2.1. Animal selection and care

Throughout the present experimental work, healthy male wistar adult strain of albino rats having 2 months of age and weighing 110±12 (g) were selected for carrying out the experiments. During pre-experimental check-up and also during the period of experiment, the animals were maintained under standard laboratory condition, 12 h light: 12 h dark cycle at 24±2°C temperature and 60±10% humidity. In each set of experiment, the animals were acclimatized for a week in laboratory environment. They were not given any medication and provided specific boiled homogenous diet (containing carbohydrate, 74.05%; protein, 10.38%; fibre, 2.20%; iron, 56 ppm; calcium, 400 ppm and sodium, 500 ppm) throughout the experimental period. The animals used in the study were maintained in accordance with the internationally accepted principles for laboratory animals' use and care (Olfert *et al.,* 1993). Our Institutional Animal Ethical Committee approved (CPCSEA Registration No-1905/PO/Re/S/2016/CPCSEA) the experimental protocol. The animals were housed in specially designed cage (6 rats per cage).

2.2. Animal grouping and exposure to hypobaric hypoxia

The animal was divided randomly into different groups. Group C served as control was maintained to normal room air (normoxia). Group HA-I, HA-II and HA-III were exposed to different barometric pressure. Different groups and their individualities represented in the Table 2.1. Exposure was carried out in a decompression chamber (Instrumentation India, India) for 7 days following the protocol of Maity *et al* (2012).

Table 2.1. Different groups and their weirdness

Name of the groups	Characteristics of each groups
C/NC/NA	Normal altitude, sea level exposure
HA-I	Moderate altitude exposure (6000 feet/1829 m) animals
HA-II	High altitude exposure (12000 feet/3657 m) animals
HA-III	Extreme high exposure (18000 feet/5486 m) animals
CP1	Supplied with Commercial probiotic **'VSL#3'**
CP2	Supplied with Commercial probiotic '**TruBiotics'**
CP3	Supplied with Commercial probiotic '**Yogut'**
CP4	Supplied with Commercial probiotic '**Propolis Plus'**
HA-I-CP	Rats were exposed to Moderate altitude (6000 feet/1829 m) with commercial probiotic supplementation
HA-II-CP	Rats were exposed to High altitude (12000 feet/3657 m) with commercial probiotic supplementation
HA-III-CP	Rats were exposed to Extreme high (18000 feet/5486 m) with commercial probiotic supplementation

The HA groups were exposed to different hypobaric pressure consecutively for 28 days 12 h/day. Other confounding variable also controlled strictly to ensure that partial pressure at HA was the true factor responsible for the alteration of gut microflora. Subsequently, end of each treatment, intense care was taken and their body weight, physiological activity and food consumption ability were monitored regularly throughout the experimental period. The control group was maintained at normal atmospheric pressure (14.68 psi) without interrupting their normal activity and daily rhythm.

2.3. Selection of probiotic and feeding procedure

Animal was provided with standard boiled rat feed with water ad libitum (Maity *et al.*,2012). While probiotic treated groups were administrated with four different known pharmaceutical probiotics, selected from local retailer medicine shop of Kolkata and Midnapur city, West Bengal, India. Product labels were scrutinized; this included an assessment of spelling of contents, if contents were clearly described (genus and species), if the number of viable organisms was stated and if there was an expiry. These probiotics were stored at 4ºC during experiments. Probiotic treated groups were administered with 1×10^9 CFU/day throughout the experiment (Venturi *et al*.,1998; Mandal *et al.,* 2013; Patra *et al.,* 2014). After completion of experiment animals from all groups were sacrificed and blood, luminal content, liver and kidney were collected. The name of probiotic with respective brand and their specification (labelled) are enlisted in Table 2.2.

Table 2.2. List of selected probiotics & its specification

Probiotic	Manufacturer	CFU count (bn)	No. of strains	Name of strains
VSL#3	Sigma Tau Pharmaceuticals	112.5	8	*Bifidobacterium breve, B longum, B infantis, Lactobacillus acidophilus, L plantarum, L paracasei, L bulgaricus, S thermophilus*
TruBiotics	Bayer	1.5	2	*L acidophilus, B animalis*
Yogut	Swiss Garniar Life Sciences	5.0	5	*L acidophilus, L rhamnosus, B bifidus, B longum, B infantis*
Propolis Plus	Essential Formulas	21.6	12	*B breve, B infantis, B longum, Enterococcus faecalis, L acidophilus, L brevis, L bulgaricus, L casei, L fermentum, L helveticus, L plantarum, Streptococcus thermophilus*

