Materials and Methods

3.1. Ethical approval

For the approval of experimental works, research synopsis was presented before the Institutional Animal Ethics Committee of Bodoland University. Ethical clearance for the experimental work was offered by the Institutional Animal Ethics Committee of Bodoland University vide its letter no. IAEC/BIOTECH/2019/3 (Appendix-I).

3.2. Morphological authentication of Doom and Ghungroo pig breeds

Two pairs each of adult male and female pig breeds of Doom and Ghungroo were morphologically identified by Dr. Sulav Sarma, veterinary officer at the state veterinary dispensary, Tangla, Assam (Appendix-II). The photos and videos of the adult pigs of the two breeds were scientifically validated and confirmed as Doom and Ghungroo by Dr. Santanu Banik, Principal Scientist of ICAR-National Research Center (NRC) on Pig located at Rani, Guwahati, Assam (Appendix-III). Dr. S. Banik observed the morphological features such as body coat colour, hair, appearance, ears, snout, belly shape, teat numbers and its position and tail features by following the protocols for identification of live animals. The verified Doom pigs were from Murmela village of Tangla, while the Ghungroo pigs were from Belguri village of Tangla, both villages under Udalguri district. Following the verification of both breeds they were allowed to mate. The litters of the pairs were used for the research work.

3.3. Selection of the sex of the litters

After the verification of Doom and Ghungroo pig breeds. Female piglings of the litter were isolated and only male litter were selected for the study. Doom and Ghungroo litters were born in the month of August, 2019. So, when pigs were 60 days old (approx. 2 months), they were shifted to the rearing site at the month of October 2019 for experimental works. The full-sibling piglets, originating from a single litter, were subsequently acclimatized and accustomed to the experimental diets. The experimental and control Doom and Ghungroo piglets were reared at Belguri village (Fig. 1.d.) located in Udalguri district of Assam.

3.4. Acclimatizing of the pig breeds

The pigs were acclimatized for 7 days (Smith and Swindle, 2006) following its shifting to the rearing site. A non-standard diet was supplied three times a day, during the acclimatization period consisting of commercially available 'grower' feed, rice-bran and polish rice. An *ad libitum* water was provided throughout the day.

3.5. Period of experiment

The period of experiment was from October 2019 to May 2020, starting on the 8th day after the acclimatization period. Therefore, the duration of rearing of pig breeds at the rearing site was 8 months (244 days). The experimental feeding period, using control and trial diet lasted for 237 days excluding the acclimatization days.

3.6. Management of the experimental pigs

Throughout the rearing period, the pigs were provided with *ad libitum* water and kept in a semi-pucca enclosure with 6 numbers of pigs in each. The open, aerated tin roof was provided. Hygienic surrounding was maintained where the area was cleaned every day to get rid of pig stools and other unwanted dirt. Minimum exposure to humans and to other animals was maintained. The pigs were handled by designated two caretakers on a daily basis for cleaning and feeding purposes (Appendix-IV, showing rearing of Doom and Ghungroo pigs).

3.7. Stages of growth observed in pigs

According to Ranjhan, S.K. (1981) three stages of growth was observed when considered to Indian pig breeds. These stages recommended by Ranjhan was based on conditions like different agro-climatic conditions and genetic makeup of the pigs that influence the body weight of the pigs. The age of pigs suggested for these three stages are tabulated in Table 3.1. Accordingly, based on these age groups, the commercially available rations are supplemented including other feed ingredients. These rations are for pre-weaned stage - pre-starter, post-weaned stage- starter, for growing stage-grower and for finishing stage - finisher rations.

Table 3.1. Stages of growth observed in pigs and their age

Stages/Phases	Weaning		Growing	Finishing
	Pre-weaned	**Post-weaned**		
In days	Birth to 25 – 27 days	28th to 61 days	62 to 153 days	154 days to slaughter age
In months	2 months		3 - 5 months	6 months to slaughter age

3.8. Allocation of pigs for experimental diets

A total of 24 pig were reared, of which 12 were Doom pigs and other 12 were Ghungroo pigs. The experimental animals were grouped into four, with six pigs in each group. Six numbers of Doom pigs were provided for control diet (C 1) and other six were for trial diet (T 1). Similarly, six numbers of Ghungroo pigs were provided for control diet (C 2) and remaining six were for trial diet (T 2). The experimental design of feeding experimental diets is tabulated in Table 3.2.

