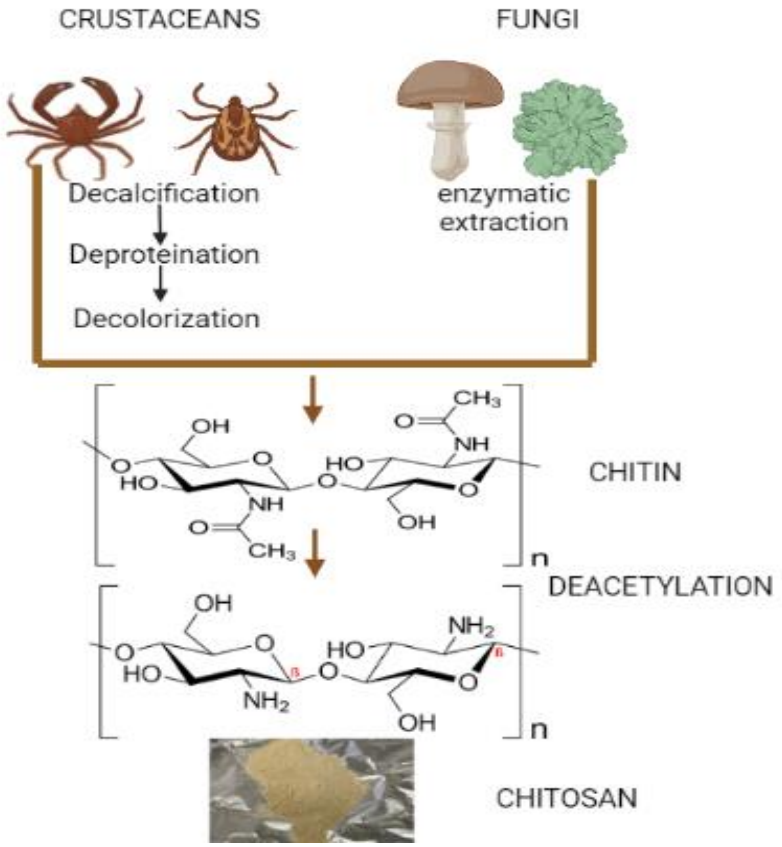


Figure:-4. Extraction of chitin and chitosan from different sources

Chitosan is soluble in dilute aqueous acidic with pH <6.5 solution such as succinic acid, acetic acid and formic acid. Chitosan forms gel at low pH when it get precipitated with alkaline solution and it also used in different application such as preparation of solution, films and fiber by being soluble in aqueous acidic solution. According to its preparation procedure and depending on the source its molecular weight ranges from 10kDa to 1000kDa (Ahmed et al. 2014). By using chromatography and light scattering the molecular weight of chitosan can be determined. It also acts as flocculants for depollution, treatment of waste water and protein recovery (Demarger-Andre and Domard, 1994). Chitosan have availability of free amino group and carries cationic charged thus it react with many negatively charged surfaces and also have a chelation with metal ion (Fukuda, 1980) like cobalt cobalt (Onsoyen and Skaugrud, 1990). Therefore it use for separation of metals. Chitosan have a free amino group which make it more effective for binding the metal ion as compare to acetyl group in chitin. This free amino group

has higher metal-ion adsorption rates. The adsorption capability of chitosan depends on many other elements like deacetylation, affinity for water and crystallanity(Kurita et al.)

Chitosan have many biological and chemical properties which relates to its application. Chitosan have some chemical properties such as cationic nature, high charged density at pH <6.5, adherence to negatively charged surfaces, gel formation ,viscosity , high molecular weight, linear polyelectrolyte, chelates certain metals, amicable to chemical modification and reactive amino/hydroxyl group (Anon 2016). Chelation is one of the most useful properties of chiotsan. Chiotsan can bind with favourable material such as cholesterol, tumor cell and fats. Chelation can also used for food preparation, water improvement and health care. Chitosan also exhibit affinity for some protein like wheat germ agglutinin and trypsin. Chiotsan also possess some other properties like it used to inhibit tumor-cells, some antifungal effect, speeding up for plant germination and wound healing.

Biological properties like biodegradability, biocompatibility, non-toxicity, mucoadhesive etc. make it more suitable for use in many applications such as food, pharmaceuticals, textile, and agriculture. The positive charged on chitosan shows electrostatic interaction with negatively charged mucus layer.

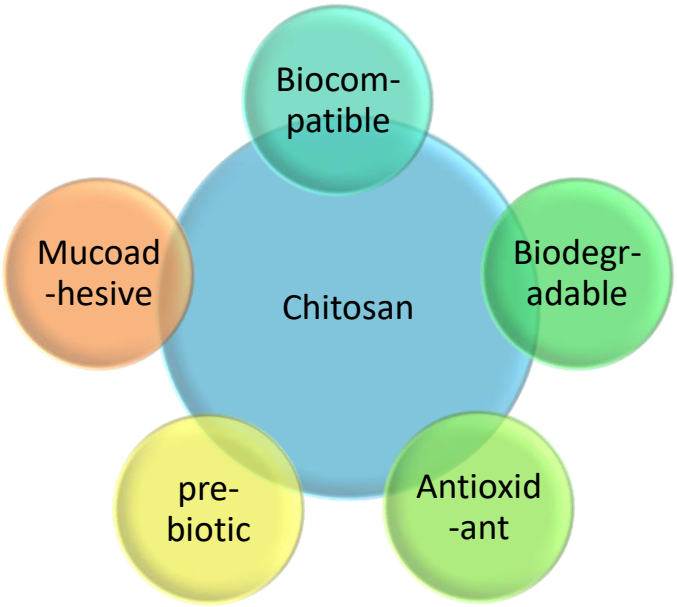


Figure:-5.Biological properties of chitosan

Chitosan microsphere used to provide targeted drug delivery, to reduce the dose of drug and to enhance the bioavailability of degradable substances across the epithelial layer. Chitosan microsphere can be prepared by using various strategies such as cross-linking with anions, precipitation, glutaraldehyde cross-linking, thermal cross- linking, modified emulsification etc. By measuring the zeta potential of chitosan microsphere the electrostatic interaction during mucoadhesion can be obtained. Zeta potential is measure the size and charge of microsphere. It has been found that by using emulsification ionotropic gelation method for chitosan microsphere formulation be more mucoadhesive as compared with other methods (Dhawan et al. 2004).

Due to mucoadhesive properties of chitosan. It can be used for drug delivery through mucosal surface. The positive charged on chiotsanis observed a main element for its muco-adhesive properties. The cationic charged on chitosan and negatively charged mucin form a electrostatic interaction in the middle of mucus layer and it causes a good adhesion on mucosal surface. chitosan have non-toxicity properties and it can be used for nasal epithelium. It expand and create a gel layer in aqueous environment (it absorbed water from (mucus layer) in nasal cavity) which is beneficial for penetration of glycoprotein chains (into mucous) and polymers (Felt et al., 1998). Chitosan exhibit some bioadhesivecharacterstics also and decreased the clearance rate of

drug from the, nasal cavity and therefore greater the bioavailability of drugs absorb into it (Soane et al., 1999).

V. CHITOSAN MICROSPHERE IN RELATION TO GUT MICROFLORA RESTORATION –

Chitosan and its derivatives have different mechanisms of action against the bacteria. Peptidoglycans make up the gram-positive bacterial cell wall, it consists two types of teichoic acid 1. wall teichoic acids (WTAs) and 2. Lipoteichoic acid (LTAs). WTAs are covalently linked with peptidoglycan, and LTAs teichoic acid is affixed to the cell membrane of a bacterial cell via lipid (Figure 1), (Brown et al., 2013). The negative charge of the cell wall of gram-positive bacteria is due to the presence of carboxyl and phosphate groups of teichoic acid (Feng et al., 2021).

Gram-negative bacteria's cell wall is made up of two membranes separated by periplasmic space, which has a thin peptidoglycan layer. The outer membrane is identical to the cytoplasmic membrane however, it varies in that it contains lipopolysaccharide (LPS). LPS comprises three components: 1) O-antigen or O-polysaccharide, 2) core polysaccharide, and 3) lipid A. Lipid- A attached the LPS into the outermost membrane(Figure 1). The negative charge of the surface of gram-negative bacteria is due to the presence of phosphate and pyrophosphate groups of LPS (Feng et al., 2021).

According to the literature, there are mainly four mechanisms of the chitosan’s antimicrobial effect.

1. Disruption of cell membrane/cell wall

An often found mechanism is the electrostatic interaction between positively charged chitosan molecule and negatively charged cell wall of bacteria(Figure 1), which cause cell permeability to be hindered and lysis to occur (Matica et al., 2019).

The bactericidal activity of chitosan related to molecular weight, degree of deacetylation, type of bacteria, the pH, different substituents, and active compound attached to the chitosan molecule

(Vinsova and Vavrikova, 2011). The positive charge of the chitosan molecule depends on the amino group of the polymer. When the pH of an aqueous acidic solution falls below the pKa (6.3) value, chitosan becomes soluble, and the amino group of chitosan is protonated (Matica et al., 2019). At pH 7.0, chitosan's antibacterial action is likely to be lost due to the deprotonation of amine groups (Raafat and Sahl, 2009). As a result, the positive charge of chitosan electrostatically react with the negative charges of teichoic acid of gram-positive bacteria and LPS of gram-negative bacteria. Because of this, the chitosan change the shape of bacterial surface, decrease membrane integrity, and enhance intracellular material release (Martins et al., 2014). Previous studies on the effect of chitosan on *E. coli* show the enhanced alteration of the outer membrane and the disrupted inner cell membrane, resulting in cytosolic content leakage and microbial cell death (Li et al., 2010). Gram-negative bacteria are more susceptible toward chitosan because LPS is widely associated with a phosphorylated group (Ke, Deng, Chuang and Lin, 2021). Furthermore, gram-positive bacteria have peptidoglycan coating that is thicker than gram-negative bacteria, making it difficult for chitosan to adhere directly to the cell membrane (Li et al., 2010). However, another study has revealed that gram-positive bacteria are more susceptible to chitosan (Raafat and Sahl, 2009).