2.4. Sample collection

In order to search the alteration of haematological, biochemical, and oxidative stress indicators, sample were collected at 7 days' interval and for the study of the changes in intestinal microflora at different altitude sample were collected 3 days' interval throughout experiments (maximum 28 days), before as well as after probiotic administration (Table 2.3). Rat faecal samples were collected just after dropping onto clean the paper underlying the cage and collected prior to feeding at every day during the total experimental period. Fresh faecal sample was suspended in sterile phosphate-buffered saline (PBS; pH 7.0 and 9 g-1 NaCl) using a manual glass homogenizer for 5 min. After treatment, 6 animals in each group were sacrificed by deep anaesthesia with the help of chloroform. GI tract was aseptically removed from the body. The defined part of the small intestine (10-12 cm) from ileocecal valve was excised out and content were collected by repeated washing with the phosphate buffer saline (PBS; pH 7.0). The content of the caecum was also washed out and collected in a sterile tube. The eluted content was centrifuged at 5000 rpm and weighted for microbial and supernatant was also collected for the analysis of different biochemical parameters. The pellet was suspended in sterilized carrier solution [containing peptone 10%; glycerol 5% (v/v) and pH was adjusted to 7.0±0.2] and preserved at -20°C until the analysis. After collection of intestinal content, the lumen was opened longitudinally and the epithelial layer was scrapped out and transferred in chilled PBS. Blood samples were collected by hepatic artery punch under diethyl chloroform anaesthesia, using 21 gauge (21 G) needles mounted on a 5 ml syringe (Hindustan syringes and medical devices ltd, Faridabad, India.) into heparin coated sample bottles for analysing haematological parameters (Patra *et al.,* 2011; Mandal *et al.,* 2017).

Table 2.3. Preparation of different types of sample for different experiments

		Type of study	Preparation of sample	References
Type of samples for study	Blood	For the study of haematological, lipid profile, electrolyte, toxicity study, antioxidant enzymes study, oxidative stress marker and uremic profile	Blood samples were collected by hepatic artery punch under diethyl chloroform anesthesia, using 21 gauge (21 G) needles mounted on a 5 ml syringe (Hindustan syringes and medical devices ltd, Faridabad, India.) into heparin coated sample bottles for analysed Hematological parameters.	Mandal *et al.,* 2017; Roy *et al.,*2014; Arpita *et al.,* 2012; Burtisand Ashwood, 1999; Beers and Sizer, 1952.
	Intestine	Microbial, biochemical, faecal enzymes and Immunoglobulins	The define part of the small intestine (10-12 cm) from ileocecal valve to the ileum was excised out and content were collected by repeated washing with phosphate buffer saline (PBS; pH 7.0). The content of the caecum was also washed out and collected in a sterile tube. The eluted content was centrifuged at 6000 rpm and weighted for microbial and supernatant was collected for analysis of different biochemical parameters. The pellet was suspended in sterilized carrier solution [containing peptone 10%; glycerol 5% (v/v) and pH was adjusted to 7.0±0.2] and preserved at -20°C until analysis.	Maity *et al.,* 2012, Paul *et al.,* 2013; Guang-Can *et al.,* 2008).
		SEM	fixed in the Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid).	(Adak *et al.,* 2014)
	Tissues (kidney, liver)	For biochemical analysis	Livers and kidneys were immediately dissected out, washed and stored in ice cold normal saline (0.9% NaCl) for different biochemical examination. After that tissues were homogenized	(Mandal *et al.,* 2017; Samanta et all., 2018 Sabbagh *et al.,* 1988 Marklund

			separately in 0.05 M Tris-HCl buffer (Merck, India) solution (pH 7.0) at a tissue concentration of 50 mg/ml. These homogenates were centrifuged separately at 10,000g at 4 ºC for 10 min for analysis	& Marklund, 1974)
		Histological analysis and SEM	2-3 cm of isolated large intestine was fixed in the Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid). After 2 h fixation, tissues were dehydrated in graded alcohol and embedded in paraffin and tissue block was prepared and cut into 3-5 µm thin sections and transferred onto the clean glass slide.	Mani, 2010; Khorsandi and Orazizadeh, 2008; Adeneye *et al.,* 2008; Lee *et al.,* 2006
	Feacal sample	Microbial analysis	Fresh faecal samples were collected from individual animals. Samples were re-suspended in phosphate buffer saline at approximate concentration of 0.03 g/ml. Serial dilutions were prepared, and 0.1 ml aliquots from dilutions of 10-6 and 10-7 were cultured. For the human samples were transported in a sterilized carrier solution containing peptone, 10 % (w/v); glycerol, 5 % (v/v). pH was adjusted to 7.0±0.2, and the samples were stored at 4°C until the analysis.	Maity *et al.,* 2012, Paul *et al.,* 2013, Adak *et al.,* 2014

2.5. Human sample collection

For the study on human, twelve young healthy Indian army male soldiers (base level residents) within the age group of 25-30 years were selected for this study; their body mass index (BMI) was approximately 24.55 kg/m^2 (±0.84). They all were healthy, not under the treatment of any medication, and they were not suffering from any bacterial or viral infections. They consumed army-specific homogenous diets throughout the experiment. The sea level (i.e. Base line or '0' day) study was carried out at Delhi (barometric pressure 740 mm Hg). After recording the physiological parameter and collecting the samples, the subjects were flown to an altitude of 3,505 meter (m) at Leh (barometric pressure 483 mm Hg) in the Western Himalayas. The subjects arrived at a 3500 m altitude in the morning and the day of arrival was taken as day one at 3500 m.