Table 3.2. Allocation of pigs for experimental diets

Total nos. of male pigs = 24			
Doom		**Ghungroo**	
12		12	
Control diet (C1)	Trial diet (T1)	Control diet (C2)	Trial diet (T2)
6	6	6	6

3.9. Experimental diets

The experimental diets supplied to the rearing pigs was prepared according to the stages of growth observe in Indian native pigs (Table 3.1.). As the male piglets were reared from the month of October 2019, two months after their birth, they had already completed the weaning stage. Therefore, experimental diets were supplied from grower stage and continued till finisher stage. At the grower stage, the pigs were supplied with experimental diets from the 8th day, after acclimatizing for 7 days. The experimental diets consisted of a control and trial diets. While preparing the experimental diets, the amount

of the feed ingredients particularly, the mineral mixture (not more than 2.5 kg) and common salt (0.5 kg) was maintained as suggested in Bureau of Indian Standard (BIS), 2001. While the quantity of rice-bran and rice polish was randomly balanced to make it a total of 100 kgs. The trial ration feed (kitchen waste) according to BIS should not exceed 10 %; hence, to ensure compliance with these standards, the experimental trial ration supplied to experimental pigs was set at 5 % (5 kg) only.

3.9.1. Control diet

The feed ingredients of control diet were rice-bran, rice-polish, mineral mixture, common salt and commercially available 'grower' feed. The grower feed is provided as a control diet (Table 3.3. and Table 3.4.). All the feed ingredients were combined to make a total of 100 kg, then mixed with 1 litre of clean water.

3.9.2. Trial diet

In Assam, many households from ethnic communities efficiently utilize kitchen waste as pig feed. This practice serves as a cost-effective alternative to expensive concentrated feeds while also contributing to environmental sustainability by reducing waste pollution. This traditional practice not only supports rural livelihoods but also aligns with eco-friendly waste management principles.

Therefore, in this study kitchen waste is supplied as an experimental trial ration. The feed ingredients of trial diet were same as that of control, only the experimental control ration was replaced by trial ration, which is the kitchen waste. All the feed ingredients were combined to make a total of 100 kg, then mixed with 1 litre of clean water (Table 3.3. and Table 3.4.). Both the control and trial diets were supplied to Doom and Ghungroo pig breeds at grower and finisher stages. These diets were provided three times a day with *ad libitum* water.

A statistical comparison of growth-related parameters was conducted between Doom and Ghungroo pig breeds. The analysis was performed separately for the grower and finisher stages using an independent t-test at a 95 % confidence level.

3.9.3. Preparation of kitchen waste feed for pigs

The kitchen waste was collected in a clean container; major part of the kitchen waste consists of leftover rice and others were vegetables. Kitchen waste (fresh weight) is later mixed with the feed ingredients as mentioned in Tables (3.3. and 3.4.) and served to pigs three times a day. According to Bureau of Indian Standards (BIS, 2001), the kitchen waste

Table 3.3. Ingredients of feed and its composition for growing stage (3 to 5 months).

Ingredients of feed (Kg)	**C 1 (Doom)**	**C 2 (Ghungroo)**	**T 1 (Doom)**	**T 2 (Ghungroo)**
Rice bran	50	50	50	50
Rice Polish	42	42	42	42
Mineral mixture	2.5	2.5	2.5	2.5
Common Salt	0.5	0.5	0.5	0.5
Experimental rations	5 (grower)	5 (grower)	5 (kitchen waste - fresh weight)	5 (kitchen waste - fresh weight)
Total	**100**	**100**	**100**	**100**

Table 3.4. Ingredients of feed and its composition for finishing stage (6 to 8 months).

Ingredients of feed (Kg)	**C 1 (Doom)**	**C 2 (Ghungroo)**	**T 1 (Doom)**	**T 2 (Ghungroo)**
Rice bran	50	50	50	50
Rice Polish	42	42	42	42
Mineral mixture	2.5	2.5	2.5	2.5
Common Salt	0.5	0.5	0.5	0.5
Experimental rations	5 (finisher)	5 (finisher)	5 (kitchen waste - fresh weight)	5 (kitchen waste - fresh weight)
Total	**100.35**	**100.35**	**100.35**	**100.35**

products as animal feed should not to exceed 10 %; hence, to ensure compliance with these standards, this threshold was maintained and the experimental trial ration supplied to experimental pigs was set at 5 % only.

3.10. Molecular characterization of the pig breeds

Molecular characterization of the Doom and Ghungroo pig breeds was performed based on mitochondrial DNA using '*cytochrome b*' gene as a molecular marker.

