Chapter III

Materials and Methods

3.1: Drugs and consumables

Linezolid and cefdinir in the form of active pharmaceutical ingredient (API) were obtained as gift samples from Macleod Pharma (Mumbai, India). Moxifloxacin API was kindly gifted from Unimark Pharma (Ahmedabad, India). Synthesized oligonucleotides (IDT, USA), emerald green PCR master mix (Takara, South Korea), cDNA synthesis kit (Thermo Scientific, USA), QIAamp Stool DNA Kit (Qiagen, Germany), biochemical reagent kits (Accucare Ltd), SYBR green master mix (Takara, South Korea) were purchased. Real time PCR compatible 96 well plates and optical adhesive films (Applied Biosystems, USA) were used.

3.2: Diets and experimental animals

3.2.1: Diet preparation

Experimental diet included normal rodent diet (CD) (Amrut agro foods, Mumbai) for control group having 65% of the starch along with many other essential components. High sucrose diet (HSD) contains 65% of sucrose instead of starch was prepared in house for the induction of diabetes **(Jena et al, 3817).** The composition of experimental diets is presented in Table 3.1**.**

Table 3.1: Experimental Diet Composition

Ingredients (%)	**Control diet (100 gms)**	**High Sucrose diet (100 gms)**
Starch	65	0
Sucrose	0	65
Casein	20	20
Groundnut Oil	5	5
Wheat Bran	5	5
Mineral Mix	3.5	3.5
Vitamin Mix	1	1
D-Methionine	0.3	0.3
Choline Chloride	0.2	0.2

3.2.2: Experimental Animals

Around 8 to 10 weeks old healthy male wistar rats, weighing about 150–200 gms were purchased from the animal research facility (Cadila Pharmaceutical Limited, Ahmedabad, India) with the approval of the Institutional animal ethics committee with protocol no (IS/BT/PhD11-12/1004) following CPCSEA guidelines. They were maintained in the animal house of institute of pharmacy (Nirma University, India). All experimental animals having similar weight were segregated randomly. The animals were divided into six different experimental groups with 4

animals each which is as follows: non-diabetic group with CD, diabetic group with HSD, diabetic group HSD along with linezolid (10 mg/kg), diabetic group HSD along with cefdinir (10 mg/kg), diabetic group HSD along with moxifloxacin (8 mg/kg) and diabetic group HSD along with chitosan polymer (60 mg/kg). The antibiotics dosage was determined by following the US Food and Drug administration (USFDA) manuals. The chitosan was selected due to its prebiotic properties and its dose was determined based on their antimicrobial activity (Dose which gives optimum antimicrobial activity). The detail experimental groups are mentioned in **Table 3.2**. Animals were fed their respective diets and antibiotic APIs for 80 days. Body weight gain was observed weekly and presented in form of final change in body weight.

Table 3.2: Experimental animal groups

Group Name	Specification	Dose	No. of animals
Control Diet (CD)	Normal Rodent diet	-	4
High Sucrose diet (HSD)	High sucrose diet (65%)	-	4
HSD+GP	High sucrose diet+ Linezolid	10 mg/kg	4
HSD+GN	High sucrose diet+ Cefdinir	10 mg/kg	4
HSD+BS	High sucrose diet+ Moxifloxacin	8 mg/kg	4
HSD+CS	High sucrose diet+ Chitosan	60 mg/kg	4

3.3: Oral glucose tolerance test (OGTT) assay

Animals were subjected to an oral glucose tolerance test OGTT) assay one day prior to the completion of the experiment. OGTT was performed after 12 hrs of the fasting. Briefly, after overnight fasting, animals were given a glucose load (2 g/kg) orally. Blood samples were collected from the animal tail vein at 0 min (before glucose administration), 15, 30, 60, 90 and 120 min after glucose administration. Glucose concentration was determined using a CareSensN

blood glucose monitor and glucose strips. Area under the curve for glucose ($AUC_{glucose}$) was calculated using the trapezoidal rule **(Harlass et al, 147).**

3.4: Blood, fecal and tissue sample collection

The experimental animals were sacrificed at the end of the study by euthanasia method. Blood was collected through heart puncture, from which serum and plasma were separated. Fecal samples were collected a day before euthanasia from all the animals and stored at -80ºC until DNA isolation. The liver, adipose tissue, proximal small intestine and colon from each animal were excised, weighed, dried and stored at -80ºC until further analysis.

