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Gut microbe characterization of mutant substrains of wistar/NIN rat

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Abstract
Obesity is an increasing burden on the health system, where several animal models and treatments have been tested. However, it is clear that an appropriate model is needed to understand the development of metabolic syndrome and its progression. Animal models are always helpful in discovering new means to diagnose problems or solve health issues. In our institute, two sub-strains of WNIN mutant rats have been identified, maintained and physically and physiologically characterised as animal models for the study of obesity and diabetic diseases. This study sought to characterise the composition of the gut microbiota population and its correlation with the phenotypes established at ICMR-National Institute of Nutrition, Hyderabad, India. Samples were obtained from three substrains (N=6) of Wistar/NIN rats (lean control, Ob/ob, Gr/ob). Haematological and serum biochemical parameters were analysed to obtain baseline data for blood parameters. PCR was performed to determine the population of intestinal microbes. Interestingly, it was found that there were significant changes in the gut microbiota of these substrains (Ob/ob, Gr/ob and WNIN-lean control) compared to the WNIN-lean control group. This study points to the role of gut microbiota in relation to physiological and pathological conditions in these mutant strains that lead to the development of obesity under controlled environmental conditions such as diet and water. The Firmicutes are comparable in all groups, while the changes in the other gut microbiota in Ob/ob, Gr/ob clearly demonstrate that these animals are more prone to develop obesity and diabetes.

Keywords: Wistar rat, obese rat, gut microbes, wistar/NIN-Ob/ob

Introduction
Obesity is a multifactorial disease in which inherited allelic variations together with environmental variations determine the individual predisposition to develop the disease. The rate of obesity and obesity-related diseases is increasing day by day, leading to unnecessary burdens on the health care system, which in turn affects the gross domestic product (GDP) of countries. Obesity is one of the three (triple) burdens of malnutrition that affects the productivity of countries in terms of labour output and reproductive performance. Obesity is a multifactorial eating disorder that can be caused by various risk factors and dietary habits or patterns.

Several developmental pathways have been discovered in obesity, and yet obesity remains a mystery to us. The reason for this could lie in the changes in genetic predisposition and genotypic variation in a randomised population. Thus, genetic predisposition could be the reason for changes in the body's metabolism and dietary habits that lead to changes in the gut microenvironment, which in turn causes dysbiosis in the gut microbiome. Studies have not yet deciphered the composition of the gut microbiota in inherited allelic variation and its association with body weight and/or phenotype. This study aims to investigate the composition of the gut microbiota in WNIN mutant rat strains in a controlled environment, such as food and water, and its possible correlation with phenotype.

Materials and Methods
The mutant WNIN substrains were obtained from the ICMR-NIN animal facility and the experiment was performed after obtaining IAEC approval (No. ICMR-NIN /IAEC/2021-1/005). 10-week-old male animals of three phenotypes (n=6/substrain) WNIN-Ob/ob (obese and euglycaemic), WNIN-Gr/ob (obese and impaired GTT) and WNIN-Lean (control) were used. The animal studies were conducted in accordance with the CCSEA guidelines.

WNIN rat strains have been kept at the ICMR-NIN animal facility for over 7 decades. Physical and physiological characterisation of the two sub-strains of WNIN (i.e. Ob/ob and Gr/ob) was

carried out (Figure 1). All animals were housed in polycarbonate cages with controlled environmental conditions such as temperature (22+/-2 °C, humidity 45 - 55% and 12 hr: 12 hr day and night cycle). Water and feed (AIN -93G) were provided ad-libitum to all study groups. Feed intake was assessed daily and the body weight of the animals was measured every week using a standard electronic scale (brand: Sartorius, Germany, with an accuracy of 0.1 grams).

