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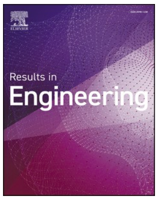

Relationship between the heterogeneity in mechanical properties, bone density and composition parameters of cortical bone to design and develop bone scaffolds and implants: Analysis of bone microstructure

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ABSTRACT

Bone is heterogeneous and anisotropic because its mechanical characteristics vary by anatomic location and loading direction. Previous research established a correlation between bone density, mineral, organic content, and porosity and mechanical qualities. Medical and bioengineering researchers can learn about bone heterogeneity and anisotropy by analysing bone density and later composition parameters across the bone diaphysis in both longitudinal and transverse orientations. This research examines bone density and composition parameters in bovine femoral cortical bone from higher, middle, and lower diaphysis regions and longitudinal and transverse orientations. At three homogeneous diaphysis sites, these properties did not alter in longitudinal or transverse orientations. At the upper and lower diaphysis locations, mineral and organic content were statistically different in both longitudinal and transverse orientations, contributing to anisotropy. At the middle location, all measured parameters were not statistically different. Transverse bone density and water heterogeneity were greater, while longitudinal values were higher for the other metrics. Upper and lower sites had increased bone variability due to %water, although mineral content was more homogenous. The longitudinal and transverse organic content and apparent density heterogeneity was greater at the middle position. At the later site, bone density and mineral content were more homogenous longitudinally and transversely. Lamellar bone microstructure with canalicular/vascular network, cavities, and holes contributed to bone materials' %water content. Collagen fibres contributed to bone material's organic composition, whereas intrafibrillar and extra fibrillar minerals contributed to its mineral content.

1. Introduction

Bone is classified as a graded material because its composition, structure, and mechanical characteristics can vary in discrete or continuous stages from one anatomic location to another. Because of the hierarchical and heterogeneous nature of bone, it is a tremendously complicated material type that cannot be defined by a single value for a specific material feature. Significant technological advancements in experimental approaches for investigating the mechanical characteristics of complex biological materials have been made in recent years; yet,

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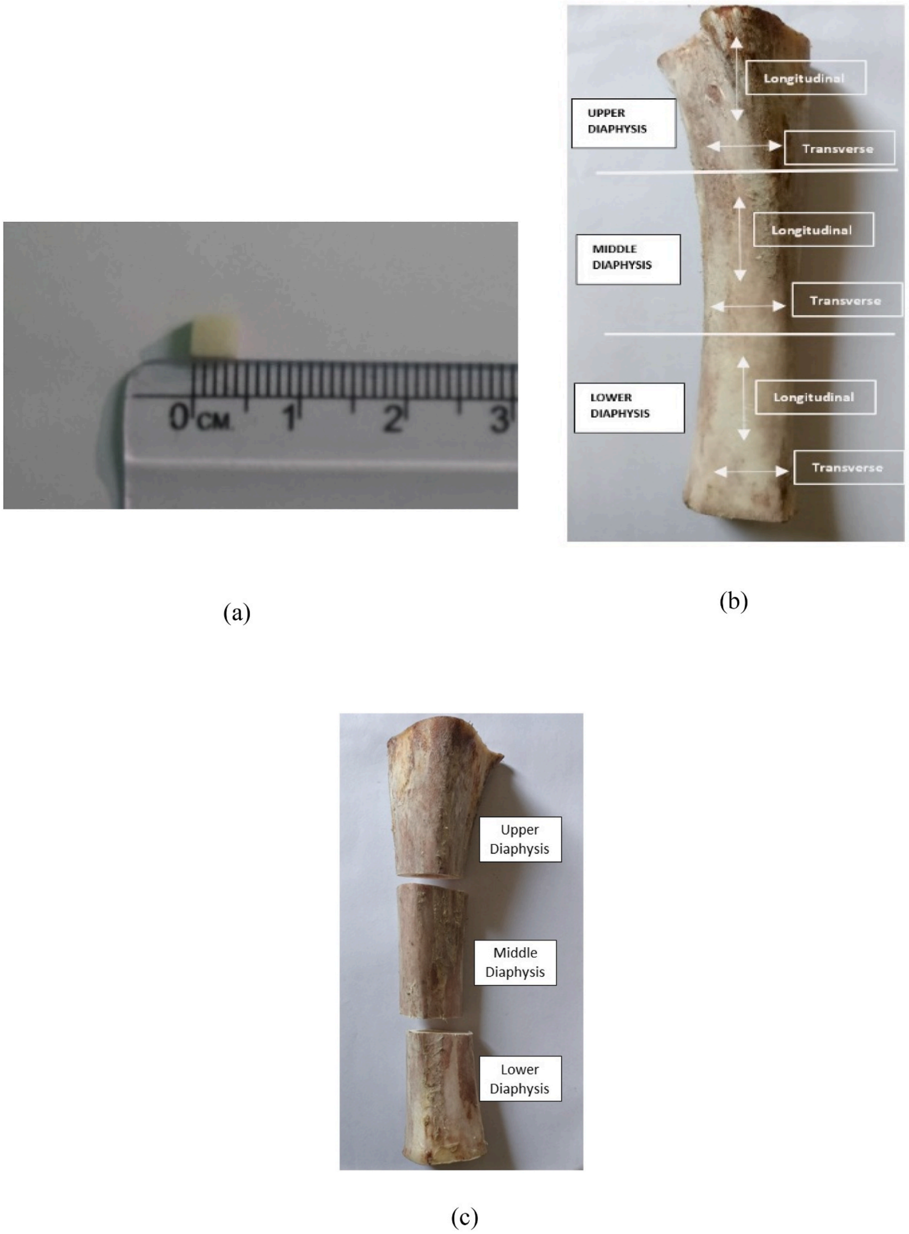