3.4.1: Blood biochemical analysis

Approximately, 1 ml of the blood samples was collected from the retro-orbital plexus of the animals under the mild anesthesia using diethyl ether. Blood was then transferred to the microfuge tubes for separation of serum at 0 day (start of the experiment) and the last day of the experiment. The fasting blood glucose, total cholesterol (TC) and triglycerides (TG) levels were analyzed from the plasma using reagent kits (Accucare, India). The liver pathophysiology evaluations were carried out by estimating the biomarkers, such as ALT (Serum Glutamate Pyruvate Transaminase, SGPT) and AST (Serum Glutamate Oxaloacetate Transaminase, SGOT) from serum samples using reagent kits (Accucare, India).

3.4.2: FFAs and LPS estimation

The plasma FFAs were estimated using chloroform and the triethyalanomine extraction method by measuring the absorbance at 440 nm. The stearic acid was used as standard fatty acid and detailed protocol was performed as mentioned in **Yadav et al (69).** LPS, an important integral part of the outer membrane of the Gram negative bacteria is a crucial component for the initiation of the inflammatory signals for the pathogenesis of the T2D. The LPS from the plasma samples of the experimental animals was analyzed using Pierce LAL Chromogenic Endotoxin Quantitation Kit, USA as per the protocol mentioned by the manufacturer.

3.4.3: Microflora estimation by culturable method

The gut microbial diversity in the fecal samples of the test animals was determined using culturable method using various media. For quantification of microflora, 1gm fecal sample was suspended in 10 ml of saline solution, homogenized and 10 fold serial dilutions were prepared. The appropriate dilutions were spread plated onto to different selective agar medium, such as MRS medium for *Lactobacillus* and Bifidobacterial agar for *Bifidobacterium* spp.; MacConkey agar for *Escherichia coli* and SPS agar for *Clostridium* spp. The plates were incubated either anaerobically or aerobically for 48 h at 37°C. The resultant colonies were counted in CFU/gm of feces.

3.4.4: Fecal collection and DNA extraction

Fresh fecal samples were isolated from each experimental animal and stored at -20°C for DNA isolation. DNA extraction from 200 mg of fecal samples was performed using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the instructions given by the manufacturer. The DNA quantity was determined by taking the absorbance at 260 nm (A_{260}), while quality was estimated by determining the A_{260}/A_{280} ratio with a nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

3.4.5: Standard bacterial strains

Standard strains of *Lactobacillus casei* (MTCC 1423), *Escherichia coli* (MTCC 443) and *Clostridium perfringes* (MTCC 450) were obtained from the microbial type culture collection (MTCC), whereas, *Bifidobacterium bifidum* (NCDC229) was obtained from the national collection of dairy culture. The strain of *Bacteroides vulgatus* (ATCC 25285) was obtained from the american Type Culture Collection. The standard strains were used for construction of standard curves for qPCR. Anaerobic culture methods were maintained by using O_2-free anaerobic glass chamber with air absorbing capsules (Himedia, USA) for the cultivation and maintenance of *Clostridium perfringes.*

3.4.6: Microbial Quantification by Real-time qPCR

The specific 16s rDNA primers targeting the different bacterial phyla and genera were used to quantify the fecal microbiota by real-time qPCR (Table 3.3) **(Ritchie et al, 145).** Real-time qPCR experiments were performed in applied biosystems 7500 Fast Real-Time PCR system using the SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus) from TAKARA Bio Inc, Japan. Each qPCR reaction was carried out in duplication with a final volume of 20 µL containing 1 µL of each fecal

isolated DNA and 10 μM of each primer as shown in (Table 3.3). The thermal cycling conditions used were as follows: an initial DNA denaturation step at 95ºC for 30 sec, 40 cycles of denaturation at 95ºC for 3 sec, primer annealing and extension at 60ºC for 30 sec.

Standard curves for all the bacteria were constructed for each experiment using serial tenfold dilutions of isolated bacterial genomic DNA (of known concentration) from pure cultures, corresponding to 10^1–10^{10} 16S rRNA gene copies/ gram of feces. The specific bacterial concentration of each fecal sample was calculated by comparing the Ct values obtained from the standard curves with the applied biosystems 7500 Fast Real-Time PCR System software v2.0.6. S The copy numbers of bacterial genome was calculated using the avogadro constant and assuming the mean molecular weight of a base pair to be 660 g/mol. The data presented are the mean values of duplicate qPCR analysis.