After a brief medical counselling, the faecal samples (~10 g) were collected in a pre-sterilized spatula–container at Delhi, and these were considered as the 'Base Line' (or '0' day sample) samples. Thereafter, the samples were collected on the first, fourth, and the seventh days at Leh, Jammu and Kashmir, India (~3500 m) during acclimatization. The samples were transported in a sterilized carrier solution containing peptone (10 %); glycerol (5 %), pH was adjusted to 7.0±0.2, and the samples were stored at 4°C until the analysis.

Blood samples were collected from subjects using 21-Gauge needles (21G) mounted on a 5-ml syringe (Hindustan Syringes and Medical Devices Ltd, Faridabad, India) into heparin-coated sample bottles for the analysis. Samples were collected on '0' (base line), during the first, fourth, and seventh day of experiment.

2.6. Analysis of physiological indices

Observed different wellness parameters of rats for the limit test at different altitude for different time periods (Skin and fur, Eyes, Sleep, Diarrhoea, Morbidity and Mortality). The body weight, body length (nose-anus length) and food intake capacity were measured regularly during experiment and following parameters were evaluated (Diniz *et al.,* 2005; Adak *et al.,* 2014).

i. Elevation/diminition in body growth (g%) = (Final body weight-initial body weight (g)/initial body weight)×100

i. Body mass index (BMI) (g/cm^2) = body weight (g)/$length^2$ (cm^2)

2.7. Quantification and diversity of microflora

2.7.1. Microbial Analysis

The cultivable microflora was enumerated on agar plates on the basis of colony-forming units (CFU/g). The CFU represent the actual number of bacteria present in the faecal samples. These CFU values were converted to their logarithmic value and tallied with the corresponding experimental set of specified conditions. The total aerobic and anaerobic faecal bacteria were enumerated by a standard pour-plate technique in a single-strength trypticase soya agar (HiMedia, India) and reduced Wilkins Chalgren agar (supplemented with sodium succinate, hemin, vancomycin, menadione, oleandomycin phosphate polymyxin B and nalidixic acid) respectively. The Anaerobic condition was maintained in CO_2 incubator filled with 10% of CO_2 and H_2 gases (Adak *et al.,* 2013). *Escherichia coli, Bacteroidetes* sp.*,* total Lactic acid bacteria, *Bifidobacterium* sp.*,* and *Salmonella* sp. were cultured on Mac-Conkey, bacteroides bile esculin agar (supplemented with gentamicin 100 mg/L), De Man Rogosa

Sharpe agar (MRS), *Bifidobacterium* and Brilliant green agar modified (HiMedia, India) respectively (Wehr and Frank 2004; Maity *et al.,* 2012; Adak *et al.,* 2013).

2.7.2 Growth direction index (GDI)

The CFU/g of bacterial populations (in 10 logarithmic values) of C group was compared with the HA and HA-CP group. When control log_{10}CFU/g was higher in comparison to test log_{10}CFU/g, the GDI was denoted as negative and reverse event was followed as positive. This will show a straight forward portrait about expansion and contraction of a microbial population in a particular ecosystem (Maity *et al.,* 2012; Adak *et al.,* 2013).

2.8. Metabolic activities of gut microflora

2.8.1. Measurement of gas volume

One hundred microlitres of faecal aliquot will add in 30 ml of MacConkey broth, and the volume of gas formation was measured by volumetric inverted Durham's tube after 24 h incubation at 37°C. Gas formation ability will express in millilitre/milligram of wet faeces (Adak *et al.,* 2013).

2.8.2. Faecal enzymes activities

The luminal content (1 gm) was vortex well and aliquot was centrifuged at 10,000 rpm for 5 min, and supernatant was collected for assay of different enzymes.

2.8.2.1. Assay of α-amylase

The activity of α-amylase was measured by dinitro salicylic acid method (Miller, 1959). In this method 500 µl starch solution was taken in which 200 µl supernatant was mixed and allowed to incubate at 37°C for 20 min. The reaction was stopped by addition of 1 ml of 3, 5, dinitrosalicyalic acid reagent and boiled for 7 min and the absorbance was taken at 540 nm in UV-Vis spectrophotometer (Systronics, India).