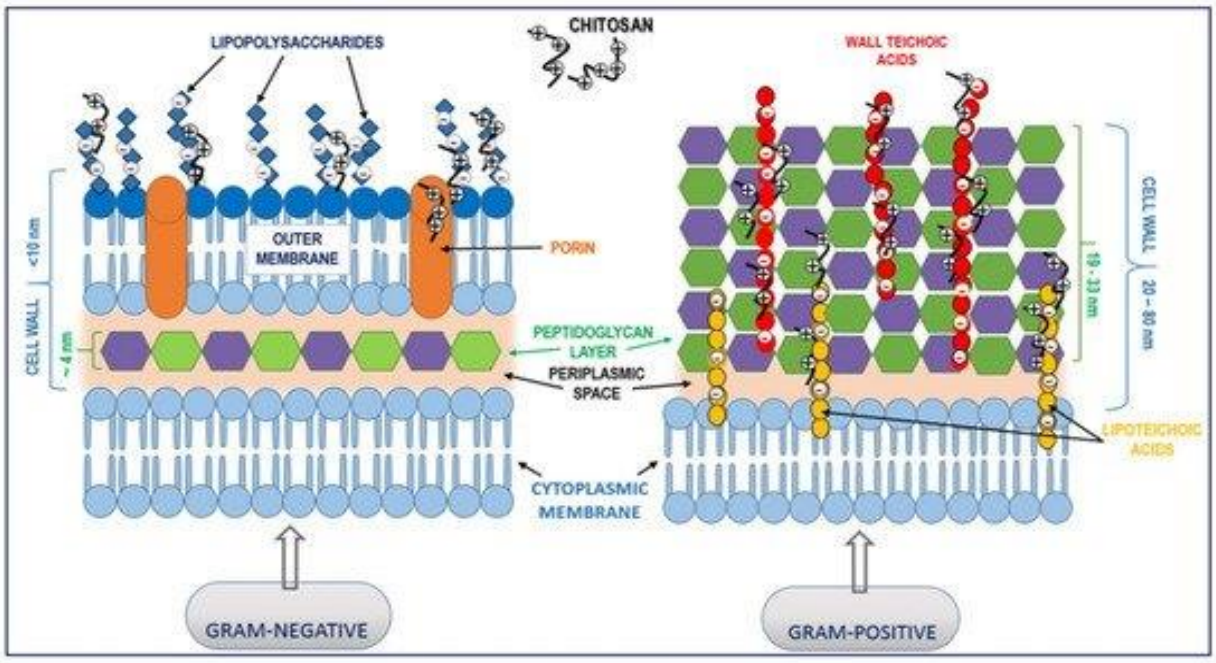


Figure:-6.Interaction between chitosan and bacterial cell wall (Matica et al., 2019).

2. Interaction with microbial DNA

Low molecular weight chitosan and chitosan hydrolysis products bind to microbial DNA and inhibit the production of mRNA and protein of the microorganism (Yan et al., 2021).

3. On the surface of cell dense polymer film forms

When high molecular weight chitosan accumulates on the cell surface of bacteria, it can form a dense polymeric film and interfere with nutrient and oxygen utilization, this impairs the bacterial growth. The cell wall becomes thicker and vesicular structure are formed due to chitosan deposition. The extra layer of the vesicular structure prevents nutrients from entering cells and prevents metabolic products from being excreted (Matica et al., 2019).

1. Chitosan acts as a chelator of nutrients

The amino group of the chitosan has the potential to chelate metal ions (e.g., Ni2+, Zn2+, Co2+, fe2+, and Cu2+) present on bacterial surfaces when the electrostatic force outweight the pH of

the mixture compared to the pKa of the chitosan. The bacterial cell membrane can be stabilized by divalent cations. Gram-positive bacteria possess wall teichoic acids (WTAs) that can bind divalent metal ions, which makes the polymer structure more stable as well as increase the integrity of the cell wall. Cations bound to WTAs can help to mitigate osmotic pressure fluctuation inside and outside of a microbial cell. The LPS membrane of gram-negative bacteria is composed of various negative charged phosphate groups. The stability of the bacterial outer membrane is maintained by minimizing repulsive force between negative phosphate groups aggregated with divalent metal cations. Chelating properties are one of the properties of chitosan. Chitosan or derivatives of chitosan can donate electrons to metal ions on the surface of the gram-positive and gram-negative bacterial cell surface to form complex when the pH of the medium is above the pKa value of the chitosan and its derivatives. Chitosan's positively charged amino groups can compete with divalent cations on the cell membrane surface for phosphorus groups in LPS or WTAs. Consequently, such a chelation reaction can result in an unstable cell surface potential because negatively charged phosphate groups repel one another, and thus rupture the cellular membrane. Furthermore, bivalent cations were reported to inhibit the activity of chitosan in the order Ba2+ > Ca2+ > Sr2+ > Mg2+. As a result of chitosan, "pores" are formed in cell membranes, and ca2+ bound to cell surfaces is subsequently released prior to the leakage of cytosolic content (Yan et al., 2021).

Antifungal activity of chitosan

The healthy human gut contains many fungi, mainly Candida yeasts, and sometimes environmental sources (like mold) may affect gut flora. The cell wall of the fungi is composed of chitin, D-glucans outside the fiber of the chitin, and mannoproteins as the cell wall's outer layer (fig.3). Multiple studies have clearly demonstrated that chitosan can bind to the outer layer of fungi resulting in the disruption in the plasma membrane and release of intracellular materials (Ke, Deng, Chuang and Lin, 2021).

Oligo-chitosan can cross the cell wall and cell membrane of the fungal cell and affect the mitochondrial function too. Chitosan's minimum lethal concentration against fungi depends on the molecular weight and degree of acetylation of the chitosan, pH value, and the type of fungi it targets. Fungicidal activity is positively related to acetylation and negatively related to molecular weight. Those fungi that are chitosan sensitive have higher level of unsaturated fatty acid in their

cell membrane, resulting in increase in membrane fluidity, negative charges, and permeability. Furthermore, it inhibits the germination of spores and the growth of the mycelium of fungi (Yan et al., 2021).

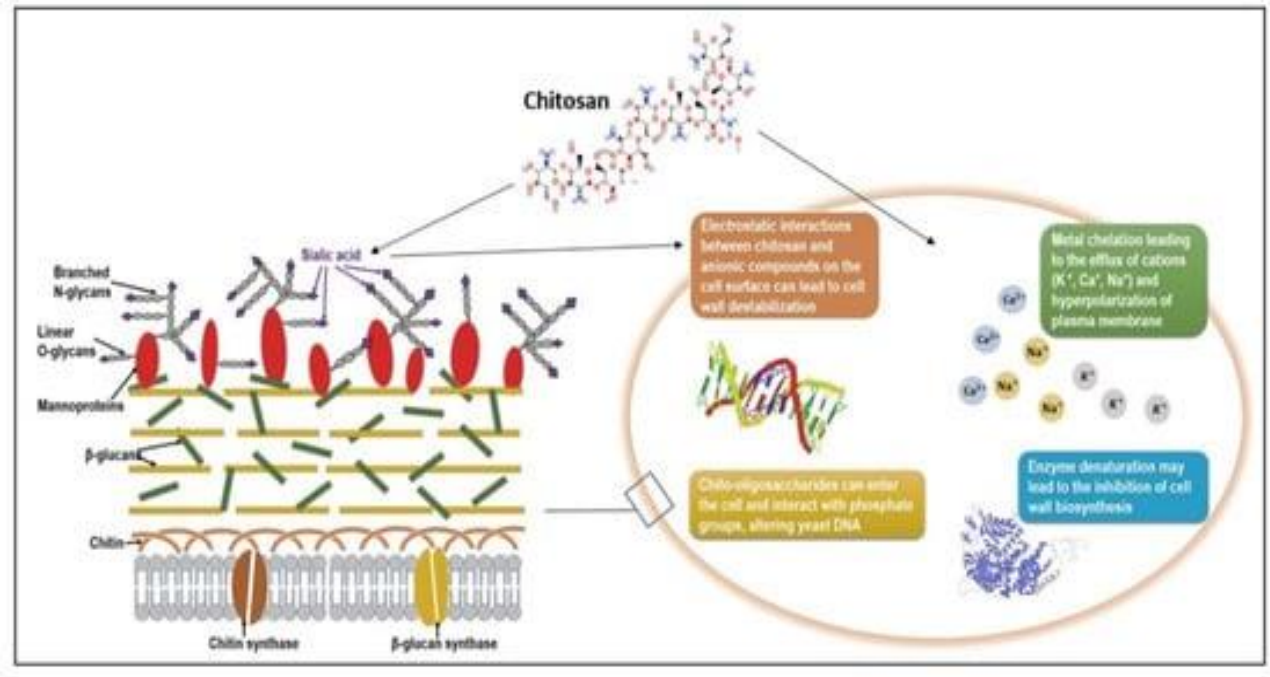


Figure:-7. Interaction between fungal cell wall and chitosan (Matica et al., 2019).

Chitosan as a prebiotic

Prebiotics is a non-digestible food component that increases the growth of beneficial gut microbiota. The important dietary component is fiber which consists great prebiotic activity, and chitosan is a polysaccharide that can be degraded by colon bacteria so iit's used for the prebiotic purpose. It is reported that the proportion of Bacteroidetes phylum increases and the proportion of firmicutes phylum decreases in mise treated with 300mg/day of chitosan. Additionally, the fraction of chitosan polysaccharide N-acetylglucosamine act as a soluble decoy that allowed bound bacteria to be removed from the body, inhibits pathogen attachment to host cells, and promotes the attachment of helpful species. Clostridia were the most hindered bacterial group in the presence of chitosan, with a 65 to 85 percent, inhibition of adhesion; whereas lactobacilli/enterococci, were less hindered with a 13 to 59 percent restriction of adhesion.

Microorganisms live in mucus, thus it's a prime location for them to survive. Mucus glycoprotein is consumed when dietary fiber is consumed without the gut microbes, causing permeability to increase. Prebiotics protect the mucus layer by preventing bacteria from consuming it. Chitosan, for example, promotes mucus structure in either way. Short-chain fatty acids (SCFAs) are favorable for mucus structure and promote the growth of the intestinal mucosa in some species.