Fig. 1. (a) Specimen shape; (b) Specimen location and orientation; (c) Bone Diaphysis Cut View.

it remains difficult to anticipate and completely comprehend the mechanical behavior of entire biological entities such as bone due to the variation in its structure and composition variation. Many scientists have worked in the field to determine the various composition factors of cortical bone for bone specimens extracted from different species [1,2], gender [6], ages [3], and other factors. The composition parameters include Fatty acids [4], protein content [1,5], mineral content [3–10], microstructure [2], porosity [11], chemical composition [12], ash content [5], collagen fibrils [13–23] and bone-marrow culture [6]. Different techniques and methods have been used to find out these composition values for different types of bones [3,6,24] such as Raman spectroscopy [25], nanotechnology [26], spectroscopy [8], electron energy loss spectroscopy [14] and X-ray [9]. The effect of composition parameters on different mechanical [5–7,11,12,25,27], chemical [2,3] and biological [6,9,24,28,29] properties of a bone has also been studied in various previous studies [35–38]. The measured composition values are also been used in the application of tissue engineering to design scaffolds [12,13] for bone defects.

In the present study, the variation in the composition characteristics of cortical bone has been studied and compared while considering specimens from three different locations of the bone diaphysis and two orientations with respect to the long axis of the whole bone. The Scanning Electron Microscopy (SEM) technique was used to analyze the microstructure and bone morphology at different anatomic locations and along two directions of bone. The outcome of this study may be helpful for the treatment of bone disease as well as for the understanding of the relationship between the heterogeneity in mechanical properties, bone density, and composition parameters to design and develop bone scaffolds and implants.

2. Materials and methods

2.1. Preparation and preservation of bone samples

A whole femoral bone was obtained from about 48-month-old bovine in the current study. The bone was extracted from the animal after it