3.10.1. Sample collection

Blood samples were collected with help of the certified veterinarian of Tangla, Udalguri, Assam. The blood samples of all the reared pigs were collected at 5th month. A half inch (1/2) needle of 20- gauge was used to draw blood samples from the pig breeds. Blood from the auricular vein of the ear was collected in EDTA vials. A total of 24 blood samples were collected (12 from Doom and 12 from Ghungroo pig) following protocols as mentioned in Reicks et al. (2006). The blood samples were stored in -20°C, until further analysis.

3.10.2 Isolation of genomic DNA

Genomic DNA was extracted from blood samples following the manufacturers protocol, provided along with the DNeasy Blood and Tissue (QIAGEN, Germany) extraction kit. The buffers and enzyme in the kit were Wash buffers (aW1 and aW2), Lysis buffer (Buffer AL), Elution buffer (Buffer AE) and a Protease enzyme.

- In 1.5 mL of Eppendorf tube, 20 µL of QIAGEN protease enzyme was mixed with 200 µL of sample.
- 200 µL of lysis buffer was added to the tube and vortexed for 15 seconds and incubated at 56° C for 10 minutes.
- After this, few seconds of spinning was performed to remove the droplets stuck in the lid.
- 200 µL of ethanol was added and the mixture was vortexed for another 15 seconds, followed by a quick centrifugation again to remove droplets from the lid.

- Next, the mixture was transferred to a spin column placed in a 2 mL collection tube and centrifuged at 8000 rpm for 1 minute.
- Collection tube was discarded and spin column was then moved to a new collection tube, and after adding 500 µL of buffer AW1, it was centrifuged again at 8000 rpm for 1 minute.
- Again, collection tube was discarded and spin column was placed in a new collection tube of 2 mL.
- 500 µL of buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes.
- Spin column was placed in a 2 mL Eppendorf tube and the old collection tube was discarded.
- 200 µL of buffer AE was added and incubated at room temperature for 1 minute, then centrifuged at 8000 rpm for 1 minute.
- The spin column was removed and DNA was eluted.
- For quality assurance, the extracted DNA was analyzed on a 0.8 % agarose gel containing ethidium bromide. The DNA was stored at -20°C until further use.

3.10.3. DNA Quantification

The quantity and purity of the isolated DNA of 24 samples was analysed in UV-VIS Spectrophotometer (Shimadzu Corporation, Model no. 80865). The DNA samples were diluted in nuclease free water and absorbance was measured at wavelengths 260 nm (A_{260}) and 280 nm (A_{280}) respectively. Ratio of A_{260} and A_{280} provided the purity of DNA and only A_{260} provided the quantity of DNA. The calculation for DNA quantity and purity was done following the formula:

DNA quantity (µg/mL) = A260 × 50 × DF (dilution factor)

DNA purity = A_{260}/ A_{280}

If A_{260}/ A_{280} ≥ 1.8, it designates pure DNA.

3.10.4. Amplification of PCR products

Cytochrome b gene sequences were amplified using universal primers listed in Table 3.5. PCR amplification was performed in a MiniAmp Plus Thermal Cycler (Applied BioSystems). PCR amplification was conducted in a final volume of 25 µL with

100 ng of extracted DNA, using 2.5 µL of 10X PCR buffer, 0.5 µL of 10 mM dNTPs, 5 pmol/µL of each forward and reverse primer and 3 units/µL of Taq DNA polymerase (Applied Biosystems). The PCR was performed using the following conditions: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 50 s, extension at 72°C for 1 minute with a final extension of 72°C for 7 minutes. The PCR products were loaded on to 2 % agarose gel containing ethidium bromide. The gel was visualized at Gel documentation system (Life Technologies).

Table 3.5. Universal primers for the amplification of '*cytochrome b*' gene

***Cytochrome b* gene primer sequences**	**Size of the primer**	**Reference**
Forward primer: 5'TACCATGAGGACAAATATCATTCTG3' Reverse primer: 5'CCTCCTAGTTTGTTAGGGATTGATCG3'	437 bp	Verma and Singh, 2003

3.10.5. Agarose gel electrophoresis of PCR amplified products

The amplified DNA were run on a horizontal electrophoresis at 100 V for 40-45 minutes. In a 2 % of agarose gel containing ethidium bromide the gels were visualized in a Gel Doc (Gel Documentation System) (Life Technologies). The photograph of the gels was recorded for documentation.

3.10.6. DNA sequencing

The PCR products were sequenced using Automated DNA Sequencer (Model 3730XL, from Applied BioSystems, USA) at CSIR-Centre for Cellular and Molecular Biology (CCMB), Laboratory for Conservation of Endangered Species (LaCONES), Hyderabad, India. Prior to sequencing of the PCR products, the concentration of the PCR product is determined by running it on an 2 % agarose gel. Purification of the PCR products was done before sequencing reaction by ExoSAP-IT PCR cleanup product.