In this regard, demonstrated that pigs fed a dietary supplementation with chitosan exhibited increased levels of SCFAs, mostely acetate, due to enhanced Bifidobacterium spp. colonization. In addition, SCFA modulates M1 and M2 polarization directly. Butyrate decreases pro-inflammatory cytokines such as NO, IL-6, and IL-12 by inhibiting the enzyme histone deacetylation. In this perspective, prebiotic nature of chitosan could promote further polarization of M2 Macs generated by SCFAs, as the biopolymer can increase arginase activity in Macs (figure 3). After chitosan delivery, the intestinal microenvironment undergoes a number of favorable metabolic changes, including an increase in SCFAs concentration. In ovariectomized rats, chitosan administration improves LPS biosynthesis, the sulfur relay system, and the formation and breakdown of ketone bodies, and also promote the branched-chain amino acids biosynthesis like valine, leucine, and isoleucine in the intestine.

By modifying endogenous defensins in young hosts, all of these metabolic alterations may improve disease resistance and intestinal health (Moine, L., et al., 2021).

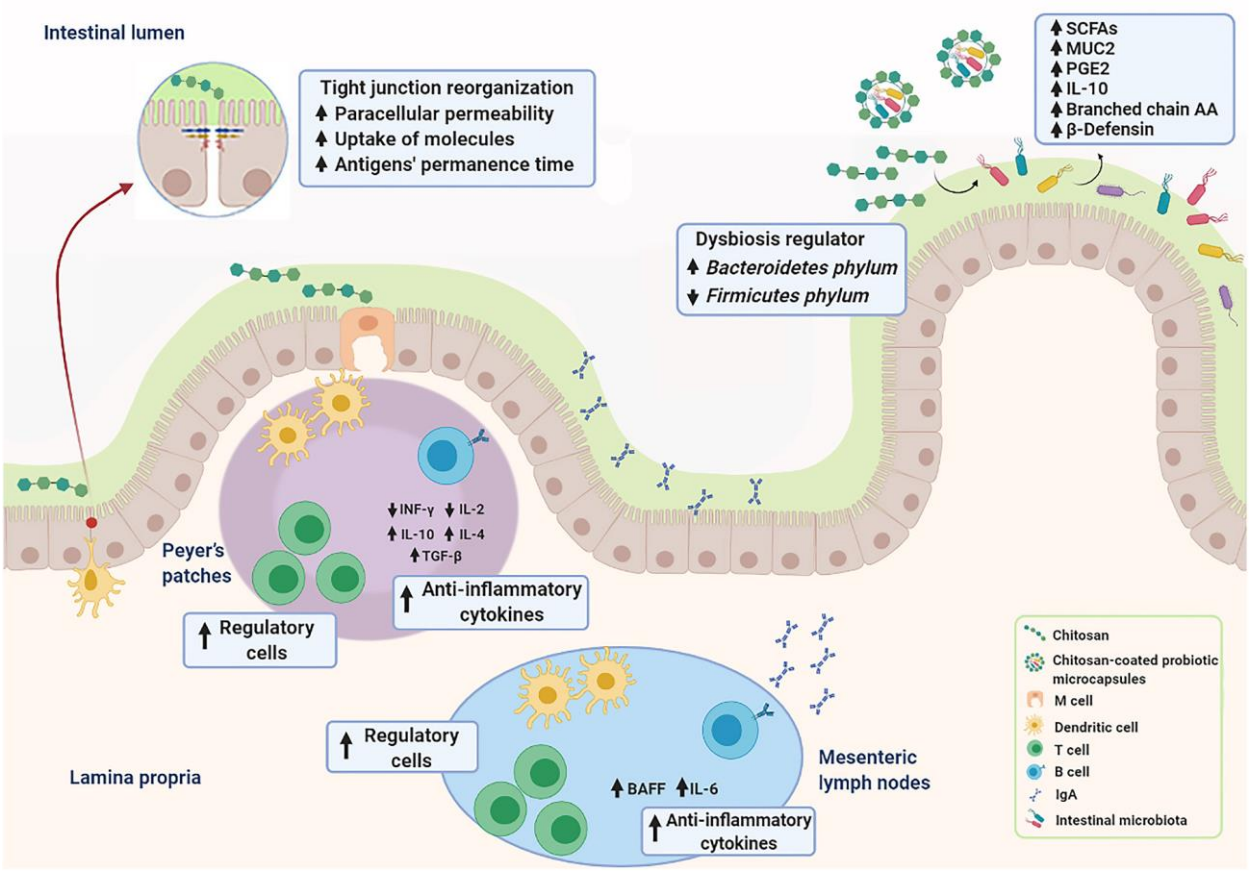


Figure:-8. Prebiotic effect of chitosan (Moine, L., et al., 2021).

VI. MODE OF ACTION OF CHITOSAN MICROSPHERE –

Hypercholesterolemia and hyperlipidemia is a major cause of type 2 diabetes. Studies have showed that the chitosan have high hypocholesterolemic and hypolipidemic activity. Chitosan have positive charge in acidic solution, therefore the positive charge of chitosan interacts with negative surface of cholesterol and bile. The chitosan cholesterol complex is transferred to intestine and changes into an insoluble gel that can be hydrolysed by pancreatic or intestinal enzymes and excreted in feces due to which the absorption of cholesterol is decreased.

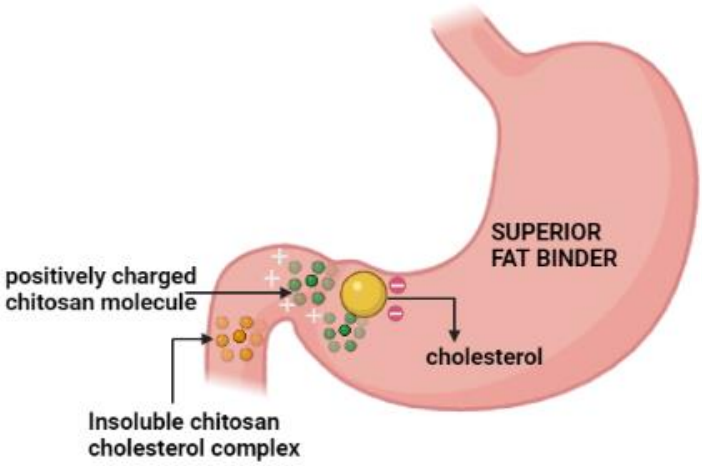


Figure:-9. Mechanism of action of chitosan – A natural Fat binder

Although more research has shown more complex mode of action for chitosan, it reported that supplementation of chitosan with different dietary treatments strategies decreased the dietary intake in mice. Basal diet with 1200 ppm chitosan decrease the feed intake as well the body weight (Walsh et al., 2013).Different form of chitosan have been investigated as an antimicrobial material against a wide range of target organisms like bacteria, yeast and fungi. Chitosan is considered as a bactericidal (kill the live bacteria or some fraction there in) or bacteriostatic (hinders the growth of bacteria but not kill the bacteria).

It has been stated that chitosan show stronger effects for gram-positive bacteria than gram-negative bacteria. So, during the comparison with hydrophilicity the gram-positive bacteria have significantly low hydophilicity as compare to gram- negative bacteria that make them more sensitive towards the chitosan. According to that in *vitro* experiments confirmed that the gram-negative bacteria appears very sensitive to chitosan and show some morphological changes during treatment when compared to gram-positive. The charge density on the surface of cell is a specific factor to entrench the quantity of adsorbed chitosan. More, adsorbed chitosan will clearly shows greater changes in membrane permeability and the structure.

.VII. MODE OF ACTION OF METFORMIN-

Metformin is an anti-hyperglycaemic drug which is used to improve the metabolism of glucose and increase glucose tolerance in type 2 diabetes patients.Metformin being effective in controllinghyperglycaemia, it also reduce the hepatic glucose output, improves the action of insulin by increasing the peripheral uptake of glucose and it's utilization and it inhibits the intestinal absorption of glucose as well.

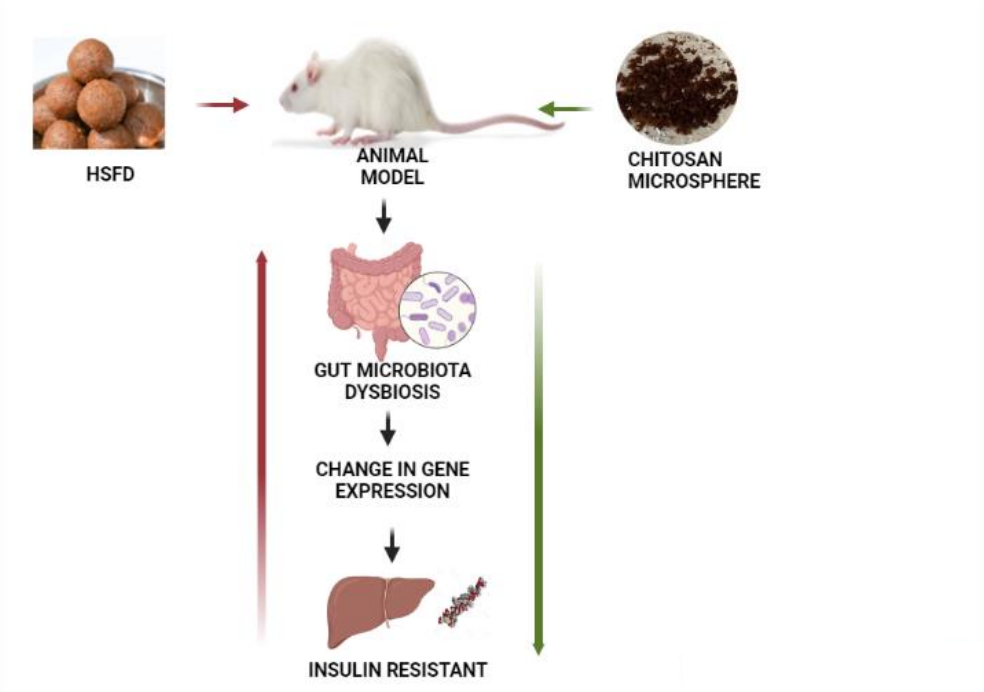
Metformin mainly works by blocking the mitochondrial mediated respiratory chain complexes promoting in lower production of glucose in liver.

Besides an advantageous clinical agent in type 2 diabetes, metformin does not cause any side effects like hypoglycaemia in patients or as such in normal condition, neither effects on body weight gain nor cause any cardiovascular complications.

With the help of metformin, the secretion of insulin remains same but as it works as an insulin sensitizer for insulin resistance, it helps in lowering the fasting insulin level in plasma and improves inflammation (Rena et al.,2017).