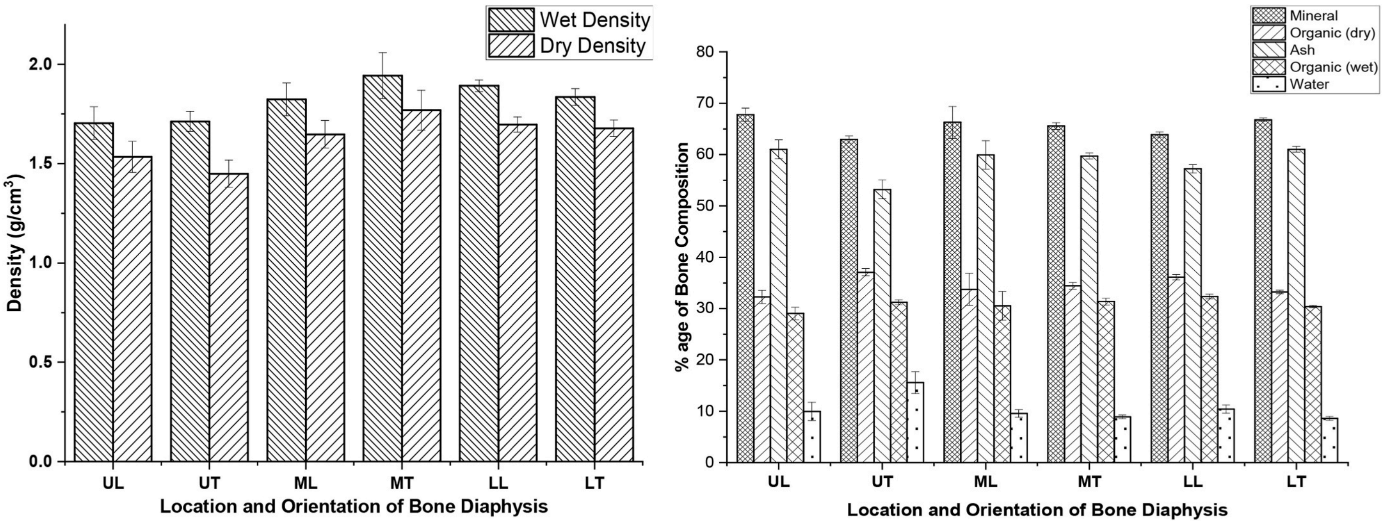


Fig. 2. Composition parameters of a cortical bone; (a) Wet and Dry density and (b) Other Compositions Parameters.

Table 1
Apparent density and composition parameters of cortical bone.

Location and Orientation of Diaphysis	Wet density	Dry density	%Mineral	%Organic (dry)	%Ash	%Organic (wet)	%Water
Upper Diaphysis Longitudinal	1.70 ± 0.21	1.53 ± 0.208	67.75 ± 3.492	32.25 ± 3.492	61.03 ± 4.887	29.03 ± 3.324	9.94 ± 4.702
Upper Diaphysis Transverse	1.71 ± 0.133	1.45 ± 0.181	62.94 ± 1.893	37.06 ± 1.893	53.21 ± 4.871	31.21 ± 1.271	15.58 ± 5.663
Middle Diaphysis Longitudinal	1.82 ± 0.218	1.65 ± 0.184	66.26 ± 8.251	33.74 ± 8.251	59.92 ± 7.297	30.52 ± 7.373	9.56 ± 1.921
Middle Diaphysis Transverse	1.94 ± 0.305	1.77 ± 0.267	65.56 ± 1.792	34.44 ± 1.792	59.72 ± 1.559	31.38 ± 1.759	8.91 ± 0.913
Lower Diaphysis Longitudinal	1.89 ± 0.077	1.70 ± 0.102	63.88 ± 1.413	36.12 ± 1.413	57.24 ± 2.122	32.35 ± 1.236	10.41 ± 2.135
Lower Diaphysis Transverse	1.84 ± 0.112	1.68 ± 0.110	66.76 ± 0.937	33.24 ± 0.937	61.02 ± 1.457	30.38 ± 0.633	8.60 ± 1.023
Mean Value	1.81 ± 0.09	1.63 ± 0.11	65.52 ± 1.81	34.47 ± 1.81	58.69 ± 3.02	30.81 ± 1.12	10.5 ± 2.57

The specimens are further compared both location and orientation wise using statistical tools i.e., paired *t*-test at $p < 0.05$.