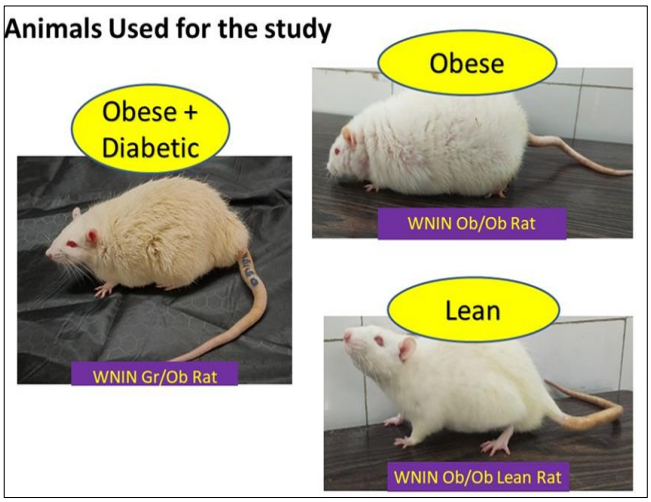

Fig 1: NIN/Wistar Rat Mutant Substrains used for this study

Haematological and Biochemical assay

Haematological and biochemical parameters were analysed in circulating blood. Blood was collected via the retroorbital vein in a normal tube for biochemical serum tests and in EDTA-coated tubes for haematological tests. Serum levels of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides (TG) were determined using a clinical automated analyser (Cobas C311, ROCHE).

Blood was collected in an EDTA tube and subjected to haematological analysis with an automatic blood cell counter (Advia 120, 5-part automatic haematology differential analyser, Siemens). Haemoglobin content (Hb), total white blood cell count (WBC), red blood cell count (RBC) and differential leucocyte count (DLC) were analysed to assess the effects of the gut microbiome on blood parameters. The animals were euthanised with a CO_2 asphyxiation chamber and the caecum was removed for gut microbiome analysis.

Extraction and purification of DNA from Cecal content

The (QIA amp Fast DNA Stool Mini Kit) (Qiagen 51604) was used to extract DNA from the caecal contents of all rats according to the manufacturer's instructions (Figure 2). The quality and quantity of isolated DNA were determined at Å260 nm/Å280 nm using a Nanodrop spectrophotometer (Thermo Scientific NanoDropTM 2000/2000c). The DNA samples were stored at -20 C until further processing for PCR.

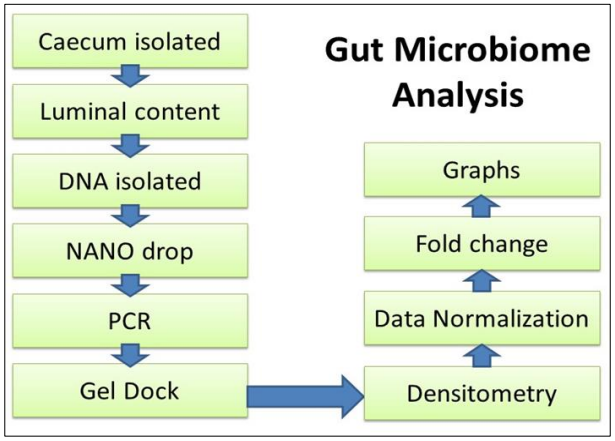

Fig 2: Steps in gut microbiome analysis

The composition of the gut microbiota was investigated by semi-quantitative PCR and DNA amplification with standard primers (Table 1) adapted from earlier publications [1, 2]. PCR products were analysed on agarose gel electrophoresis and amplicons were recorded using the gel documentation system (Syngene, G Box) (Figure 3). The amplicons were quantified densitometrically using Image J software (Fiji version) and graphed (Figure 4).

Table 1: Primers used in the study for PCR on caecal microbiota

Target organism	Primer	Sequence (5 ⇒ to 3 ⇒)	PCR product bp
Bacteroides-Prevotella group	BacF	GAAGGTCCCCCACATTG	410
	BacR	CAATCGGAGTTCTTCGTG	
Lactobacillus group	LacF	AGCAGTAGGGAATCTTCCA	341
	LacR	CACCGCTACACATGGAG	
Bifidobacterium	BifF	GCGTGCTTAACACATGCAAGTC	126
	BifR	CACCCGTTTCCAGGAGCTATT	
Firmicutes	FirF	AGYATGTGGTTTAATTCGAAGCA	126
	FirR	AGCTGACGACAACCATGCAC	
All bacteria	UnivF	TCCTACGGGAGGCAGCAGT	466
	UnivR	GACTACCAGGGTATCTAATCCTGTT	