2.8.2.2. Assay of Proteinase

Proteinase activity was analysed by using 0.8 ml of 1% (w/v) azocasein as the substrate and 0.2 ml of faecal aliquot and incubated for 45 min. The reactions were stopped by equal volume of 5% (w/v) trichloroacetic acid and 1 ml of 1 (N) NaOH was added, the absorbance was taken at 450 nm (Hutadilok-Towatana *et al.,* 1999).

2.8.2.3. Assay of Alkaline phosphatase

Alkaline phosphatase activity was measured by taking 0.1 ml of 50 mM P-nitrophenyl phosphate and 50 μl faecal aliquot and 0.7 ml of 50 mM tris buffer (pH 8.5) and incubated for 1 h at 37°C. The reaction was stopped by adding 0.1 ml of 1(M) perchloric acid and 2.5 ml of 0.50 mM NaOH. The absorbance was taken at 420 nm (Hubscher & West, 1965).

2.8.2.4. Assay of α-glucuronidase

The activity of β-glucuronidase was determined by using 0.7 ml of 1.2 mM phenolphthalein glucuronide and 0.7 ml of 100 mM sodium acetate buffer (pH 3.8) and 0.1 ml faecal aliquot. It was mixed and incubated at 37°C for 30 min. Thereafter, 5 ml of 200 mM glycine buffer (pH 10.4) was added and absorbance was taken at 540 nm (Fishman *et al.,* 1948).

2.8.2.5. Calculation of specific activity

Total protein in the faeces was estimated (Bradford, 1976) and the enzyme activities were expressed in specific activity (U/mg of protein).

2.9. Health profile analysis

2.9.1. Haematological parameter

2.9.1.1. Total WBC count by haemocytometer

The blood specimen is diluted (usually 20 times) with white cell diluting fluid which does not remove the red cells but allows the white cells to be counted under 400X magnification in a

known volume of fluid. Finally, the number of the cells in undiluted blood is calculated and reported as the number of white cells/µL of whole blood by using haemocytometer counting chamber under compound microscope.

Equipment: Blood Sahli pipette, Coverslip, Microscope and haemocytometer, counting chamber.

Reagents: WBC Diluting Fluid

Procedure: Dilution Procedure of Blood in WBC Pipette. Blood is collected in the WBC pipette upto the mark 0.5. Then it is diluted by WBC diluting fluid in the same pipette upto the 11 mark. At the time of collection of blood as well as the time of dilution the volume should be maintained upto the mark very carefully. The mouth part along with the rubber tube of WBC pipette is blocked by placing a finger and the WBC pipette is moved by rotation as well as up and down very slowly for the proper mixing of the collected blood with WBC diluting fluid. This is the protocol for total count of WBC in haemocytometer. Haemocytometer is placed under the low power objective of the compound microscope and any one of the WBC counting square area is focused. The counting chamber is covered by a specific cover slip and the diluted blood is allowed to charge at the junction between the slide and cover slip by a pasture pipette very carefully by a single effort in such a way the mixture should spread on the counting square evenly. For the fixation of WBC inside the counting chamber 2-3 minutes' time has been lapsed. Then carefully switched to high power objective and moved the chamber so that the smaller upper left corner smaller (with 16 smaller squares) is completely in the field of vision. Count the number of white cells seen on the small square ($0.2 \times 0.2 = 0.04$) of the upper left corner which is divided into 16 smaller squares to facilitate counting. Any corpuscles lying on the lines were counted and those include on the right or

downward smaller square rejected as well. Addition of a total number of counted cells in 4 squares were made (Mukherjee and Dierolf, 2010; Mandal *et al.,* 2013a).

Calculation:

$$\text{Numberof WBCs } (\mu l) = \frac{\text{Number of white cell counted} \times \text{Dilution}}{\text{Areacounted} \times \text{Depth of fluid}}$$

2.9.1.2. Total RBC Count by haemocytometer

The blood specimen is diluted (usually 200times) with red cell diluting fluid which does not remove the white cells but allows the red cells to be counted under 400X magnification in a known volume of fluid. Finally, the number of the cells in undiluted blood is calculated and reported as the number of red cells/μl of whole blood by using haemocytometer counting chamber under compound microscope.

Equipment: Blood Sahli pipette, Coverslip, Microscope and haemocytometer, counting chamber.