3.10.6.1. Procedure for PCR cleanup (for 96-well plate)

ExoSAP-IT reagent was kept on ice throughout the process. 50 µL ExoSAP master mix and 50 µL PCR product was added in each well followed by short spin.

Followed by incubation at 37°C for 15 minutes to degrade left-over nucleotides and primers, another incubation was done at 80°C for 15 minutes to inactivate ExoSAP-IT reagent. The obtained PCR product was sequenced.

3.10.6.2. Procedure for Sequencing

For sequencing process, a Big Dye buffer premix was prepared. In a 1.5 mL Eppendorf tube, 25 µL of Big Dye dissolved in 175 µL of distilled water. 100 pM stock solution of sequencing primer was diluted with sterile distilled water in ratio 1:10. 2 µL of the diluted sequencing primer was pipetted into PCR plate. Then 1.8 µL of the Big Dye Ready Reaction Termination Mix was pipetted into each plate. The plates were placed in thermal cycler. The duration and temperature conditions maintained during the PCR sequencing is presented in Table 3.6.

Table 3.6. Duration and Temperature conditions maintained during PCR sequencing.

Temperature	Duration
96 ° C	10 seconds
50 ° C	5 seconds
60 ° C	4 min
4 ° C	10 min

3.10.6.3. DNA precipitation and post-processing for Sequencing

A precipitation mixture of 3 mL 100 % ethanol with 250 µL of sodium acetate was prepared. 25 µL of the precipitation mixture was added into each well, followed by brief vortex of the mixture and storing at room temperature for 30 minutes. The tubes were allowed to spin for 20 minutes at 4000 rpm in a micro centrifuge. The supernatants were aspirated completely in a separate pipette tip for each sample. 100 mL of 70% ethanol was added to the tubes and briefly vortexed, followed by centrifugation at 3500 rpm for 20 minutes and again the supernatants were aspirated. The plate was spinned inverted at 200 rpm for 30 seconds. The plates were air dried for an hour. After the complete drying of the plates, the wells were suspended with 25 µL of Hi-Di formamide. Followed by placing the plate in an Automated DNA sequencer.

3.10.6.4. Processing of raw data

The Automated DNA Sequencer designed to sequence and detect the amplified DNA converts the data into a graph that is easy to interpret is called electropherogram. The peaks resulted in an electropherogram are saved as ABI files. ABI files were edited with CodonCode software. To obtain the data in FASTA format, the following steps were followed:

- In the CodonCode software, ABI files were opened, sample files were clicked creating a new project option.
- New project options show the electropherogram of the samples depicting the peaks and tracks of each nucleotide of the DNA sequence.
- Height of the peak was adjusted in the software with zoom option.
- Strong and poor quality of peaks present at the ends of the trace were observed and noted.
- The corresponding nucleotide was edited according to the compliment strand sequence.
- Consensus sequence was generated where samples were sequenced, in forward and reverse directions, checking the accuracy of the sequence.
- The edited sequences were saved in the project file and exported in FASTA format.

3.11. Submission of nucleotide sequences to NCBI

After the amplification, the PCR products were sequenced in a DNA Sequencer. The data obtained in the electropherogram was later converted to FASTA format with the help of Codon-Code software. The *cytochrome b* gene sequences of Doom and Ghungroo pig breeds obtained in FASTA format were submitted to NCBI (National Centre for Biotechnology Information) for generating accession number.

3.12. Analysis of nucleotide sequences

BLASTN (Basic Local Alignment Search Tool for Nucleotide) search was performed on the Doom and Ghungroo pig *cytochrome b* gene sequences to generate similarity scores (i.e., 90 to 100 %) with other suidae sequences available at NCBI. The

cytochrome b gene sequences samples of present study along with sequences retrieved from gene bank were run for multiple sequence alignment and pairwise sequence alignment using Clustal W (Thompson et al., 1994).

3.12.1. Nucleotide variation sites of the sequences

Among all the models tested using MEGA 11, the General time reversible (GTR+G) using discrete Gamma distribution model has the lowest Bayesian Information Criterion (BIC) score. Therefore, it is regarded as the most suitable model for nucleotide substitution to determine the variation sites.

3.12.2. Genetic distance analysis of the sequences

Kimura 2-parameter model was used to generate the genetic distance between the samples of the current study and all the suidae sequences retrieved from gene bank (Kimura, 1980; Tamura et al., 2021).