Table 3.3: 16S rDNA gene specific primers for bacterial quantification

Primers	Orders	Sequence	Amplicon size (bps)
		Phylum	
Firmicutes	Forward	ATGTGGTTTAATTCGAAGCA	126
	Reverse	AGCTGACGACAACCATGCAC	
Bacteroidetes	Forward	CATGTGGTTTAATTCGATGAT	126
	Reverse	AGCTGACGACAACCATGCAG	
Proteobacteria	Forward	CATGACGTTACCCGCAGAAGAAG	195
	Reverse	CTCTACGAGACTCAAGCTTGC	
		Genus	
Lactobacilli	Forward	TGGAAACAGRTGCTAATACCG	224
	Reverse	GTCCATTGTGGAAGATTCCC	
Bifidobacteria	Forward	GCGTGCTTAACACATGCAAGTC	136
	Reverse	CACCCGTTTCCAGGAGCTATT	
Clostridia	Forward	GCACAAGCAGTGGAGT	239
	Reverse	CTTCCTCCGTTTTGTCAA	
Escherichia	Forward	CATGCCGCGTGTATGAA	126
	Reverse	CGGGTAACGTCAATGAGC	

3.4.7: Bile salt hydrolase (BSH) enzyme bacterial quantification

Feces (0.1 gm) was used to extract bacterial genomic DNA using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) and quantified by the Nanodrop (Nanodrop Technologies). The BSH specific 16S rDNA primer was used for Real time qPCR **(Kim et al, 782).** After qPCR, a dissociation step was included to analyze the melting profile of the amplified products. In

parallel, qPCR was performed using ten-fold serial diluted synthetic DNA fragment containing BSH gene with known concentrations. The sample DNA concentrations were calculated using standard curves of the diluted synthetic DNA fragment.

Primer	Order	Sequence	Amplicon size (bps)
BSH	Forward	AGTCCATATGTGCACAGCCGTTCGTTTTG	305
	Reverse	AGTCAAGCTTCACTTGCTCTGCAACTGG	

3.4.8: Estimation of SCFAs (metabolites)

The SCFAs from fecal samples of experimental animals (acetate, propionate, and butyrate) were determined by HPLC analysis (ZORBAX, eclipse plus Phenyl-Hexyl, Agilent, USA) **(Cleusix et al, 59).** 0.1 M phosphate buffer solution having pH 2.5 was used as a mobile phase. Fresh fecal samples from experimental animals were homogenized with mobile phase, centrifuged at 10000 RPM for 15 min at 4°C and filtered through a 4mm HPLC filter with a 0.45 mm nylon membrane (Millipore) directly in vials, which were immediately preserved and analyzed. The analysis was performed at a flow rate of 0.5 mL/min at 25 °C, with 10 mM H_2SO_4 as eluent and an injection volume of 50 µL. The mean metabolite concentrations were expressed in millimolar (mM). Standard fatty acids were used as a reference. The sodium acetate, sodium propionate and sodium butyrate (Sigma-aldrich, USA) were used as internal standard for the SCFAs estimation.

3.4.9: Tissue Biochemistry

3.4.9.1: Liver glycogen

After the euthanasia, liver from each experimental animal was dissected out, washed with physiological buffer or saline solution, blotted dry and weighed before being used for tissue biochemical analysis for glycogen, total cholesterol and triglycerides. The liver glycogen was determined as described by **(Vander-Vies, 413).** In brief, liver tissue (200 mg) was finely grounded with 20 mL of 5% trichloroacetic acid in a homogenizer. The clear filtrate was taken for analysis by filtering out the protein precipitates. Then filtrate was analyzed with anthrone reagent. The colour was developed and the OD was read within 2 hrs at 650 nm.

3.4.9.2: Liver total cholesterol

Liver cholesterol content was determined after homogenization with folch solution (chloroform/methanol ratio = 2:1) **(Robinet et al, 3366).**

3.4.9.3: Liver triglycerides

The lipids in the frozen liver were extracted according to **Robinet et al, (3377).** Triglyceride was first hydrolyzed in a basic solution (0.5N KOH in ethanol) and then measured using commercial enzymatic triglyceride analysis kit **(Mendenhall, 179).**