died naturally and with the institution's permission. After being extracted, the bone was cleansed to remove any remaining tissues before being bathed in saline solution and wrapped in a saline-soaked towel. The bone tissue was further wrapped in an airtight plastic bag and kept in a refrigerator at a temperature of −20 °C in a sealed container until sample preparation. The bone was kept hydrated throughout all stages of sample processing after being removed from the refrigerator using a saline solution. Soft tissues, epiphyses, and diaphysis are all removed. A water jet was used to separate the cortical bone from the bone marrow, and the whole bone diaphysis was carefully sectioned into three parts namely the higher, middle, and lower parts. Wet sandpaper was used to remove machine damage after the bone incisions were made with a hacksaw blade and a hacksaw wire. An ISOMET 5000 diamond cutter was used to make fine cuts, and the sample is flattened with a belt sander. To avoid overheating, bone samples are immersed in a saline solution during various cutting and machine operations. After being sliced and machined, small bone samples were kept hydrated in a saline solution.

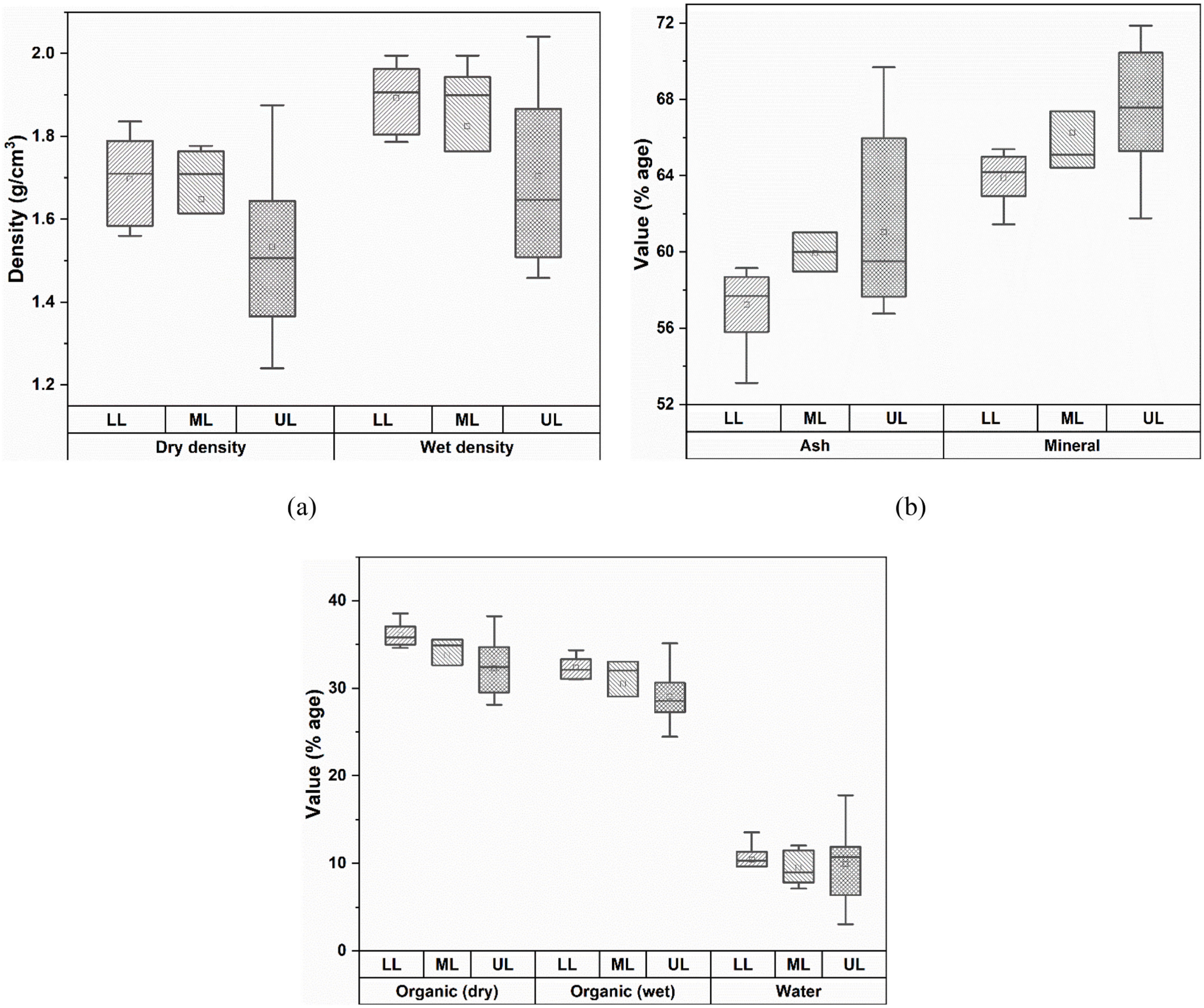
For composition testing, specimens with lengths of 4 mm, breadths of 4 mm, and widths of 4 mm were created, as shown in Fig. 1(a). The samples were collected from three distinct locations, namely the upper, middle, and lower diaphysis, with Longitudinal (parallel to the long axis) and Transverse (perpendicular to the long axis) orientations, as shown in Fig. 1 (b). The cut view of bone diaphysis for three locations is shown in Fig. 1(c).