Reagents: RBC Diluting Fluid

Procedure: Dilution Procedure of Blood in RBC Pipette. Blood is collected in the RBC pipette upto the mark 0.5. Then it is diluted by RBC diluting fluid in the same pipette upto the 101 mark. At the time of collection of blood as well as the time of dilution the volume should be maintained upto the mark very carefully. The mouth part along with the rubber tube of RBC pipette is blocked by placing a finger and the RBC pipette is moved by rotation as well as up and down very slowly for the proper mixing of the collected blood with RBC diluting fluid. This is the protocol for total count of RBC in Haemocytometer. Haemocytometer is placed under the low power objective of the compound microscope and any one of the RBC counting square area is focused. The counting chamber is covered by a specific cover slip and

the diluted blood is allowed to charge at the junction between the slide and cover slip by a Pasture pipette very carefully by a single effort in such a way the mixture should spread on the counting square evenly. For the fixation of RBC inside the counting chamber wait for 2-3 min. Carefully switch to high power objective and move the chamber so that the smaller upper left corner smaller (with 16 smaller squares) is completely in the field of vision. Count the number of red cells seen on the small square ($0.2 \times 0.2 = 0.04$) of the upper left corner which is divided into 16 smaller squares to facilitate counting. Any corpuscles lying on the lines must be counted and those include on the right or downward smaller square should be rejected. Make a total of all the cells counted in 5 squares (Chakravarthy and Dierolf, 2010; Mandal *et al.,* 2013a).

Calculation:

$$\text{Numberof RBCs } (\mu l) = \frac{\text{Numberofredcellcounted} \times \text{Dilution}}{\text{Areacounted} \times \text{Depth of fluid}}$$

2.9.1.3. Haemoglobin estimation by colorimetric method

Principle: Cyanmet haemoglobin method is a colorimetric procedure for determining haemoglobin concentration. An aliquot of whole mixed blood is taken and reacted with a potassium cyanide and potassium ferricyanide (called Drabkin's solution). The chemical reaction yields a product of stable colour – the cyanmethaemoglobin. The intensity of colour is proportional to the haemoglobin concentration (Chakravarthy and Dierolf, 2010; Mandal *et al.,* 2013).

Equipment: Sectro-Photometer with 540 nm filter, Blood pipette, Test tubes

Reagents: Drabkin's reagent is composed of 50 mg Potassium cyanide, 200 mg Potassium ferricyanide ($KFeCN_6$) and 1000 ml distilled water. Cyanmethaemoglobin used as standard.

Procedure: Label the test tubes as B (blank), T (test sample) and S (standard)and then pipette 5 ml of Drabkin's solution is taken into the marked test tubes. Then 0.02 ml of standard solution is transferred to the test tube marked as 'S'. After that 0.02 ml well mixed anticoagulated blood is transferred to the test tube marked as 'T'. Mix the content of the test tubes thoroughly and wait for 10 min. After that read the absorbance of test and standard against blank at 540 nm.

Calculation:

$$\text{Haemoglobinconcentration (gm/dL)} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times 15.06$$

2.9.2. Lipid profile

2.9.2.1. Blood Cholesterol level

Total cholesterol was evaluated by enzymatic colorimetric method. Cholesterol esters were cleaved through the action of cholesterol esterase producing free cholesterol and fatty acids. Cholesterol oxidase catalyzed the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of oxidase, the hydrogen peroxide formed affects the oxidative coupling of phenol and 4-aminoantipyrine, forming a quinone-imine red dye. The color intensity is directly proportional to cholesterol concentration, and the absorbance reading was 512 nm.

2.9.2.2. Total HDL Level

HDL was analyzed by homogeneous colorimetric enzymatic method. In the presence of magnesium ions, dextran sulfate selectively forms water soluble compounds with LDL, VLDL and chylomicrons, which are resistant to polyethylene glycol-modified enzymes. Under the influence of the cholesterol enzyme, the cholesterol esters are quantitatively decomposed into free cholesterol and fatty acids.

2.9.2.3. Total triglyceride level

Triglycerides were measured by colorimetric enzymatic method, which utilizes the lipoprotein lipase for rapid and complete hydrolysis of triglycerides into glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminophenone and 4-chlorophenol under peroxidase catalytic action to form a red dye. The concentration of triglycerides is proportional to the intensity of the color generated and measured photometrically (Daniela *et al.,* 2014; Florian *et al.,* 2009).

2.9.2.4. Total LDL level

LDL was estimated by the Friedewald formula:

LDL = total cholesterol-HDL-VLDL (Triglycerides/5).