HYPOTHESIS

HSFD induces the alterations in composition of gut microflora which leads to inflammation causing insulin resistance in cells. This study aims to evaluate the anti-diabetic role of chitosan microsphere in diet induced T2D. chitosan have many biological properties which helps in restoration of microflora altered in T2D. it also works for restoring the microbial population of gram positive bacteria, thus helps in reduction of inflammation and insulin resistance. So,We are hypothesizing that on administration of the chitosan microsphere the gut microbiota dysbiosis which occurs in type II diabetes can be restored.



OBJECTIVES

1.To prepare the chitosan microsphere and its characterization.

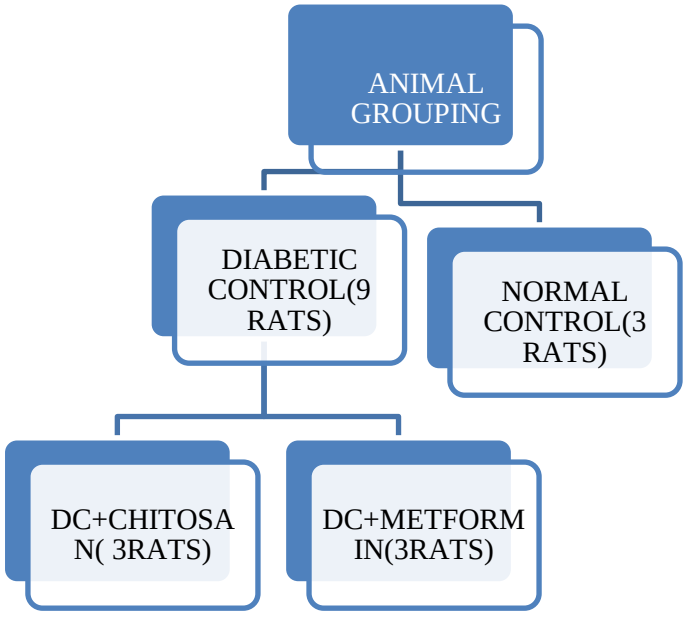
2.To understand the alterations in gut microbiota and diabetic characteristics following administration of chitosan microsphere in diet induced diabetic rats.

MATERIAL AND METHODS

1.Experimental Animals:-

Male Wistar rats of 10-12 weeks of age and weight 250-300 gm were purchased from Zydus Pharmaceuticals and housed at Central Animal Facility, Institute of Pharmacy, Nirma University, Ahmedabad. Proper conditions were provided to the animals with controlled temperature and humidity. Animals were fed with respective diet and water according to their animal grouping. . The animal studies were conducted in accordance with the ethical guidelines for the care and use of laboratory animals of the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad under the CPCSEA guidelines of Ministry of Environment and Forest, New Delhi (Protocol No. IS/PHD/27/2020/032).

2.Experimental groups of Animals-



TOTAL NUMBER OF RATS=12 RATS

Animal protocol no : IS/PHD/27/2020/032

3.Diet Preparation:-

Animals of the diabetic control were fed with 65% sucrose dosing and 4% fructose water for consecutive 3 days and in between 1 day R.O. water provided continuously for 3 months for induction of diabetes and while animal of the control group were given standard chow pellets. Oral Glucose Tolerance Test(OGTT) was carried out after fasting the animal for 12 hours.

Constituents	Normal control diet (prepared for 100gm)	High Sugar Fat Diet(HSFD) (prepared for 100gm)
Corn starch	65gm	0
Wheat bran	5gm	5gm
Dalda	0	150gm
Casein	20gm	20gm
D-methionine	0.3gm	0.3gm
Salt mixture	3.5gm	3.5gm
Vitamin mixture	1gm	1gm
Choline chloride	0.2gm	0.2gm
Sugar	0	65gm

(Jena at al. 2014)

4.Treatment strategy:-

HSFD induced diabetic rats were treated with chitosan microsphere at a dose of regimen 40mg/kg body weight (Prajapati et al,2016)and metformin as an standard anti-diabetic drug was given at a dose of 100mg/kg body weight(Attia et al,2009). Both chitosan microsphere and metformin were given by orally injecting to the diabetic rats in phosphate buffer saline(PBS). The treatment time period was 75 days.

5.Chitosan Microsphere Formulation:-

Appropriate quantity of crude chitosan was dissolved in 2% Glacial acetic acid on keeping it on a magnetic stirrer with continuous stirring for 10 minutes at 27c at 1500 rpm Added this chitosan solution into the paraffin oil along with span 80(work as a surfactant) dropwise and kept on magnetic stirrer at 1500rpm for. Glutaraldehyde which works as a cross linking agent added to the solution dropwise and kept for 3-4 hours on magnetic stirrer.Thechiotsan microsphere were successfully formulated and then collected after washing and filtering with petroleum ether upto 2-3 times. The chitosan microsphere then dried in hot air oven at 55C. After drying, the microsphere were crushed gently and given to the diabetic rats in the form of dose by dissolving in PBS(Phosphate buffer saline) .

6.Administration of Metformin as an standard anti-diabetic drug:-

A group from diabetic control was segregated as diabetic control +metformin group .in which Metformin as standard anti diabetic drug was given at a dose of 100mg/kg body weight of animal during entire treatment time period of 75 days.

7.Body weight and Organ weight:-

Body weight was taken on consecutive 5 days interval during the induction as well as treatment period.at the time of Autopsy Small intestine, Large intestine, Liver and Adipose Tissue of rats in each group were taken and weighed.

8. Fecal sample collection:-

Fresh fecal sample were collected on the basis of 5 days of interval from day one of treatment to at the end of treatment period. Also, colonic fecal (cecal) sample were collected during autopsy and stored at -80°C and were used further for microflora quantification.

9. Oral Glucose Tolerance Test(OGTT):-

OGTT was performed in all groups under fasting condition and diet was removed from the cages for 12hrs prior performing the test to check the blood glucose level and therefore to check whether the animal is diabetic or not. Fasting blood glucose level was estimated and later

according to their body weight 2gm/kg glucose was administered to the animals and estimation of glucose was done by using caresensN glucometer and glucose strip at 0, 30, 60 and 120 min. Area under the curve for glucose ($AUC_{glucose}$) was calculated.

10. Autopsy Schedule:-

Animals were sacrificed of all treatment groups including NC, DC, DC+C and DC+M at 76^{th} day after treatment time period. At the time of autopsy organs such as liver, small intestine, large intestine and adipose tissue were collected. All the organs were stored and preserved at -20°C until RNA isolation. Liver was used for gene expression analysis.

11. Blood collection and serum separation:-

Blood was collected from the animals through puncturing the heart of animal for estimation of biochemical parameters. The blood was then transferred in microcentrifuge tubes, incubated at room temperature for 2-4 hrs and serum was separated by centrifugation.

12.Histopathological analysis-

A small section of liver , small intestine , large intestine and adipose tissue was taken from rats of each group after autopsy and fixed in 10% formalin. A section of 5µm was taken using microtome and slides were prepared and stained with Hematoxylin and Eosin (H.E.)stain and then observed using microscope and digital photographs were taken using Cat-cam 3.0 MP.

13.DNA isolation from fecal sample:-

QIAamp (Qiagen) DNA stool mini kit was used for DNA extraction .following the protocol mentioned on the kit Fecal DNA was successfully isolated.

14.Estimation of Short Chain Fatty Acids (SCFAs) :–

HPLC was used to determine SCFAs (acetate, butyrate and propionate) from fecal samples of animals of each group . 1M phosphate buffer saline was used as mobile phase with pH 7.4. 100 mg fresh pooled fecal samples from experimental rats of each group was homogenized with mobile phase , centrifuged at 10000rpm for 15 min at 4˚C and then filtered using syringe filters in another eppendorf which were then analyzed . the analysis was performed at a flow rate

of1mL at 25˚C and injection volume 10µL. Standard SCFAs (acetate , butyrate , propionate and mix including all three) were used as standards.

15.Biochemical parameters analysis from serum:-

REAGENTS-Accucare serum glucose reagent set , Accucare serum triglycerides reagent set, Accucare direct HDLc reagent set , Accucare direct LDLc reagent set, Accucare SGOT reagent set , Accucare SGPT reagent set.

Serum biochemical parameters such as glucose , triglycerides , HDLc (High Density Lipoprotein cholesterol) , LDLc (Low Density Lipoprotein cholesterol) , SGOT and SGPT were estimated using Accucare reagent kits following the protocol given by manufacturer on kit .serum was separated from blood samples through ultracentrifugation.

16.Microbiota profiling by PCR:-

Specific primers targeting 16 rRNA gene sequence of different bacterial Phylum and genera were used to characterize the fecal microbiota by PCR**.**

Table:-2.16S rRNA gene specific primers for PCR:-

S.No.	Target Groups	Oligonucleotide sequence
1.	*Bacteroidetes*	F-CATGTGGTTTAATTCGATGAT R-AGCTGACGACAACCATGCAG
2.	*Lactobacillus*	F-TGGAAACAGRTGCTAATACCG R-GTCCATTGTGGAAGATTCCC
3.	*Firmicutes*	F-ATGTGGTTTAATTCGAAGCA R-AGCTGACGACAACCATGCAC
4.	*Bifidobacteria*	F-GCGTGCTTAACACATGCAAGTC R-CACCCGTTTCCAGGAGCTATT
5.	*Escherichia coli*	F-CATGCCGCGTGTATGAA R-CGGGTAACGTCAATGAGC

TAKARA PCR Kit was used to run PCR following the protocol mentioned on it by manufacturer.

17.Gene Expression Analysis:-

a. Total RNA Isolation:

Tissue was extracted from animals and then processed to obtain total RNA. 100mg of tissue was taken to the homogenizer tube and 1000µl of Trizol Reagent was added to it and homogenized. The homogenate was transferred to the Eppendorf tube and centrifuged at 12000rpm for 5 min. at 4℃. The upperpart was taken to the 1.5ml Eppendorf tube and then 200µl of chloroform was added to it. It was vortex gently (milky solution obtained) and incubated at room temperature for 5 minutes and then centrifuged again at 12000rpm for 15 minutes at 4℃. The upperlayer (50-100µl) was taken to a other tube (for Adipose tissue, the whole upperpart was taken to a new tube). Chilled Isopropanol of about 0.5-1.0x of the volume was added to the Eppendorf in which supernatant was taken and mixed gently. Tubes were then incubated on ice for 10 minutes and centrifuged at 12000rpm for 10 minutes at 4℃. The upperpart was discarded and the pellet obtained was washed with 100µl of ethanol. The sample was centrifuged at 8000rpm for 5 minutes at 4℃. The supernatant was discarded and the pellet was air-dried to let ethanol evaporate. The pellet obtained was dissolved in 50µl nuclease-free water and then was loaded onto the gel and observed on UV/Vis spectrophotometer. Concentration and purity were checked in Nanodrop and were further used for cDNA synthesis and gene expression studies.

b. Primer designing for gene expression studies:-

Primers for the different genes were designed using Integrated DNA Technologies. Primers for various genes like TLR-2, TLR-4, and NF-κb, were designed. The nucleotide sequence for each gene was blasted with the primer sequence to check the complete alignment of the primer with the mRNA sequence of the respective genes.