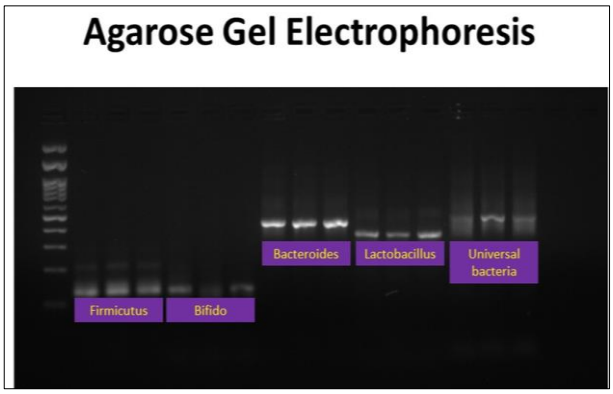

Fig 3: Gut Microbiome Gel Electrophoresis Image captured using the Gel Dock system

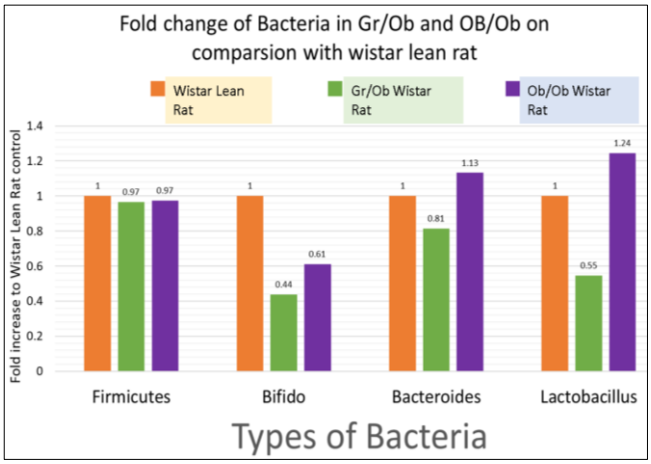

Fig 4: Densitometry of Gut Microbiome in Fold Change

Results and Discussion

Obesity is a chronic disease caused by an unhealthy diet, lack of exercise and family eating habits. Although earlier researcher from our group has published significant data on these mutant strains of obesity [3], here in the present unique data on gut microbiota as it shows a unique pattern unveiling the answers for phenotypes. the physical and physiological parameters of WNIN mutant strains were further investigated and the most striking feature of these animals is the variation in body weight (Figure 5).

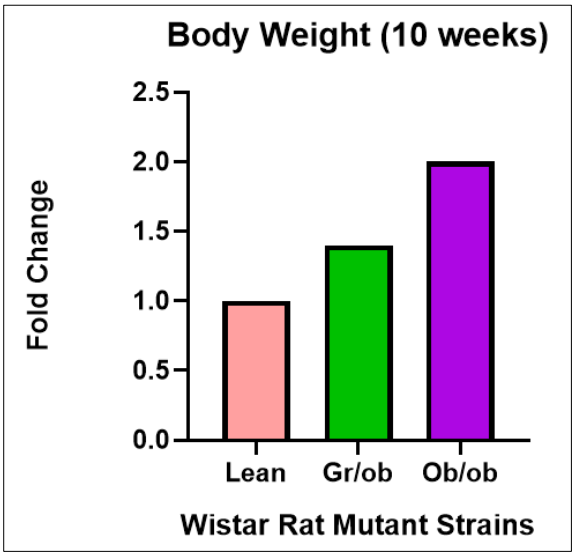

Fig 5: Body weight of Wistar mutant strains (in fold change)

Nevertheless, it was observed that the Ob/ob and Gr/ob animals gained more weight at a younger age compared to the WNIN-lean control animals, even though they were kept in the same environment and fed the same diet (1.5 times more for Gr/ob and 2 times more for Ob/ob). Additionally, haematological profiling revealed that there is a active and chronic inflammation observed in obese mutant strains of rat (Table: 2).