2.2. *Composition test*

A total of 48 specimens were recovered from these locations and orientations, with 8 specimens from each of the following categories: UL (Upper longitudinal), UT (Upper transverse), ML (Middle longitudinal), MT (Middle transverse), LL (Lower longitudinal), and LT (Lower transverse) to investigate and addressed the variation in bone composition and apparent density as a function of location and orientation. The volume of these samples was calculated by measuring their dimensions with a digital caliper. The samples were hydrated overnight and weighed the next day to determine their wet weight. After determining the wet weight, the specimens were immersed in acetone overnight before being placed in a 60 °C oven with silicate gel for 24 h to remove any remaining moisture. These samples' dry weight was determined by weighing them after the later process. The samples were then heated for 24 h in a furnace at 600 °C. After being removed from the furnace, the specimens were placed in the desiccator to cool to room temperature before being weighed to determine the amount of residue (ash). The composition parameters were calculated using the equations proposed in the previous studies [30,31].

2.3. *Structural characterization*

The SEM analysis was conducted on selected samples of bone obtained from three different locations and along two directions of a long bone axis. These samples were obtained from the specimens tested under compressive loading for a separate study. The selected samples were first cleaned in acetone and later dried in a vacuum oven at 60 °C. the remaining moisture was further removed by placing them with silicate gel inside the desiccator for about 4 h. The samples were gold coated and examined under the JEOL JSM-IT500 instrument equipped with automatic correction function and capable of EDS functions. It is capable of providing resolution up to 15 nm with the acceleratin voltage of 0.3 kV to 30 kV. For the current study, the samples were observed at 15 keV accelerating voltage with a resolution ranging from 100 μm to 200 μm.



(a)

(b)

(c)

Fig. 3. Variation in Composition parameters along the longitudinal direction.