2.9.3. Measurement of electrolytes

2.9.3.1 Biochemical estimation of plasma sodium, potassium and chloride

Sodium is sediment as a triple salt with magnesium and Uranyl acetate. The excess of Uranyl ions is reacted with ferrocyanide in an acidic medium to develop a brownish color. The strength of the colour formed is inversely proportional to the concentration of sodium in the sample. Potassium conjugates with sodium tetra phenyl boron in standard buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the

concentration of potassium in the plasma sample. Quantification of plasma sodium and potassium by Standard Kit methods by using plasma samples (Sunderman, 1959; Pradhan *et al.,* 2013). The electrolytes analyser system (Systronic, India) uses indirect (or diluted) I.S.E. (ion selective electrode) methodology to determine chloride concentration in blood. Chloride is measured using an Ag/AgCl electrode, while the ion-selective electrode uses a transducer to convert the activity of the chloride ion into an electrical potential, chloride with other components produce colored solution and then measures color density to determine chloride concentration (Tavallali *et al.,* 2012).

2.9.4. Toxicity study by biochemical assay of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) activities

2.9.4.1 Assay of SGOT

Principle:

Glutamate oxaloacetate transaminase (GOT) catalysed the transfer of amino group between L-aspertate and α-ketoglutarate to form oxaloacetate and glutamate. The formed oxaloacetate reacts with NADH in the presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD was measured as a decrease in absorbance which was proportional to the GOT activity in the sample (Goel, 1988).

Reagents:

i. GOT Enzyme/coenzyme

ii. GOT Buffer substrate

Preparation of working reagent: Into the bottle containing No. (1) GOT reagent was fully transferred 50 ml of No. (2) GOT reagent and then mixed gently to dissolve completely. Allow for 5 min before use.

Preparation of serum and tissues:

Whole blood sample was centrifuged at 10,000 g for 5min at 4ºC and serum was separated. Kidney and liver tissues were separately homogenized in 0.1 M phosphate buffer at a tissue concentration of 50 mg/ml. The homogenates were centrifuged at 1000 g for 20 min and the supernatents were used for the assay of GOT. Absorbance was measured at 340 nm for 2 min at an interval of 30 sec.

Assay procedure:

i. In a spectrophotometric cuvette 1.0 ml of working GOT reagent was added, then added 0.05 ml of plasma samples.
ii. Mixed and was then incubated for 60 secs in a room temperature.
iii. Absorbance was measured at 340 nm for 2 min at an interval of 30 sec.

SGOT activity was expressed in term of unit/lit of serum using mM extinction coefficient of NADH at 340 nm. Tissue GOT activity was expressed in term of unit/mg of tissue using mM extinction coefficient of NADH at 340 nm.

2.9.4.2 Assay of SGPT

Principle:

Glutamate pyruvate transaminase (GPT) catalysed the transfer of amino group between L-alanine and α-ketoglutarate to form pyruvate and glutamate. The formed pyruvate reacts with NADH in the presence of lactate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD was measured as a decrease in absorbance which was proportional to the GPT activity in the sample.

Reagents:

i. GPT Enzyme/coenzyme

ii. GPT Buffer substrate

Preparation of working reagent: Into the bottle containing No. (1) GPT reagent carefully was transferred 50 ml of No. (2) GPT reagent and then mixed gently to dissolve completely. Wait for 5 min before use.

Preparation of serum:

Whole blood sample was centrifuged at 10,000 g for 5 min at 4°C and serum was separated. Kidney and liver tissues were separately homogenized in 0.1 M phosphate buffer at a tissue concentration of 50 mg/ml. The homogenates were centrifuged at 1000 g for 20 min and the supernatents were used for the assay of GPT. Absorbance was measured at 340 nm for 2 min at an interval of 30 sec.

Assay procedure:

i. In a spectrophotometric cuvette 1.0 ml of working GPT reagent was added, then added 0.05 ml of plasma sample.
ii. Mixed and was then incubated for 60 secs in a room temperature.
iii. Absorbance was measured at 340 nm for 2 min at an interval of 30 sec.

SGPT activity was expressed in term of unit/lit of serum using mM extinction coefficient of NADH at 340 nm were measured according to the method of Goel (Goel, 1988). Tissue GPT activity was expressed in term of unit/mg of tissue using mM extinction coefficient of NADH at 340 nm.

2.9.5. Assessment of novel antioxidant enzymes

2.9.5.1 Biochemical assay of catalase activity (CAT)

Catalase (CAT) activity was measured biochemically. For the evaluation of CAT activity in blood, liver and kidney samples were homogenized separately in 0.05 M Tris-HCl buffer

(Merck, India) solution (pH 7.0) at a tissue concentration of 50 mg/ml. These homogenates were centrifuged separately at 10,000g at 4 ºC for 10 min. In a spectrophotometric cuvette, 0.5 ml of hydrogen peroxide (H_2O_2) and 2.5 ml of distilled water were mixed, and a reading of absorbance was noted at 240 nm. Tissue supernatants and plasma were added at a volume of 40 µl separately, and the subsequent and 12 following readings were noted at 30-second intervals (Beers, 1952).