3.4.10: Gene expression using Real time PCR

Total RNA was isolated from fresh tissue samples (proximal small intestine, colon, liver, and adipose tissue) using TRI reagent (Sigma-Aldrich, USA), according to the manufacturer's protocols. RNA was quantified by determining optical density at 260 nm, while purity was determined by calculating the ratio at 260 nm and 280 nm using nanodrop. The mRNA expression of various inflammatory genes, such as TLR2, TLR4, NLR1, NLR2, NF-κB, TNF-α and IL-6 was performed. The mRNA expression of glucose transporter, such as GLUT4 and incretins, such as GLP-1 and DPP-4 were also analyzed. Gene expression analysis of GPCRs such as GPR43 was also studied. The mRNA expression of various adipokines, such as leptin, adiponectin and resistin was also analyzed from the adipose tissue. The expression of PPARs such as PPAR-α and PPAR-γ was also performed. Reverse transcription (RT) was performed using a first strand cDNA synthesis kit (Thermo Scientific, USA) according to the mentioned protocol. β-actin was served as an internal control. The primer sequences are shown in Table 3.4 **(Kurland et al, 569).**

The RT-PCR reaction was performed on Applied Biosystems 7500 Fast Real-Time PCR System to check the relative expression using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio Inc, Japan). Relative quantification of gene expression was performed by using the comparative threshold cycle method ($\Delta\Delta$CT), in which the expression level of each target gene was normalized to the levels of β-actin. Data output was expressed as fold changes of mRNA expression levels.

Table 3.4: mRNA specific primers for gene expression analysis

Primers	Accession Number	Sequence	Amplicon size (bp)
TLR2	NM_198769	F-TGCAGAGCAACGATGGAGAAA	222
		R-ACAGCAGCGTCAGGGTGAAG	
TLR4	NM_019178	F-GGCTGTGGAGACAAAAATGACCTC	272
		R-AGGCTTGGGCTTGAATGGAGTC	
NLR1	XM_575485	F-GCTCATCCGGACCAAAACTA	189
		R-CTGCCCAGGTTTTCATTGTT	
NLR2	XM_226330	F-ATCCCTCGGTTACTATGTTG	241
		R-GCTTCCTGAATACTCCTCCT	
NF-κB	NM_199267.2	F-CCCCACGAGCTTGTAGGAAAG	130
		R-CCAGGTTCTGGAAACTGTGGAT	
GLUT4	D28561.1	F-GGGCTGTGAGTGAGTGCTTTC	150
		R-CAGCGAGGCAAGGCTAGA	
GLP-1	NM_012728.1	F-TCTCTTCTGCAACCGAACCT	350
		R-CTGGTGCAGTGCAAGTGTCT	
GPR43	U92802.1	F-TCACCTGGATGAGCTTCGAC	359
		R-GACAAGGACCACTGCGAAGA	
DPP-4	NM_012789.1	F-TTGTGGATAGCAAGCGAGTTG	140
		R-CACAGCTATTCCGCACTTGAA	
Leptin	NM_013076	F-GGTCCAAGAAGAAGAAGACC	191
		R-GAAATGTCATTGATCCTGGT	
Adiponectin	NM_144744	F-AATCCTGCCCAGTCATGAAG	214
		R-CATCTCCTGGGTCACCCTTA	
TNF- α	NM_012675.3	F-TCGAGTGACAAGCCTGTAG	230
		R-GTTGGTTGTCTTTGAGATCC	

PPAR-α	NM_013196	F-GCCATTCTGCGACATCA R-AGCCCTCTTCATCTCCAA	483
PPAR-γ	NM_013124.3	F-CCCATTCCTTTGACATCAAACC R-ATTGTGAGACATCCCCACAGC	349
β-actin	NM_031144.3	F- GGAATCCTGTGGCATCCATGAAAC R-TAAAACGCAGCTCAGTAACAGTCCG	315

3.5: Drugs and polymers

Linezolid and cefdinir in form of active pharmaceutical ingredient (API) were obtained as gift samples from Macleod Pharma, Mumbai. Eudragit series of the polymers were chosen for their pH specific drug release behavior. Eudragit L100-55 (EL100) and Eudragit S100 (ES100) were obtained as a gift sample from Corel Pharma chem. Pvt. Ltd (Ahmedabad, India).

3.6: Solvents and reagents

The acetone, sorbitan sesquioleate 80 (Span 80), paraffin light liquid and petroleum ether were purchased from Sigma–Aldrich (Ahmedabad, India). The de man Rogosa Sharpe (MRS) agar, bifidobacterial selective agar, luria agar and clostridial agar were purchased from himedia (India). All the solvents and reagents used were of analytical grades.