3.12.3. Phylogenetic analysis of the sequences

Neighbor-Joining tree method was applied for phylogenetic construction, bootstrapping at 1000 replications using Kimura-2 parameter using MEGA 11 (Tamura et al., 2021).

3.13. Measurement of body weight and other growth parameters

The changes observed in body weight and other growth parameters of Doom and Ghungroo are recorded during the time of rearing. Other growth parameters are chest girth (CG), height at withers (HAW), paunch girth (PG) and body length (BL).

Chest girth (CG) is the circumference of the chest located directly behind the forelegs. Height at wither (HAW) is the height from the ground to the highest point of its withers, which is the ridge between its shoulders. Paunch girth (PG) is also the circumference of pig taken just in-front of hind legs. Body length (BL) is the measurement of the straight line from the occipital bone (midpoint between ears) to the base of the tail. The live body weight of pigs was taken using a digital weighing balance and other growth parameters like CG, HAW, PG and BL was measured with measuring tape (Appendix-

V). The changes observed in body weight and other growth parameters were measured at three stages of growth (weaner, grower and finisher).

The first measurement was taken when the piglets were 15 days old. The first measurement was taken when the piglets were 15 days old. During the grower stage, two measurements were taken: the first at 8 days after a 7-day acclimatization period, and the second at 154 days of age. The third measurement was taken at 244 days of age.

3.14. Slaughtering of pig breeds

The pigs were slaughtered at the age of 8 months with live weights of approximately 49.27 kg for Doom and 63.23 kg for Ghungroo pigs. Prior to slaughter, the pigs were fasted for 12-16 hour with *ad libitum* supply of water. Pigs were slaughtered in a commercial abattoir for poultry and swine in Tangla market, Assam, with the slaughtering process adapted for pigs guided by a certified veterinary officer. By applying low-voltage electric shock on head, the pigs were stunned, then restrained by the left leg and bled out while suspended vertically on an overhead rail. Following that, the pigs underwent scalding at 65°C, after which their hair was removed using an automatic dehairing machine. After the slaughter, pig carcasses underwent scraping, washing, splitting, evisceration, and chilling in accordance with standard commercial practices. Samples were collected from both the sides of the carcass i.e., left and right, identified and stored at freezer of - 20°C until the analysis of parameters.

3.14.1. Collection of tissue samples

For meat quality analysis in muscles and viscera, following the slaughter of the pig breeds, six muscles and six viscera were dissected. The six muscles selected for this study was based on popular primal pork meat cuts and high demand by local pork consumers. The weight of dissected muscles and viscera are given in Table 3.7.

Table 3.7. Weight of six dissected muscles and viscera

Muscles	Anatomical region	g (mean ± SEM)
Triceps brachii	Shoulder	291.91± 42.0
Latissimus dorsi	Shoulder	313.3 ± 9.46
Biceps femoris	Ham	305.95 ±17.79

Gracilis	Ham	306.73 ±19.73
Tensor fasciae latae	Ham	300.7±10.42
Longissimus dorsi	Loin	169.23±9.4
Viscera		
Heart	-	41.66±1.45
Liver	-	900.33±63.80
Kidney	-	201±1.52
Spleen	-	98.66±0.88
Small intestine	-	562.66±31.52
Large intestine	-	1696±54.30

The dissected tissues were stored at freezer of -20 °C. 24-hour post-mortem, the subcutaneous fats from all the samples were trimmed off by knife, and homogenized using a mechanical grinder. Following removal of the fats, samples were again stored at -20°C until further analysis.

3.15. Methods of biochemical analysis of the samples

3.15.1. Preparation of kitchen waste sample

Kitchen waste samples were randomly collected from neighbouring households once daily throughout the experimental period. Kitchen waste mainly consists of leftover rice and vegetables. Similar types of leftover vegetables were maintained during the collection. Immediately after collection, the wastes were homogenized thoroughly for subsequent analysis. 200 g of the kitchen waste was dried in hot air oven at 60° C overnight. Following its complete drying, it was grinded in a mortar. The sample was sieved in 1 mm mesh size sieve according to 950.02 (AOAC, 1990) method and analysed for proximate compositions.

3.15.2. Analysis of moisture and dry matter in kitchen waste

AOAC (2002) method was applied to estimate the moisture content in the kitchen waste sample. 2 g of the grinded sample was taken in triplicates and shaken for even distribution throughout the container. Then the samples were oven dried at 105°C for 2 hours. Before calculation, the samples were placed at desiccator for cooling. After

obtaining the results of moisture content, dry matter was estimated by subtracting with 100.