Table 2: Haematological profiling of Wistar mutant strains

Species	Lean	Gr/ob	Ob/ob
PLT (10E3/mm3)	1054.5±229	987.8±138.9	743.3±117
Neutrophils (%)	12.8±2.9	21.3±3.6	28.2±6.6
Lymphocytes (%)	79.9±4.5	68.8±5.1	57.5±3.6
Monocytes (%)	5.4±1.6	8.2±1.7	10.5±3.9
Eosinophils (%)	1.6±0.9	1.4±0.2	3.5±0.3
Basophils (%)	0.3±0.2	0.2±0.1	0.2±0.1

WBC (10E3/mm3)	8.7±1.3	7.7±1.2	8±1.9
RBC (10E6/mm3)	6.9±0.2	7.2±0.5	6.7±0.3
HGB (g/dl)	13.9±0.3	14.4±1	13.8±0.3
HCT (%)	44.6±1	46.3±3	45.1±1.8
MCV (µm3)	64.7±2	64.4±0.9	67.3±3.8
MCH (pg)	20.1±0.3	20±0.1	20.5±0.7
MCHC (g/dl)	31.1±0.9	31.1±0.4	30.5±0.8

The reason for this could be allelic variation, as they are mutants of the WNIN rat. In this study, the composition of the gut microbiota in the caecum was found to be different in these mutant animals.

Bacteria, especially from the gut, are known to have a strong influence on obesity through various mechanisms such as energy utilisation and secretion of short-chain fatty acids (SCFAs) [4]. Strain strength or strain ratio has been observed to cause significant changes in obesity by affecting the gut barrier [5] and metabolic outcomes [6, 7].

Of the four organisms studied, the Firmicutes were comparable and showed no changes in the three rat strains. Bifidobacteria were reduced to half in both Gr/ob and Ob/ob rat strains when compared to their lean counterpart. Bacteroides and Lactobacillus were reduced in Gr/ob and increased in Ob/ob compared to lean rats. No change in Firmicutes in these animals suggests that they are known to maintain a healthy gut by producing an important substance butyrate, a short-chain fatty acid. However, bifidobacteria were reduced by half in both the Gr/ob and Ob/ob rat strains. Bifidobacteria are important bacteria in the gut and play an important role in digesting fibre, helping to prevent infection and producing vitamins and other important chemicals [8, 9]. Therefore, possibly due to the decrease in bifidobacteria, these two strains have shown an increase in obesity and a decrease in lifespan to 50% compared to WNIN-lean. Interestingly, the results of this study are consistent with many other studies reporting the role of these bacteria in promoting longevity [10] and obesity [11].

Interestingly, Gr/ob and Ob/ob showed changes in the opposite direction in Bacteroides compared to the WNIN-lean control. The literature suggests that Bacteroides are particularly abundant in obese individuals and that their abundance is positively correlated with BMI [12]. In terms of phenotype, Gr/ob is relatively less obese compared to Ob/ob. On the other hand, Lactobacillus decreased by almost 50% in the Gr/ob group compared to the WNIN-lean control group, while Ob/ob showed an increase of about 20%. A number of researchers have claimed that Lactobacillus alleviates diabetes through multiple mechanisms such as the regulation of glucose, lipids and gut microbiota [13]. Thus, the decline in Gr/ob rats indicates that they are more susceptible to diabetes.

The variation in the gut microbiome suggests that the phenotype in these mutant stains of obesity has a strong association with body weight development and no change in firmicutes between the three rat strains indicates a protective effect. Another point of interest is the reduced Lactobacillus in the prediabetic rat mutant, which is more prone to develop diabetes compared to the other two strains studied.

Summary and Conclusions

As we know, there are various communicable and non-communicable diseases that affect our daily lives in one way or another. Sometimes they can even lead to death. The most common factor, as noted by a researcher, is obesity, where fat is deposited under the skin, called obesity. In layman's terms, obesity is a condition in which one is very fat or overweight. However, in scientific parlance, a BMI of 30 or higher is considered obese. Several causes of our obesity are thought to exist, but it is a great mystery to scientists whether the egg or the chicken came first. Again, the question is: is it genetics first or is it nutritional disorders?

Several parameters in the serum biochemistry are very high or low, but clinically no signs of disease or obesity-related comorbidity were observed in the initial phase. The mechanism preventing obesity-related organ damage in these strains is interesting and should be further explored. The bacterial population changes significantly in the three sub-strains of the same strain (Wistar). The decline of Lactobacillus and Bacteroides in Gr/ob could be the cause of diabetes, or it could be the effect of overeating, this needs further investigation. The data collected here is unique and will further unravel the pathways of protection and susceptibility to obesity.

Conflict of Interest

The authors have no conflict of interest in publishing this original manuscript.

Acknowledgements

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