3.7: Fourier transform infrared spectroscopy (FT-IR)

The FT-IR analysis of the physical mixtures of polymers and drug as well as enteric coated microspheres was carried out to find the possible interaction amongst them during the formulation process. FT-IR spectra were obtained in KBr pellets using a JASCO BX-FTIR spectrophotometer in the frequency ranges from 4000- 400 cm^{-1} **(Oelker and Grinstaff, 39).**

3.8: Formulation of microspheres

Two antibiotic (Cefdinir and linezolid) APIs were taken in their different drug to polymer ratio (1:1,1:2,1:3) and were added in an 5 ml acetone organic phase (internal aqueous phase) having 10% w/v ES100 or EL100 and sonicated in an ultrasonicator (JY92-11DN, Syclon, Japan) for 10 min. This solution was then added drop by drop through a disposable syringe into an external aqueous phase containing paraffin light liquid and was emulsified containing the emulsifier, span 80 (2% v/v). The mixture was stirred constantly using a mechanical stirrer at 1200 RPM and 38 ± 0.5°C for 5 hrs to allow the complete evaporation of the solvent. The paraffin was decanted off, the microspheres were washed repeatedly 3 times with petroleum ether (40-60° C), collected by filtration and finally dried at 40°C for 1 hr **(Shivani et al, 265).**

3.9: Physicochemical characterization of microspheres

3.9.1: Particle size analysis

The average particle size distribution of the optimized microspheres was estimated through the optical microscopy method using both calibrated stage micrometer and eyepiece. The particle sizes of around 100 microspheres and median diameter was calculated **(Chawla et al, 536).**

3.9.2: Scanning electron microscopy (SEM)

A scanning electron microscope (ESEM TMP with EDAX, Philips, holland) was used to characterize the surface morphology of formulated microspheres. The surface was scanned and images were taken at 30 KV accelerating voltage and taken at magnification of 200x for the drug-loaded microspheres. The samples for SEM study were prepared by lightly sprinkling the formulation on a double-adhesive tape stuck to an aluminum stub. The stubs were coated with gold to a thickness of ~300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken with a scanning electron microscope **(Swathi et al, 172).**

3.9.3: Production yield

The product yield was calculated by dividing the weight of the collected dried microspheres by the weight of all the non-volatile components used for the preparation of microspheres and expressed in term of percentage **(Jena et al, 3818).**

Yield (Å) Í Û Ü Ü Û Ü Ü / Ü Ü Ü ó È

3.9.4: Percentage entrapment efficiency (EE)

The accurately weighed amount of the ES and EL coated microspheres was taken and added into respective phosphate buffers containing pH 7.0 and 5.5, respectively. The mixture was continuously agitated on a magnetic stirrer for 5 to 6 hrs. Further, the solution was filtered and drug content of the filtrate was determined by UV spectrophotometer at respective range according to the antibiotics. The percentage drug EE was calculated using below formula **(Kuchekar et al, 491).**

Û (Å) ÚÏ Ü / Ü ÚÏ Ü ó È

3.10: *In vitro* drug release study

The accurately weighed formulated microspheres containing 300 mg of equivalent drugs were filled in the dialysis bag and dispersed in 500 ml of 0.1 N HCl for initial 2 hrs. The dissolution media containing phosphate buffer with an appropriate pH in the USP apparatus were maintained at 37ºC and 100 RPM. After 2 hrs, the microspheres were transferred to the pH 5.5 phosphate buffer for 2 hrs. Consequently, the pH of the buffer was increased by the further addition of Na_2HPO_4 to pH 7.0 and maintained till the completion of study. 5 ml of the sample was withdrawn hourly replacing each withdrawn sample with the fresh release medium. The samples were filtered and analyzed for drug content spectrophotometrically. The drug content in the withdrawn samples from 0.1N HCl, pH 5.5 and pH 7.0 phosphate buffers was measured by spectrophotometer at 294 nm, 288 nm, and 290 nm, respectively **(Jena et al, 3818).**

3.11: Release kinetic modeling

The cumulative drug release *vs.* time data were entered into various kinetic models to find out the mechanism of the drug release from the enteric coated antibiotic microspheres. The data were calculated for the various kinetic models, such as zero order, first order, higuchi model and korsmeyer-peppas model. The model co-efficient and regression co-efficient values for all the kinetic models were deliberated using DDsolver tool. The co-efficient and regression values were observed for all the models and appropriate values were further considered **(Modi and Anderson, 3079).**