Table:-3.Primers for gene expression studies:-

Gene	Forward primer	Tm (°C)	Reverse primer	Tm (°C)
NFκB	CCCCACGAGCTTGTAGGAAAG	66.8	CCAGGTTCTGGAAACTGTGGAT	66.2
TLR2	TGCAGAGCAACGATGGAGAAA	68.1	ACAGGAGCGTCAGGGTGAAG	67.9
TLR4	GGCTGTGGAGACAAAAATGACCTC	68.2	AGGCTTGGGCTTGAATGGAGTC	69.5

RNA was converted to cDNA using reverse transcriptase provided in the cDNA synthesis kit (Thermo Scientific, USA). To check the transcriptional level expression of TLR2, TLR4, and NF-Kb PCR was performed (TAKARA).

c. Densitomtric analysis:-

OmniDOC Gel Documentation Version1.3.3.4 software was used to generate graph of bands observed after PCR for both DNA as well as RNA through that Area Under Curve was calculated and abundance was measured through statistical analysis.

d. Statistical analysis:-

Results were presented as mean ± SE. A statistical difference between the means of thevarious groups were analyzed using a one-way analysis of variance (ANOVA). The statistical significance was then evaluated by T-test using GraphPad prism software (V 5.0).

RESULTS

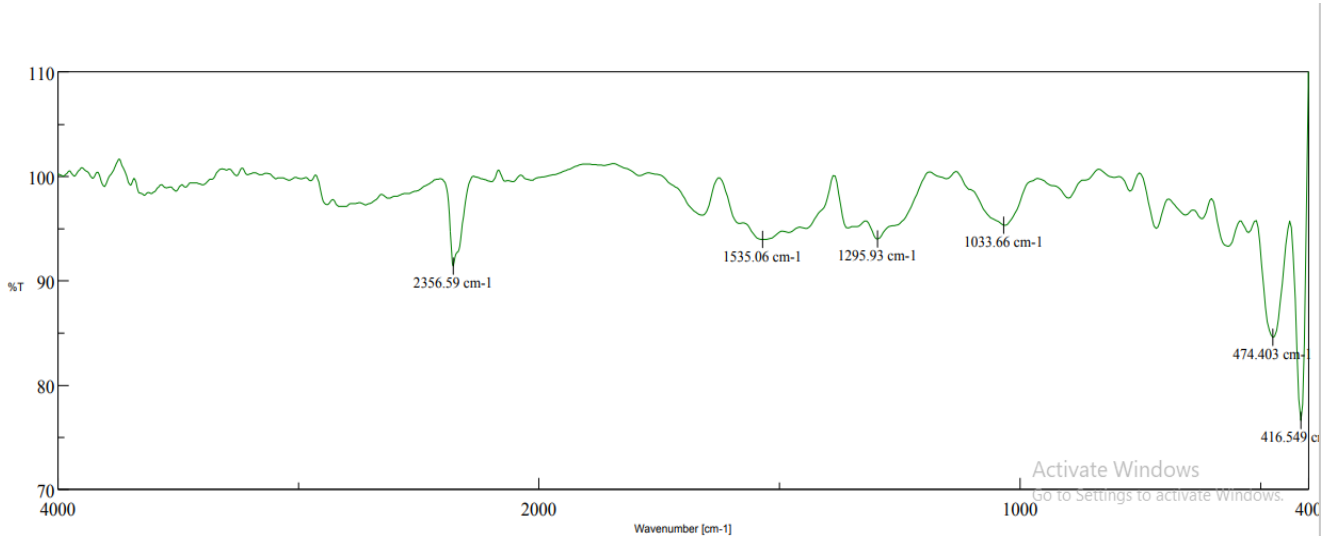
1. CRUDE CHITOSAN AND CHITOSAN MICROSPHERE CHARACTERIZATION:-

a. ZETA Potential and Particle Size Analyze-

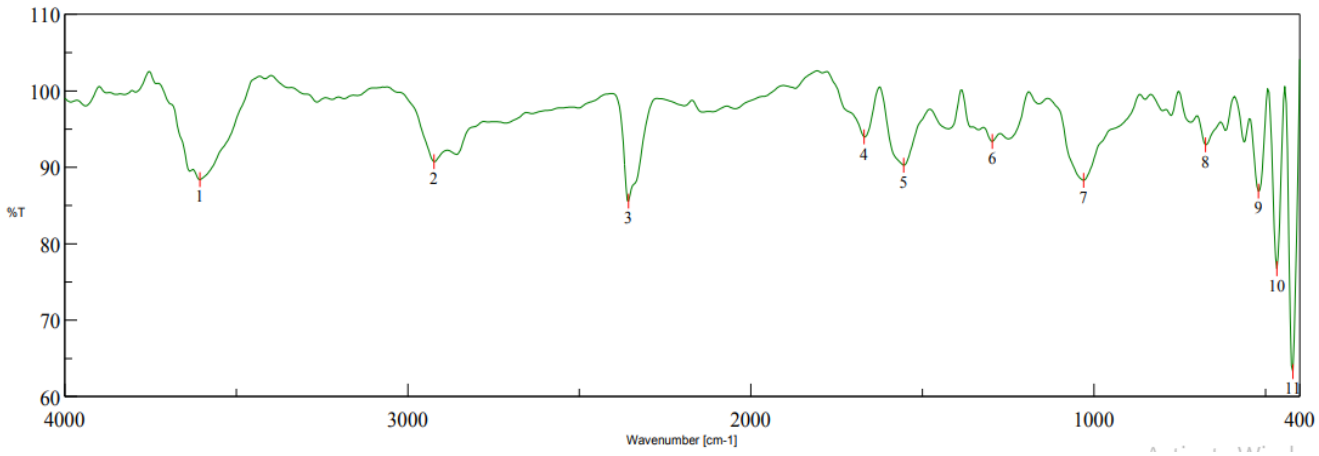
The data of ZETA potential and particle size analysis have shown that the chitosan microsphere is positively charged with size of molecule 24.5nm.

b. Fourier-Transform Infrared Spectroscopy (FTIR)-

The FT-IR was performed to find out possible chemical bonds present in Crude chitosan and Chitosan microsphere .IR spectrum of crude chitosan was characterized by absorption peaks at 2000cm^{-1}(Alkyne), 1500 cm^{-1}(C=C), 2850 cm^{-1}-3300 cm^{-1}(C-H),1000 cm^{-1}-1300 cm^{-1}(C-O),3230 cm^{-1}-3550 cm^{-1}(O-H),2500 cm^{-1}-3300 cm^{-1}(Acids).IR spectrum of Chitosan microsphere was characterized by absorption peaks at 2356 cm^{-1} (NH component),1535.06 cm^{-1}(amide group),1295.93 cm^{-1}(C=N, C=C).



Graph:-1.FT-IR Spectra of Crude Chitosan with Absorption peaks at different wavelengths.

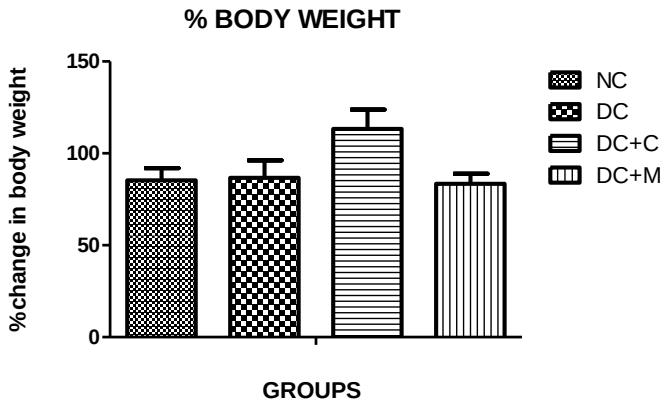


Graph:-2. FT-IR Spectra of Chitosan Microsphere with absorption peaks at different wavelengths.

1 3606.23cm^{-1}, 2 2923.56cm^{-1} ,3 2356.59cm^{-1}, 4 1670.05cm^{-1} ,5 1554.34cm^{-1} ,6 1295.93cm^{-1}, 7 1029.8cm^{-1}, 8 674.963cm^{-1} ,9 520.686 cm^{-1},10 466.689cm^{-1} ,11 420.406 cm^{-1}.

2. CHANGE IN PERCENTAGE BODY WEIGHT :-

No Significant change in body weight of animals of all groups NC,DC, DC+C and DC+M. there was increase in body weight of Chitosan group as compared to DC and NC group may be due to diet intake and obesity.



Graph:-3. % Change in Body weight of animals of all groups. NC-Normal Control, DC-Diabetic Control, DC+C- Chitosan Group, DC+M-Metformin Group.

3.AVERAGE ORGAN WEIGHT:-

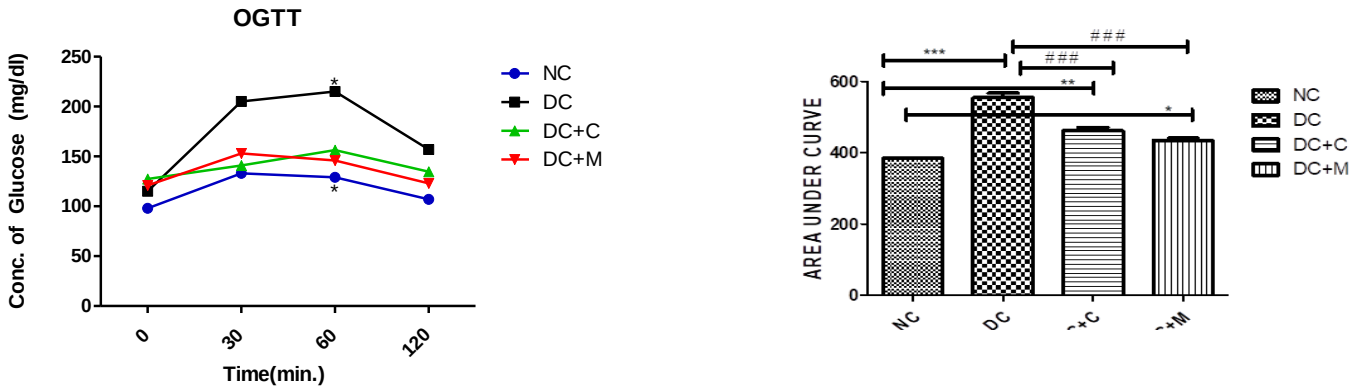
At the time of autopsy Small intestine, Large intestine, Liver and Adipose Tissue of rats in each group were taken and weighed.