Calculation:

$$\text{Moisture \%} = \frac{(W1-W2)}{W1} \times 100$$

where, W1 = weight (g) of sample before drying in grams
W2 = weight (g) of sample after drying in grams

Dry matter (%) = 100 – Moisture (%)

3.15.3. Moisture content in tissues

AOAC (2005) method was applied to estimate the moisture content in tissues (six muscles and six viscera) samples. 3 g of the sample was taken and spread equally on all sides followed by drying in hot air oven for 3 hours at 105° C. Sample was cooled in a desiccator and weighed. The differences in the weight before and after, determined the moisture loss in the sample and are expressed in percentage of the dried sample.

Calculation:

$$\text{Moisture \%} = \frac{(W1-W2)}{W1} \times 100$$

where, W1 = weight (g) of sample before drying
W2 = weight (g) of sample after drying

3.15.4. Estimation of protein content

AOAC (2002 and 2005) method was applied to estimate the protein content in kitchen waste and tissues (six muscles and six viscera) was conducted following Kjeldahl method. Kjeldahl apparatus is divided into three parts, digestion, distillation and titration. 0.2 g of the samples was weight accurately. For digestion, 3 g of catalyst and 10 mL of concentrated H_2SO_4 was added into a digestion flask and mixed well for digestion. Catalyst was prepared by dry mixing 2 g of copper sulphate and 20 g of potassium sulphate. Heater was turned 'on' for digestion process to start. Once all the digested samples turned bluish green, along with the blank, the heater was turned 'off' and allowed to cool at room temperature. After cooling down, distillation was performed by adding 30 mL of distilled water to the cooled solution. Later, to it 40 % of NaOH (as an alkali) was added. In a beaker 25 mL of boric acid was taken and 2 drops of bromocresol green

and one drop of methyl red were added. The solution was shaken until it turns pink in colour. To the pink solution, the ammonium extract that was formed after adding the alkali was allowed to fall dropwise, until the pink colour turned into colourless solution. The ammonia reacts with the acid to form ammonium ions. Followed by titration, the colourless solution that was formed was titrated using 0.1 N of H_2SO_4 where the solution turn pink again once drops of sulphuric acid is added.

Calculation:

$$\% \text{ of Nitrogen} = \frac{(A-B) \times N \times 1.4007 \times 100}{W}$$

where, A= volume (ml) of 0.1 N H_2SO_4 used in sample titration
B = volume (ml) of 0.1 N H_2SO_4 used in blank titration
N = Normality of H_2SO_4
W = weight of the sample in mL
1.4007 = atomic weight of nitrogen

% of Protein = Nitrogen % × 6.26

where, 6.25 = the protein-nitrogen conversation factor for meat/fish and its by-products.

3.15.5. Estimation of total ash

AOAC (2002 and 2005) method was applied to estimate the total ash content in kitchen waste and tissues (six muscles and six viscera) was conducted by the following method. For kitchen waste 2 g of the sample was taken and for tissues 5 g was taken and was weight in crucible in triplicates. The weight samples were placed in a muffle furnace that was pre-heated at 600˚C for 2 hours. Immediately after 2 hours samples were placed in a desiccator and weight. Total ash content of the samples was estimated by the following formula:

$$\text{Total ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

where, A = Weight of the ash sample in grams
B = Weight of the original sample in grams

3.15.6. Estimation of crude fat

Crude fat content in kitchen waste and tissues (six muscles and six viscera) was conducted by the following AOAC (2002 and 2005) methods. 2 g for kitchen waste sample and 3 g for tissue samples was placed in a Soxhlet extraction apparatus and extracted with petroleum ether at 40-60 ºC. From the obtained extract, crude fat was calculated by using the following formula:

$$\text{Crude fat (\%)} = \frac{\text{Weight of fat (g)}}{\text{Weight of sample (g)}} \times 100$$

where, A = weight of extracted fat sample in grams
B = weight of the original sample in grams

3.15.7. Available carbohydrates

After determining all the other fractions of proximate content, the available carbohydrate content was estimated by difference method by James, (1995) in kitchen waste and tissues (six muscles and six viscera).

Available carbohydrates (%) = 100 – (Protein + Ash + Moisture + Fat)

3.15.8. Nutritive value

The nutritive value or caloric value in kitchen waste and tissues (six muscles and six viscera) was determined using the calculation as mentioned by FAO (2003) in Kcal/100g.