3.12: *In vitro* antimicrobial study

The ES and EL coated microspheres as well as pure drugs were dissolved separately in distilled water to obtain the drug concentration of 5µg/mL. One ml of the overnight grown bacterial culture inoculums of *Escherichia coli* (MTCC443) and *Lactobacillus casei* (MTCC1423) and enteric coated antibiotics microspheres were simultaneously added in freshly prepared LB and MRS broths respectively. After 10 hrs of the incubation at 37°C with shaking at 150 RPM, changes in the optical density at 600 nm were recorded at every hr up to 10 hrs. The antimicrobial activities of enteric coated microspheres were compared with control microbial cells without any treatment **(Aleem et al, 537).**

3.13: Animal experimentation

Around 8 to 10 weeks old healthy male wistar rats, weighing about 150–200 gms were procured from the animal research facility (Cadila pharmaceutical limited, Ahmedabad (India) under the approval of institutional animal ethics committee with protocol no. IS/BT/PhD11-12/1005, and were maintained in the animal house of institute of pharmacy (Nirma university, India). The

animals were acclimatized at temperature 25±2°C with the relative humidity of 50–60% under 12/12 h light/dark conditions for 1 week before the initiation of the experiment. The enteric coated cefdinir and linezolid microspheres were orally administrated into the rat models on a daily basis for 7 days to check the efficacy of the formulated microspheres (Table 3.5A)**.** Another 24 animals were divided into six different experimental groups with 4 animals each to study the effect of altering the target specific gut microflora in HSD model for 12 weeks. The experimental grouping of animals was as follows: non-diabetic group with CD, diabetic group with HSD, diabetic group HSD with ES linezolid, diabetic group HSD with EL linezolid, diabetic group HSD with ES cefdinir and diabetic group HSD with EL cefdinir (Table 3.5B).

Table 3.5A: Experimental animal groups to check efficacy of formulation

Group Name	Specification	No. of animals
Control	PBS only (no drug)	4
ES Cef	ES coated cefdinir microspheres	4
EL Cef	EL coated cefdinir microspheres	4
ES Lin	ES coated linezolid microspheres	4
EL Lin	EL coated linezolid microspheres	4

Table 3.5B: Experimental animal groups for gut microflora alteration

Group Name	Specification	No. of animals
CD	Normal rodent diet	4
HSD	High sucrose diet	4
HSD+ES Cef	ES coated cefdinir microspheres with HSD	4
HSD+EL Cef	EL coated cefdinir microspheres with HSD	4
HSD+ES Lin	ES coated linezolid microspheres with HSD	4
HSD+EL Lin	EL coated linezolid microspheres with HSD	4

3.14: The *Ex vivo* antimicrobial study

The *Ex vivo* antimicrobial activity of enteric coated antibiotic microspheres was estimated from the colon and proximal small intestinal fecal samples; isolated from the test animals (as mentioned in Table 4.5A) at the end of the experiment. The antimicrobial activity was determined using culturable method through selective media. For the quantitative determination of microflora, 1gm fecal sample was suspended in 10 ml of 0.85% NaCl solution for homogenization and 10 fold serial dilutions were prepared. The appropriate dilutions were spread plated onto two selective agar medium such as; MRS medium for *Lactobacilli* and MacConkey agar for *Escherichia.* The plates were incubated aerobically for 48 hrs at 37°C and the resultant colonies were counted in cfu/mg of feces.

3.15: Histopathological analysis

The experimental animals (as mentioned in Table 3.5A) were sacrificed using the euthanasia method at the end of the experiment. Small portion of the adipose tissue was excised from animals of each group, fixed with 10% v/v formalin saline, and processed for standard histopathological procedures. Paraffin embedded specimens were cut into 5 μM sections (Yorco sales Pvt. Ltd., New delhi) and stained with hematoxylin as well as eosin (H&E). The histopathological tissue sections were viewed and digitally photographed using a Cat-Cam 3.0MP trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India) **(Kevadiya et al, 96; Chaudhary et al, 907)**

3.16: Physiological, biochemical and gene expression analysis

The estimation of physiological and biochemical parameters, metabolites estimation, microflora quantification as well as gene expression analysis were carried out for all the experimental animal groups (as mentioned in Table 5B) according to previously mentioned methodology (Point 3.3 to 3.4)

3.17: Statistical analysis

The statistical analysis was applied using graph pad prism software version 6. One way ANOVA followed by Turkey's multiple comparison tests were used to determine the statistical

significance between various groups. Differences were considered to be statistically significant when $p>0.05$. All the values were expressed as mean±SD.