ORGAN	NC	DC	DC+C	DC+M
Small Intestine	5.23±1	3.53±1.2	3.83±2	2.40±1
Large Intestine	1.06±0.4	0.96±0.5	1.30±0.5	1.43±0.4
Liver	14.26±1	17.83±3	16.70±0.3	15.40±1
Adipose Tissue	22.16±6	39.63±10	40.56±7	27.93±4

Table 4: Average organ weight. Values are presented as mean± standard error. calculated as n=3/group. Values with different superscripts are significantly different.

4.ORAL GLUCOSE TOLERANCE TEST (OGTT):-

The incremental changes in plasma glucose concentrations of rats after oral glucose administration were recorded. An increase in plasma glucose peaked at 30 min after oral administration of glucose in animals. The increase in glucose concentration of DC group was significantly higher than NC at 60 min. As shown in the graph of AUC the plasma glucose concentration of DC group was elevated approximately 44% as compared to NC group, while treated group showed a decrease in AUC of plasma glucose concentration by 20% and 27%.



Graph:-5.Values are presented as mean calculated as n = 3/group. Values with different superscripts are significantly different. *Compared with NC, #Compared with DC group, ***p<0.001, **p <0.01, *p <0.05, ### p <0.001.

5.BIOCHEMICAL PARAMETERS:-

Plasma glucose, Triglycerides, HDLc, LDLc, SGPT and SGOT

As shown in the table the plasma Glucose level of the Normal Control lies in the normal range and the Diabetic control showed higher than the NC group whereas treatment groups showed an increase in plasma glucose level.

Triglyceride is a blood test that measures the total amount of fatty substances present in the blood, which is quite evident in type II diabetes. The level of triglyceride($p<0.01$) is significantly increased in the DC group as compared to NC group and after treatment of chitosan and metformin, the level of TG is Significantly decreased as compared to DC. The level of LDLc($p<0.001$) is significantly increased in DC group as compared to NC and the treated group showed no significant difference as compared to the control group. The cholesterol and TG levels can be further correlated with insulin resistance. The level of HDLc is marked increase in DC whereas all treated group showed no significant difference as compared with NC. Serum glutamic pyruvic transaminase (SGPT), and Serum Glutamic-Oxaloacetic Transaminase (SGOT) are enzymes that are normally present in liver cells. Both are released into the blood when the liver cells are damaged. There was no considerable change found among all the groups.

S. NO.	BIOCHEMICAL PARAMETERS (mg/dl)	NC	DC	DC+C	DC+M
1	GLUCOSE	142.17±47	255.94±95	373.14±81	313.87±178
2	TRIGLYCERIDES	86.82±15	208.25±43**	130.87±1##	130.57±24##
3	HDLc	93.61±66	108.52±27	129.73±32	92.36±33
4	LDLc	36.71±6	81.47±8***	57.28±8##	40.09±3
5	SGPT	33.23±12	18.43±7	27.20±10	37.16±16
6	SGOT	109±10	92.24±14	89.13±20	143.08±35

Table:-5 Values are presented as mean±Standard Error calculated as n = 3/group. Values with different superscripts are significantly different.*Compared with NC, #Compared with DC group, ***p<0.001, **p <0.01, *p <0.05, ### p <0.001.

6. HISTOPATHOLOGICAL ANALYSIS:-

Histopathological analysis of Large intestine, Small intestine, Liver and Adipose tissue was performed after autopsy to check the effect of HSFD diet , Metformin and Chitosan microsphere administration.We observed from the slides that in Small intestine, the DC group shown the inflammation as the mucosal layer, crypts and Villi are distorted in it as compared to NC's compact layer. In treatment groups the layer is been again in shape with fine crypts and structured Villi.Inflammation has been observed in structure of Large intestine , the Crypts and Goblet cells are distorted and burst out in DC group as compared to fine structure in NC group, again in Chitosan group structure have been regained same in Metformin group.Slides of liver have also shown significant changes in tissue morphology , in DC group the hepatic cells burst out in central vein and kupffer cells have been distorted as compared with fine structured cells of NC group, in treatment groups the cells have regained their original morphology.In Adipose tissue , the Fat cells size is increased in DC group as compared to NC group due to induction of diabetes , obesity and body weight gain. The fat cells size has been increased in Chitosan and Metformin group too.

Figure:-10.Histopathological analysis of Small intestine, Large intestine , Liver and Adipose

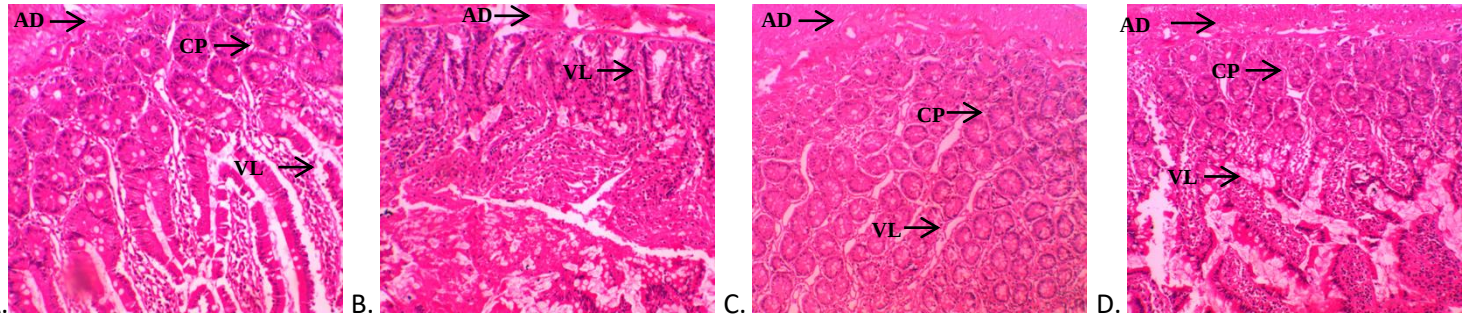


Figure:- SMALL INTESTINE: A.NC ,B. DC, C.DC+C, D. DC+M . AD- Adventitia, CP-Crypt , VL- Villi.

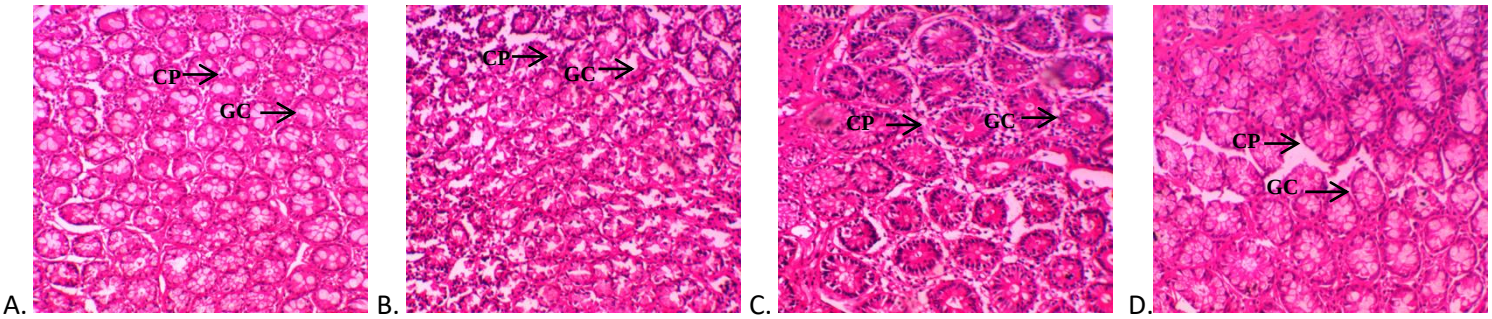


Figure:- LARGE INTESTINE: A.NC , B.DC, C. DC+C , D. DC+M. CP-Crypt, GC- Goblet Cell.

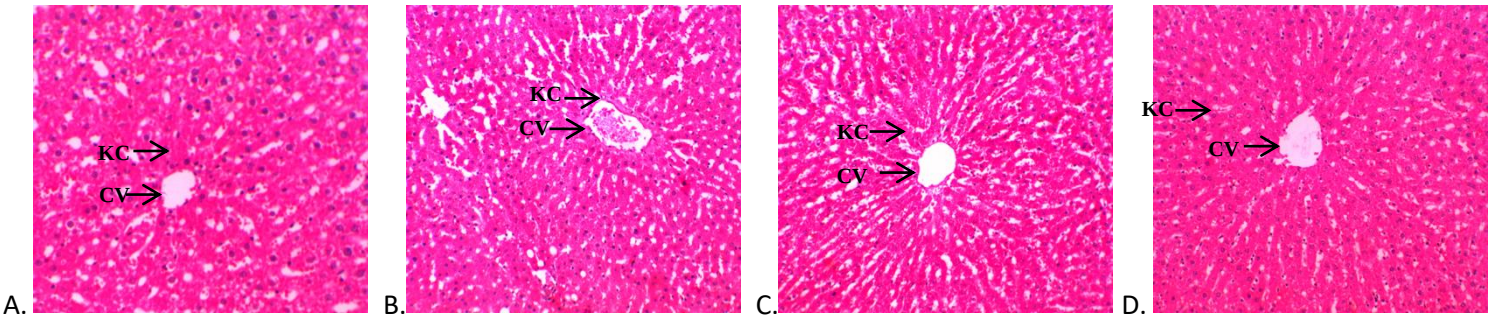


Figure:- LIVER: A.NC ,B. DC, C. DC+C, D. DC+M. KC-Kupffer Cell, CV-Central Vein.