2.9.5.2 Biochemical assay of superoxide dismutase (SOD)

Kidneys were homogenized in ice cold 100 mM Tris-cacodylate buffer (LOBA Chem, India) to give a tissue concentration of 50 mg/ml, which as centrifuged at 10,000g for 20 min at 4ºC. The superoxide dismutase (SOD) activity of these supernatants was estimated by measuring the percentage of inhibition of the pyrogallol (HIMEDIA, India) autooxidation by SOD (Marklund and Marklund, 1974). The buffer was 50 mM Tris (pH 8.2) containing 50 mM cacodylic acid (pH 8.2), 1 mM ethylenediaminetetra acetic acid (EDTA) (HIMEDIA, India) and 10 mM HCl. In a spectrophotometric cuvette, 2 ml of buffer, 100 µl of 2 mM pyrogallol and 10 µl of supernatant were added, and the absorbance was noted in a spectrophotometer at 420 nm for 3 min. One unit of SOD was defined as the enzyme activity that inhibited the autooxidation of pyrogallol by 50%.

2.9.6. Assessment of Oxidative stress markers

2.9.6.1 Malondialdehyde (MDA)

For assay, 0.1 ml of supernatant, 0.1 ml tris-HCl buffer (pH-8.2) and 0.1 ml FeSO4 were added in a test tube then 0.6 ml dH2O was added to make the volume 1.0 ml. It was incubated at 37°C for 15 min. Then 1.0 ml trichloroacetic acid (TCA) and 2 ml thiobarbituric acid (TBA) were added to the reaction mixture. Tubes were plugged and incubated for 15 min at

boiling water. Centrifugation was done at 3,000 rpm for 10 min, and absorbance was measured at 532 nm. The concentration of MDA is calculated using extinction coefficient of MDA-TBA complex and calculated as: [Absorbance×sample volume 1.56×10^5×total volume×mg protein/ml] and expressed as mM of MDA/mg protein (Lapenna & Cuccurullo, 1993).

2.9.7. Measurement of uremic biomarkers

2.9.7.1 Biochemical estimation of plasma urea

The blood was centrifuged and plasma fraction was separated. Urea level was measured by commercially available standard urea kit (Merck, Japan) by Semi-autoanalyser (Systronic, India) according to the Urease GLDH method (kinetic UV test). First 10 µl of urea standard (50 mg/100 ml) was mixed with 1000 µl monoreagent (Composed of Tris pH 7.8; 120 mM/l, 2-Oxoglutarate-7 mM/l, ADP-0.6 mM/l, rease-6ku/l, Glutamate dehydrogenase-1ku/ 1 and NADH-0.25 mM/l) to incubate for approx. 60 sec. at 25ºC and absorbance was taken at 37ºC for standardization, then 10 µl sample is used above procedure and automatic calculated reading has taken (Standard kit method Mandal *et al.,* 2017; Burtisand Ashwood, 1999).

2.9.7.2 Biochemical estimation plasma Creatinine

The blood was centrifuged and plasma fraction was separated. Creatinine level of plasma measured by commercially available standard Blood Urea Kit (Merck, Japan) and by Semi-autoanalyser (Systronic, India) as per standard protocol for phtotometric determination of creatinine based on Jaffe kinetic method without deproteinization. First 100 µl of creatinine standard (1 mg/100 ml) was mixed with 1000 µl mono reagent (Buffer: NaOH-313 mM/l + 12.5 mM/l and Picric acid- 8.73 mM/l) to incubate for approx. 0-5 min at 25ºC and absorbance was taken at 37ºC for standardization then 100 µl sample was used above

procedure and calculated reading was taken (Standard kit method Mandal *et al.,* 2017; Sabbagh *et al.,* 1988).

2.9.8. Biomarker of kidney function test

2.9.8.1 Assay of urinary kidney injury molecule- 1 (KIM-1)

Principle:

This KIM-1 (HAVCR1) solid-phase, sandwich ELISA (enzyme-linked immunosorbent assay) is designed to measure and detect the amount of the target bound between a matched antibody pair. A target-specific antibody has been pre-coated in the wells of the supplied microplate. Samples, standards, or controls are then added into these wells and bind to the immobilized (capture) antibody. The sandwich is formed by the addition of the second (detector) antibody, binding to the target on a different epitope from the capture antibody. A conjugated enzyme has been incorporated into the assay. After incubation periods and wash steps to remove unbound antibody from the plate, a substrate solution is added that reacts with the enzyme-antibody-target complex to produce measurable signal. The intensity of this signal is proportional to the concentration of target present in the original specimen (Sun *et al.,* 2017).