Calculation:

Nutritive Value = 4 × protein % + 9 × fats % + 4 × carbohydrate %

3.15.9. Estimation of alcohol content

The alcohol content in kitchen waste was determined following the method given by Caputi et al. (1968). 34 g of potassium dichromate was dissolved in 500 mL of distilled water. Then, 325 mL of conc. H_2SO_4 was added to it and volume was made up-to 1000 mL by distilled water. Absolute ethanol was used as a standard. To a test tube containing different concentration of absolute ethanol, distilled water was added to make

it up-to 5 mL. 5 mL of chromic reagent was added to each concentrated ethanol. Test tubes were incubated in a water bath at 60°C for 20 minutes. The absorbance was measured at 584 nm via UV-Vis Spectrophotometer (Shimadzu Corporation, Model no. 80865). For blank, to 5 mL of distilled water 5 mL of chromic reagent was added. For unknown sample, two different concentrations were taken and same dilution method as that of standard was applied.

3.15.10. pH (pH_{45} and pH_u)

The pH of the tissues (muscles and viscera) was analysed determining their acidity property. The pH of the tissue samples was analysed using a pH meter (Systronics μ pH system 361) following Alonso et al. (2009). Measurement of pH of the tissues were taken two times; one within 45 minutes of slaughter (pH_{45}) and secondly, final pH (pH_u) measured at 24th hour postmortem. The thickness of tissues was about 3 cm. The electrode was calibrated using pH buffers of 4.01 and 7.00, equilibrated at 35°C for measurements of 45 minutes post-mortem samples and at 4°C for measurements of final pH.

3.15.11. Amino acid analysis

Amino acid analysis in kitchen waste and tissues (six muscles and six viscera) samples was performed following procedure of Szkudzinska et al. (2017). 1 g of oven dried sample was taken for amino acid analysis. The samples were dissolved in 2 mL of milliQ-water followed by incubation at 45°C in thermomixer for 30 minutes. 8 mL of methanol was added to precipitate the proteins and incubated overnight at -20°C. The solutions were centrifuged at 4000 rpm for 30 minutes and the supernatant was transferred to another tube. The supernatant was evaporated under nitrogen gas at 60°C to complete dryness. Derivatization was done by adding 350 μL of Borate buffer, 20 μL of AccQ-Tag ulta reagent to the sample and incubated for 10 minutes at 55°C. After incubation 2 μL is loaded on the instrument and quantified using a Sigma standard. Buffer for Mobile Phase A: AccQ- Tag Ultra eluent A1 and for Mobile Phase B: AccQ-Tag Ultra eluent B were used. Amino acids were determined using a WATERS Acquity (make) UPLC system. The quantitative amino acid present in the sample was performed by comparing with the amino acid standard run simultaneously with the samples. The amino acid identification

and quantification was done based on the retention time with definite experimental conditions for each individual amino acid. For the determination of the amino acid content in mg/100g, following calculation was applied as mentioned in ASEAN (2011).

Calculation:

$$\text{Amino acid content in g/100g} = \frac{A \times B}{10 \times \text{C} \times \text{D}}$$

where, A = amount of amino acid (ng/volume sample injected)
B = Total volume of the sample
C = Volume of sample injected
D = sample weight in grams

3.15.12. Fatty acid analysis

For fatty acid analysis, the kitchen waste and tissues (six muscles and six viscera) samples were subjected to lipid extraction following Bligh and Dyer, (1959) method. 20 g of the oven dried sample was taken, to it 40 mL of chloroform and 80 mL of methanol was added, including 16 mL of distilled water. The solvents along with the sample were homogenized for 1 minute at 4◦ C at a speed of 9500 rpm. 40 mL of chloroform was added and homogenized for another 30 seconds, followed by homogenizing again for 30 seconds by adding 40 mL of distilled water. The solution was centrifuged at 2000 rpm at 4◦ C for 20 minutes and the supernatant obtained was placed in separate container and allowed to separate. The lipid part was extracted out followed by its separation.

3.15.12.1. Analysis of fame's (fatty acid methyl esters)

The lipid extracted was esterified with BF_3- methanol following Joseph and Ackman, (1992). 1 mL of the extracted lipid was added in 1 mL of concentrated Boron Trifluoride (BF_3) and 9 mL of methanol. The solution was mixed well and transferred to a separating funnel. 4 mL of hexane was added to the solution dropwise. A saturated sodium hydroxide brine was prepared and 4 mL of brine was added to the solution and shaken well. After shaking, the solution was allowed to form layers so that FAME's can be extracted. For fatty acid composition, an aliquot of FAME extract was inserted in Gas Chromatography. GC-MS analysis of sample extracts was carried out with Perkin Elmer (USA), Model: Clarus 680 GC & amp; Clarus 600C MS comprising a liquid autosampler.