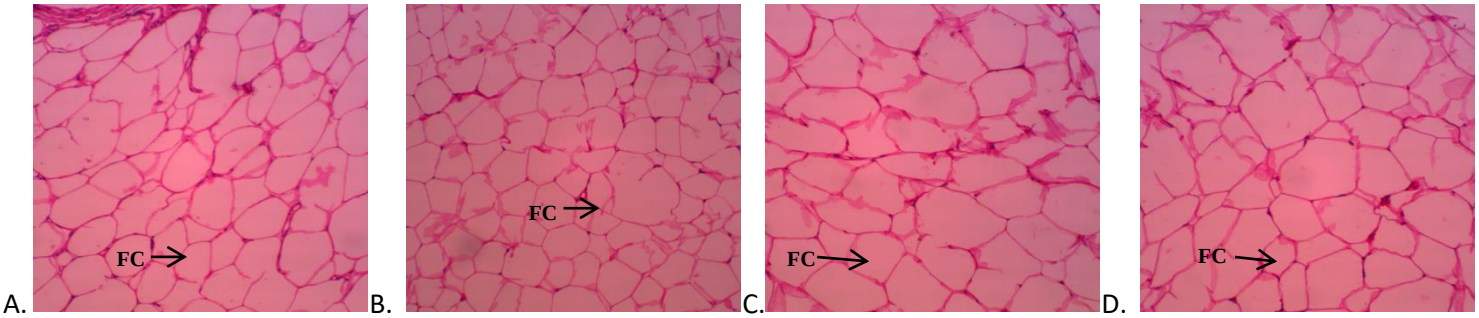


Figure:- ADIPOSE TISSUE: A.NC , B. DC, C. DC+C, D. DC+M. FC- Fat Cell.

7.GENE EXPRESSION STUDIES-

Gene expression studies were done for Liver. TLR-2, TLR-4 and NF-KB expression was checked using Densitometric analysis .TLR-4 is a receptor for lipopolysaccharide present in cell wall of Gram Negative Bacteria checked in Liver as wells as TLR-2 and NF-KB expression was also checked , TLR-2 is a receptor for peptidoglycan present in cell wall of Gram Positive bacteria and NF-KB induces various pro-inflammatory responses but no results was obtained.

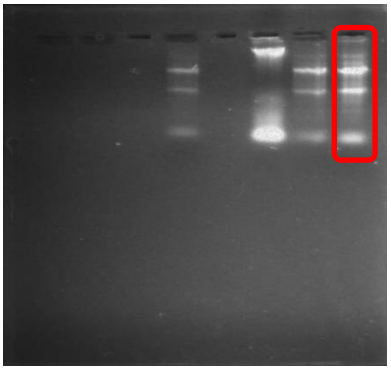
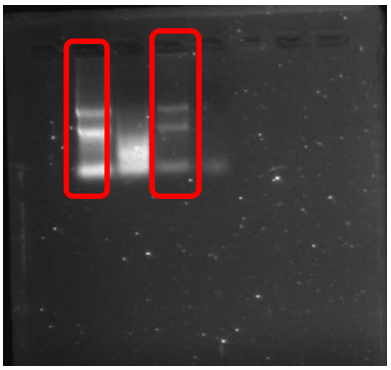
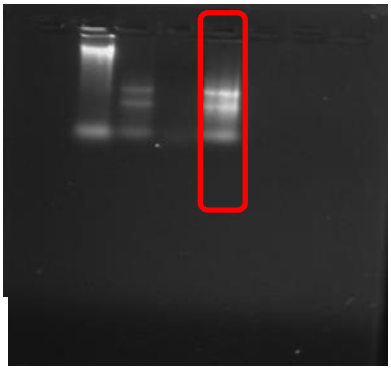
A  B.  C. 

Figure:-11.RNA isolation from NC, DC, DC+C and DC+M groups. A. lane-8 RNA bands of NC, B. lane-3 RNA bands of DC, lane -5 RNA band of DC+M , C. lane-4 RNA bands of DC+C.

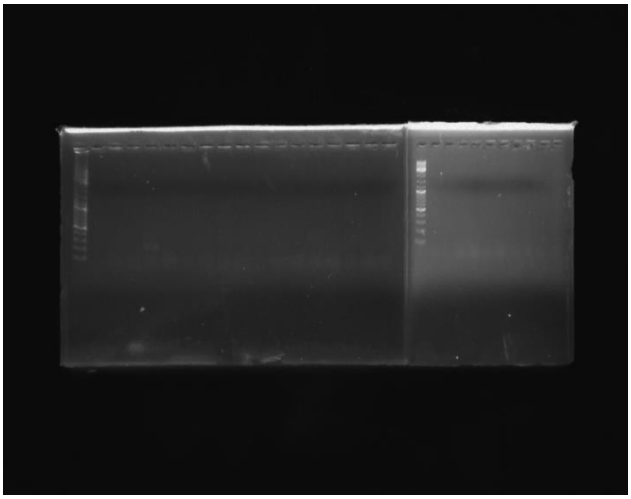


Figure:-12. Gene expression checked for TLR-2, TLR-4 and NF-KB gene from cDNA from Liver. Liver.Lane-1 ladder, Lane 2-7 TLR-2, Lane8-14 NF- KB ,next gel- TLR-4.No results were obtained.

8. MICROBIOTA PROFILING-

The common population of gut microbiota was examined from a fecal sample taken at the end of the study. Our data showed an increase in the number of *Bifidobacterium*and *E.coli* in the treatment group as compared to the Diabetic control.

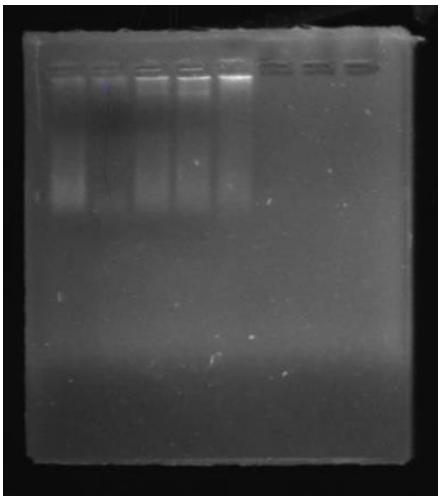
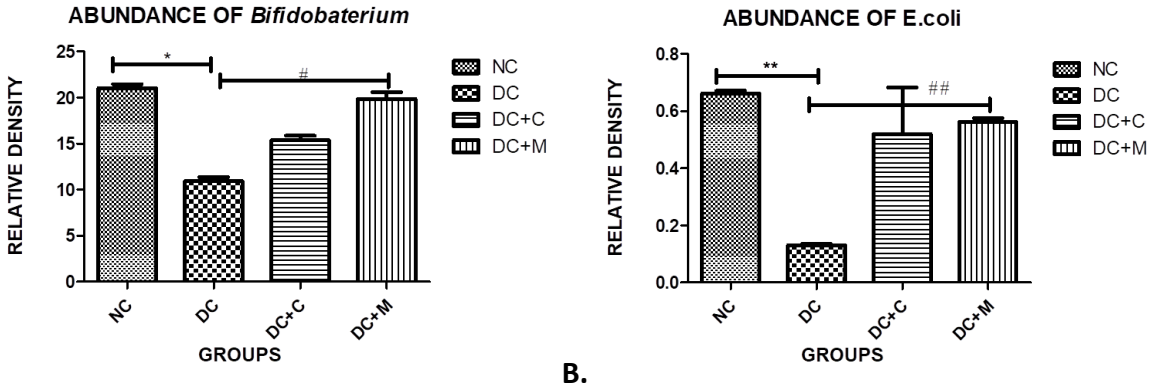


Figure:-13. DNA ISOLATION FROM POOLED FECAL SAMPLES OF NC, DC, DC+C, DC+M. Lane:1 DNA band of NC, Lane:3 DNA band of DC, Lane:4 DNA band of DC+C, Lane:5 DNA band of DC+M.

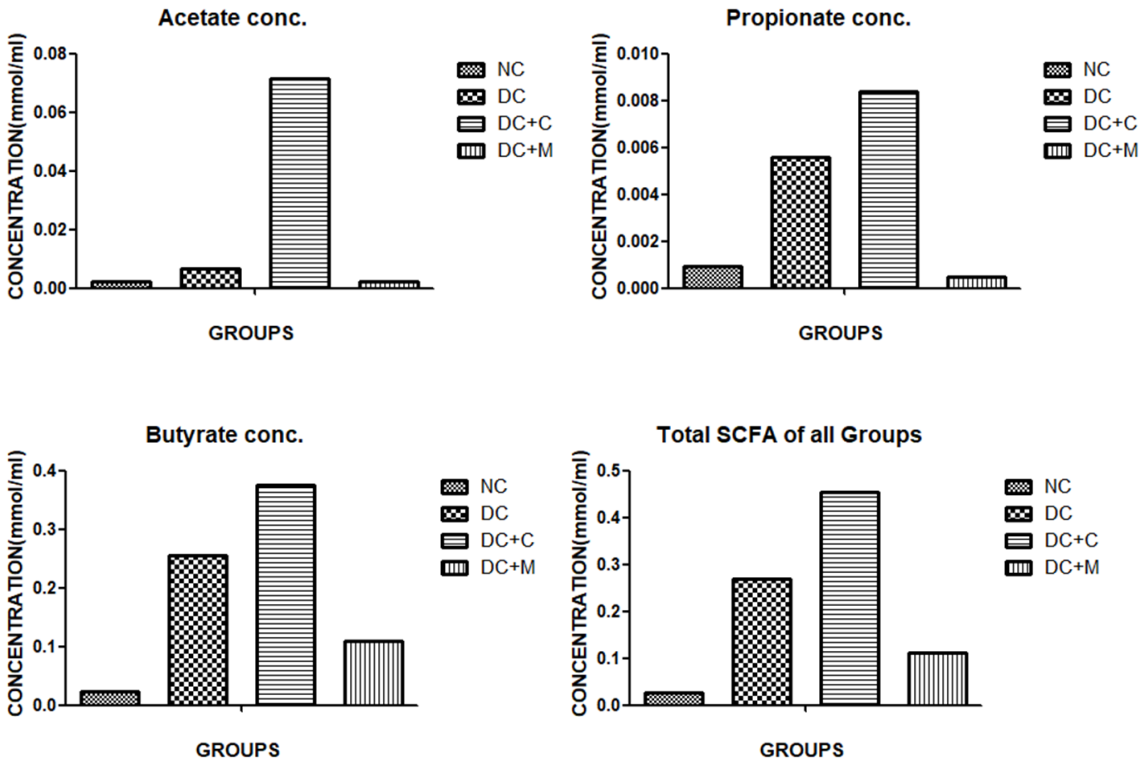


Graphs:-6. A. Abundance of*Bifidobacterium*, B. Abundance of *E.coli.* above mentioned graphs shows the abundance of bacteria in fecal samples.Values are presented as mean,Values with different superscripts are significantly different.*Compared with NC, #Compared with DC group,*$p<0.05$, **$p<0.01$,$^{\#}p<0.05$,$^{\#\#}p<0.01$.