Materials Required:

- Precision pipettors with disposable plastic tips to deliver 5-1000 µl and plastic pipettes to deliver 5-15 ml
- Ultrapure water for Wash Buffer and Standard reconstitution
- A glass or plastic 1L container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5 ml polypropylene or polyethylene tubes to prepare standards - do not use polystyrene, polycarbonate or glass tubes

- Disposable reagent reservoirs
- A standard ELISA plate reader for measuring absorbance at 450 nm and 550 nm. If a 550nm filter is not available, the
- Absorbance may be read at 450nm only. Refer to the instruction manual supplied with the instrument being used.

Procedure Summary:

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl standard and sample to wells. Cover plate and incubate at RT for 2.5 hours.
3. Wash plate four times.
4. Add 100 µl Biotinylated Antibody to wells. Cover plate and incubate at RT for 1 hour.
5. Wash plate four times
6. Add 100 µl of Streptavidin- HRP Reagent to each well.
7. Cover & incubate plate at RT for 45 min.
8. Wash plate four times.
9. Add 100 µl TMB Substrate to each well.
10. Develop plate at room temperature in the dark for 30 min.
11. Add 50 µl of Stop Solution to each well.
12. Measure absorbance and calculate results.

2.9.8.2 DNA fragmentation assay of kidney tissue

Principle:

This protocol provides a qualitative method for assessing cell death by detecting DNA fragments using agarose gel electrophoresis. One of the classic features of apoptosis is the cleavage of the genomic DNA into oligonucleosomal fragments represented by multiples of 180-200 bp. These visualizing fragments can aid in characterizing an apoptotic event. This method should always be combined with more quantitative methods in order to compare the degree of apoptosis among the experimental samples (Patra *et al.,* 2014).

Reagents and equipments:

- Agarose
- 6X gel-loading buffer
- Ethidium bromide
- Proteinase K (20 mg/ml; Ambion)
- RNase Cocktail (RNase A and T1 at 500 units/ml and 20,000 units/ml,
- respectively; Ambion)
- TAE
- TES lysis buffer
- Standard gel electrophoresis equipment
- UV transilluminator
- Wide-bore pipette tips

Procedure:

1. Transfer 5×105 cells to 1.5-ml sterile microcentrifuge tubes. Centrifuge at 2000 rpm in an Eppendorf table top centrifuge for 5 min at 4°C and remove supernatant.

2. Add 20 µl of TES lysis buffer. Mix cell pellet with TES lysis buffer by stirring with a wide-bore pipette tip.
3. Add 10 µl of RNase Cocktail and mix well by flipping the tip of the tube. Do not vortex. Incubate for 30-120 min at 37°C.
4. Add 10 µl of proteinase K, mix by flipping the tip of the tube, and incubate at 50°C for at least 90 min or, if preferred, overnight.
5. Add 5 µl of 6X DNA loading buffer and load DNA samples into dry wells of a 1-1.5% agarose gel in TAE containing 0.5 µg/ml ethidium bromide.
6. Run the gel at a low voltage, which improves resolution of DNA fragments (i.e., 35 V for ~4 hours or until loading dye has run two-thirds of the way down the gel).
7. DNA ladders are finally visualized by a UV light source and documented by photography. Apoptotic cells will form a distinct DNA ladder, whereas necrotic samples may generate a smear pattern (or no DNA fragmentation). DNA from viable cells will stay on the top of the gel as a high-molecular-weight band.

2.10. Morphometric Analysis:

2.10.1 Histological analysis

For histological study, kidney and liver of all the animals from all the groups of respective experiment were dissected out and then fixed in Bouin's solution. After fixation tissues were embedded in paraffin wax. Sections were prepared in microtome (weswox) at 5µm thickness. All the sections were finally stained with haematoxylene eosin as per standard protocol and then examined under compound microscope at 40x magnification for detection any pathological and morphological changes (Mani, 2010; Khorsandi and Orazizadeh, 2008; Adeneye *et al.,* 2008; Lee *et al.,* 2006).

2.10.2 Scanning electron microscopic analysis

Fresh intestinal tissue was rinsed with cold saline (0.9% NaCl) and cut into 5 mm×5 mm sections, fixed in 2.5% glutaraldehyde, 10% osmium, and dehydrated in sucrose solution containing PBS. Then it was gold coated and observed under scanning electron microscope (ZEISS, IIT-Kharagpur). The arrangement of microvilli, deformed and exfoliated villi and the intercellular space between epithelia were examined (Adak *et al.,* 2014).

2.11. Statistical Analysis

Statistical analysis of experimental data by sigmaplot 11.0 (USA) software. One-way ANOVA and the multiple comparisons of all possible pairs will do by Tukey t test (SPSS-10.0). The alteration in bacterial quantity was tested by Bonferroni for post hoc testing. Significant variation was accepted at the level of 5 %, i.e. $p<0.05$.