The Software used in the system was Turbo Mass Ver.6.1.2. The peaks were analyzed using data analysis software NIST-2014. The capillary column used is 'Elite- 5MS' having dimensions- length- 60 m, ID- 0.25 mm and film thickness- 0.25 µm and the stationary phase is 5% diphenyl 95% dimethyl polysiloxane. Helium gas (99.99%) was used as carrier gas (i.e., mobile phase) at flow rate of 1 mL/minute. An injection volume of 2 µl was employed in splitless mode. Injector temperature was 280°C and ion-source temperature 180°C. The oven temperature was programmed at 60°C (for 1 minute), with an increase at the rate 7°C/minutes to 200°C (hold for 3 minutes) then again increased at rate of 10°C/min to 300°C (hold for 5 min). The total run time is ~ 39 minutes. Solvent delay was kept for 8 minutes. MS Protocol Mass Spectra was taken in Electron Impact positive (EI+) mode at 70 eV. For MS scan, a solvent delay of 8 minute was provided with m/z range 50-600 amu. The data file obtained from the GC/MS assay was deconvoluted and identified by NIST mass spectral library software, by matching entries in the compound libraries to the measured mass spectra.

Calculation of FAME's following the formula given in ASEAN (2011):

$$\% \text{ Area of each fatty acids} = \frac{A}{B-C} \times 100$$

where, A = area of each fatty acid methyl ester
B = total area of all fatty acid methyl ester
C = area of fatty acid internal standard

3.15.13. Mineral analysis

For the estimation of mineral content, in kitchen waste, tissues (six muscles and six viscera) and soil samples were digested using wet digestion procedures as reported by ASEAN Manual of Food Analysis (2011). The soil samples collected from the rearing place of Doom and Ghungroo pigs were taken from the surface, at a depth of 0 cm. 1.5 g of the oven dried sample was digested in 8 mL of concentrated nitric acid (HNO_3) and 2 mL of perchloric acid ($HCLO_4$) overnight to predigest the sample. The samples were kept in oven for 5 hours at 100°C and cooled to room temperature in a fume hood. The solution was diluted up to 50 mL with deionized water and mixed well. The solution was filtered with Whatman filter paper No. 541 and analyzed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry), Model No. 7600, Thermo Fisher Scientific. The

data that were obtained were in concentration of ppm (parts per million) designated in mg/L. Therefore, to convert from liter to gram following calculation were used:

Calculation:

$$\text{Element (mg/kg)} = \frac{C_{\circ} \times V \times D \times 100}{W \times 1000}$$

where,

$C_{\circ}$ = sample concentration (mg/L)
V = total volume (mL)
D = factor for dilution
W = sample weight in mg
1000 = conversion for L to kg

3.15.14. Minerals in drinking water

The drinking water sample was collected from the rearing place of Doom and Ghungroo pigs. The drinking water sample supplied to the pig breeds at the rearing site were collected and directly analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry), Model No. 7600, Thermo Fisher Scientific.

3.16. Statistical analysis

- To compare the proximate composition between the two experimental diets of Doom and Ghungroo pig breeds, 'independent samples T-test' was performed using SPSS software (version 21.0).
- For genetic diversity and phylogenetic tree construction following analysis was performed at MEGA 11 -
    - Multiple sequence alignment and pairwise sequence alignment was carried out at MEGA 11 software (Thompson et al., 1994). The genetic distance between the wild boars and indigenous pig was generated based on Kimura 2-parameter model (Tamura et al., 2021; Kimura, 1980).
    - Neighbor-Joining tree method was applied for phylogenetic construction, bootstrapping at 1000 replications using Kimura-2 parameter using MEGA 11.
- The data of goat, sheep and beef are taken from the Standard Nutritional Database of Indian Food Composition put forwarded by Indian Council of Medical

Research (ICMR), India, (2017) and are compared with nutritional data of Doom and Ghungroo pig. Food codes provided by them are – O001 (Goat), O014 (Sheep), O025 (Beef) for proximate composition in shoulder region. O011 (Kidney), O012 (Small intestine), O010 (Spleen), O008 (Liver) and O007 (Heart) for viscera (Longvah et al., 2017).

- Spearman correlation coefficient (r) was used to construct correlation matrix between the mineral concentration of tissues (muscles and viscera) and feed, drinking water and soil.

3.17. Supervision of the experimental works

The experimental works of this study starting from the identification of adults, mating, rearing of piglets of both the pig breeds till dissecting of the tissues (muscle and viscera) and collection of blood for molecular characterization was conducted under the supervision of certified veterinarian Dr. Sulav Sarma, Veterinary Officer at the State Veterinary Dispensary in Tangla, Udalguri, BTR, Assam.