9.SHORT CHAIN FATTY ACIDS (SCFAs) ANALYSIS-

The fecal samples from each treatment group collected prior to autopsy and the cecal collected from LI during autopsy used for estimation of SCFA by HPLC. Based on standard sample peak, retention time and area of peak , samples were analyzed . the concentration of Acetate, Propionate and Butyrate in mmol/ml was calculated and plotted in bar graph.

Acetate , Propionate and Butyrate are major SCFA's produced by gut microflora. Their level shows the activity of microbial population. Butyrate is higher in treatment group i.e. Chitosan Group and in comparison to it, Acetate and propionate concentration is low as compared to butyrate concentration in treatment group shows the increase in number of gram positive bacteria producing Butyrate.



Graph:-7. HPLC of SCFA's from fecal content. Data represents the concentration (mmol/ml) of Acetate, Propionate and Butyrate in NC , DC, DC+C and DC+M groups.

DISCUSSION

The present study focuses on treatment of T2D with the help of Chitosan Microspheres (CM), a prebiotic. T2D, a chronic disorder characterized by decreased sensitivity towards insulin of targeted organs or in simple words cells becomes insulin resistance (IR). IR is also associated with obesity (Ma et al., 2019). Recent studies have shown along with modern lifestyle, environmental factors, genetic factors and dietary factors gut microflora also contributes to T2D occurrence and its development. In our study Male Wistar rats were used. Diabetes was induced by HSFD .Administration of HSFD increased the body weight by accumulation of adipose and increase in blood glucose concentration. Long-term elevated level of glucose triggers abnormal metabolic syndrome including obesity, cancer, gastrointestinal diseases, various neurological diseases and CVD (Schwartz and Porte,2005). According to the overall mechanism, macrophages and kupffer cells will decrease the sensitivity of insulin by secreting pro-inflammatory cytokines that activates the threonine kinases named as C-Jun N-termial kinase(JNK) and Inhibitor of kappa-B(IkB) kinase that leads to impaired signaling of insulin(Lackey and Olefsky,2016). But accumulation of lipid in liver targets the unfolded protein response(UPR) signaling pathway, which plays an important role in development of insulin resistance by altering the Hepatokine secretion. In addition, elevated level of the pro-inflammatory cytokines such as TNF-α, IL-6 and monocyte chemotactic protein 1 are also responsible for the insulin resistance development because they activates the JNK and IKK and causes the β-cells functioning failure resulted in insulin resistance.

In order to restore insulin sensitivity, microbes present in the gut such as the gram positive bacteria has cell wall of peptidoglycan induces activation of TLR-2 receptors that stimulates anti-inflammatory cytokines and chemokines. For example R.intestinalis can increase IL-22 production which is an anti-inflammatory cytokine that known to restore the gut microflora and insulin sensitivity. This IL-22 also promotes T-cell regulatory differentiation, that induces TGF-β and suppress the intestinal inflammation in the gut.

Due to various anti-diabetic properties of chitosan, we have formulated chitosan microsphere for colon targeted drug delivery system to prevent the degradation of CM by the microflora present in the gut and colon(Bansal et al.,2011). Before administration of formulated CM,

characterization of CM was done using FT-IR. After that, we got the possible chemical interactions among the polymers and it was appropriate and safe as a drug to treat the diseased condition. Recent study have suggested that the body weight in diabetic condition increases due to accumulation of adipose leads to obesity and administration of CM decreases body weight because of its anti-obesity property. In our results the body weight is increased in treatment group (Chitosan group) reasons are not clear but may be due to diet intake and at the time of animal grouping the rats grouped for treatment were initially of higher body weight.

OGTT was performed to test impaired glucose tolerance (IGT) which is one of the most important T2D diagnostic parameter. Our results shows an increase in level of blood glucose on administration of HSFD in DC group. On administration of Chitosan microsphere the blood glucose level significantly decreased , elevated by HSFD in DC+C group.similar results observed in DC+M group , the blood glucose level decreased.these results indicates that administration of CM improves glucose tolerance and it prevents the hyperglycemic condition in HSFD induced diabetic rats.

In our study, the serum biochemical profiling was done and we observed that administration of chitosan have shown the significant changes in TG and LDL level in treatment group. The level of TG has been significantly decreased as compared to DC, this is mainly because diabetic condition is associated with vascular destruction which is caused by the increased oxidative stress, thus the free-radical production will increased and improve the diabetic condition. The level of LDLc has been significantly decreased in treatment group as compared to DC. This clearly indicates that chitosan have significant effects on various biochemical parameters that are mainly responsible for causing T2D.According to a report, this may be possible because after giving CM to the animals, chitosan increased the fecal bile acids excretion and decreased the cholesterol level by enhancing the hepatic liver LDL receptor mRNA (Xu et al,2007).

Liver plays a pivotal role in synthesis of proteins, detoxification of body and storage of compounds forms in body. In addition, it also plays important role in metabolism of carbohydrates and lipids .Histopathological analysis shows the damage to hepatic cells, kupffer cells and central vein in DC group due to inflammation as compared to NC group. Chitosan administration reduced this damage to liver by reducing the inflammation ,positive results have been observed in DC+C group as the kupffer cells regained their original morphology which is

quite visible in our results, similar results have been observed in DC+M group due to administration of Metformin. Histopathological analysis of Small intestine shows more damage to villi , crypts and adventitia in DC group as compared to intact villi ,crypts and fine structured adventitia in NC group. In DC+C group the damage to structure have been restored through administration of CM indicating its positive effects on small intestine through microflora alterations. Similar restoration results observed in DC+M group. Colonic goblet cells have also been distorted in DC group but in DC+C such damages have been prevented in Large intestine(Jena et al.,2014). It is reported in T2D that the expression of TLR-2, TLR-4, and NF-kB increased followed by inflammation (Dasu et al. 2010). In our study, we performed gene expression studies but we did not get the results one of the reasons is that we did not have sufficient time to check the exact melting temperature (Tm) of primers by doing gradient PCR we set the temperature of PCR cycle according to the theoretical calculation.

But According to our results of the histopathology study, we observed in the SI, LI, and LIVER the cell shapes were distorted in DC is restored in treatment groups. So according to that, if we have got the results of gene expression studies so we can state the confirmation of the effective treatment. The gut microbiota plays a critical role in human health and illness. The

probiotics, prebiotics, or postbiotics to modify the commensal microbial community might be a potential therapeutic strategy. It is reported the consumption of HSFD increases the Firmicutes and Proteobacteria and decreases the Bacteroides as well as the dominant probiotic strains such as *lactobacillus* and *bifidobacteria*. Firmicutes are more efficient than Bacteroidetes at generating energy from food and this energy is used by the body and stored as fat. The Bacteroides are mainly metabolites proteins to generate energy that's why it decreases in HSFD consuming individuals. The increased gram-negative bacteria (proteobacteria) increase the level of endotoxin (LPS) in plasma circulation leading to insulin resistance (Prajapati, Rajput, Kumar Jena and Seshadri, 2015)

In our study, we observed the treatment group has increased the number of *Bifidobacterium* as compared to the DC. Demonstrating that chitosan (prebiotic) has health benefits for diabetes and *E.coli*increasedin the treatment group. From the results, we state that chitosan increases the

number of probiotics but in the given time we were not able to perform major gut microbiota profiling if we get more time we could confirm the effectiveness of treatment.

Intestinal microflora plays an important role in development of T2D. the disturbance in microflora population number in diabetic condition leads to significant lowering down of microflora stability .Researchers have shown that reduction of beneficial bacterial population or probiotics such as lactic acid producing bacteria in gut and *Bifidobacterium* is related to abnormality in glucose tolerance which hinders the absorption of glucose by cells and energy production, simultaneously supports accumulation of fat in body and occurrence of diabetic condition(Ma et al., 2019). Intestinal microflora produces organic carboxylic acids i.e. Short Chain Fatty acids (SCFAs).these are the products of microbial fermentation of undigested carbohydrates and dietary fibres. Primarily produced SCFAs are acetate, propionate and butyrate and major producers of these in gut are Firmicutes and Bacteroidetes. Amongst these three, acetate is most abundant SCFA in gut .these SCFAs plays most important role in human health and diseases. They are involved in metabolic pathways and energy production. SCFAs production and associated microflora producing the same are directly related to T2D condition. According to researches done till now, in normal condition the concentration of Butyrate is higher than acetate and propionate and Firmicutes(gram positive) producing same their population is also high and Bacteroidetes (gram negative) number is low and in diabetic condition the ratio changes Bacteroidetes increases and Firmicutes decreases along with other bacterial population producing butyrate . in our results it is observed that in DC group the concentration of acetate and propionate is low which may be due to there are many bacterial strain (gram negative) other than the one which we have used in our study producing the same and we haven't studied all but the butyrate concentration have been increased in treatment group showing positive results indicating the restoration of gram positive population.

CONCLUSION

The rationale of our study was to formulate a chitosan microsphere and the Evaluation of the Reversal potential of the Chitosan Microsphere in diet-induced T2D. The chitosan microsphere was formulated successfully and it has a positive charge with the size of a molecule of 24.5nm.

From the results of the second objective, we can observe that the administration of low molecular weight chitosan could increase the proportion of Butyrate. Alongside, Butyrate improves insulin resistance by modulation of gut microbiota and increases the production of anti-inflammatory cytokines (TLR2). The profiling of gut microbiota and gene expression study can also help us to conclude the effectiveness of chitosan treatment against T2D. Hence, we conclude that the treatment strategy with chitosan might work against T2D. The results obtained are not sufficient to confirm the reversal strategy as our gut microbiota and gene expression study haven't been completed and the serum biochemical parameters do not show significant results. If the time period of the study is prolonged.it might show the expected outcomes.

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