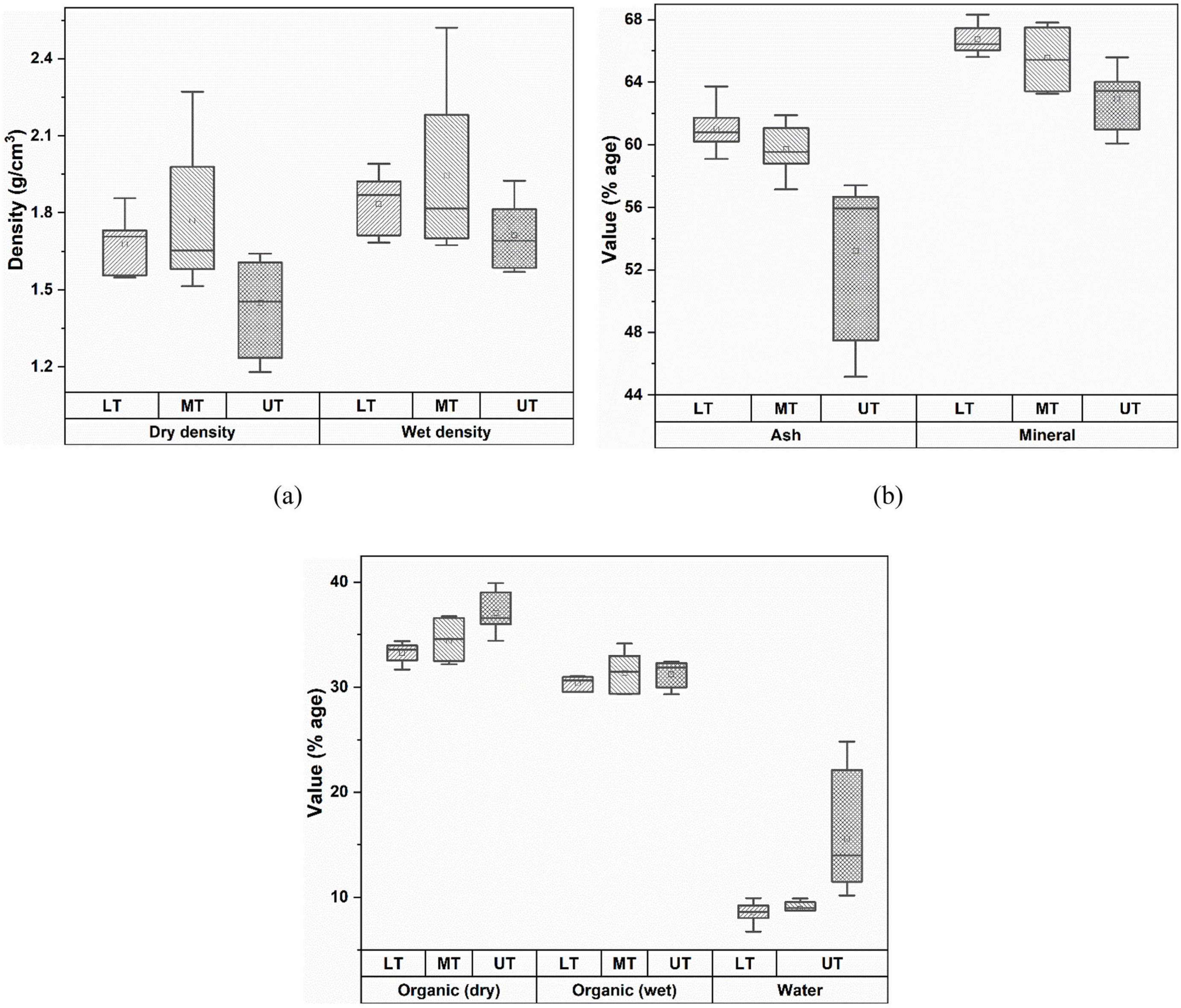
3. Results and discussion

The variation in the composition parameters and apparent density along the whole bone diaphysis at three different locations and two orientations are determined and reported in Fig. 2.

Fig. 2(a) shows the variation in the value of wet density and dry density for different locations and orientations of the bone diaphysis. It has been observed that the maximum value of wet and dry density was found for the specimens extracted from the middle diaphysis location with transverse orientation (MT). Other composition parameters have been shown in Fig. 2(b), the maximum ash content found in the specimens of Upper diaphysis longitudinal (UL), % mineral in UL, % organic (wet) in LL, % organic (dry) in UT, and % water in UT. The minimum wet density in UL, dry density in UT, ash % in UT, % mineral in UT, % organic (wet) in UL, % organic (dry) in UL, and % water in LT. The mean values of different composition parameters of cortical bone for different locations and diaphysis along with their corresponding standard deviation are reported in Table 1. The overall mean value of wet density was found to be 1.81 g/cm^3, dry density as 1.63 g/cm^3, % mineral to be 65.52, % organic (dry) as 34.47, % Ash as 58.69, % organic (wet) 30.81 and % water as 10.5.

3.1. Locational variation in composition parameters along the bone diaphysis

Fig. 3(a) shows a comparison of dry density and wet density values for specimens extracted from three different diaphysis locations along the longitudinal orientation. As per the paired *t*-test, no significant difference was observed in the density values determined for the upper, middle, and lower bone diaphysis along the longitudinal direction. This shows that the apparent value of bone density is almost homogeneous along the bone diaphysis in the longitudinal direction, however, for the upper location these values were found to be having a wider range as compared to the other two locations. As the bone density values have



(a)

(b)

(c)

Fig. 4. Variation in Composition parameters along transverse direction.

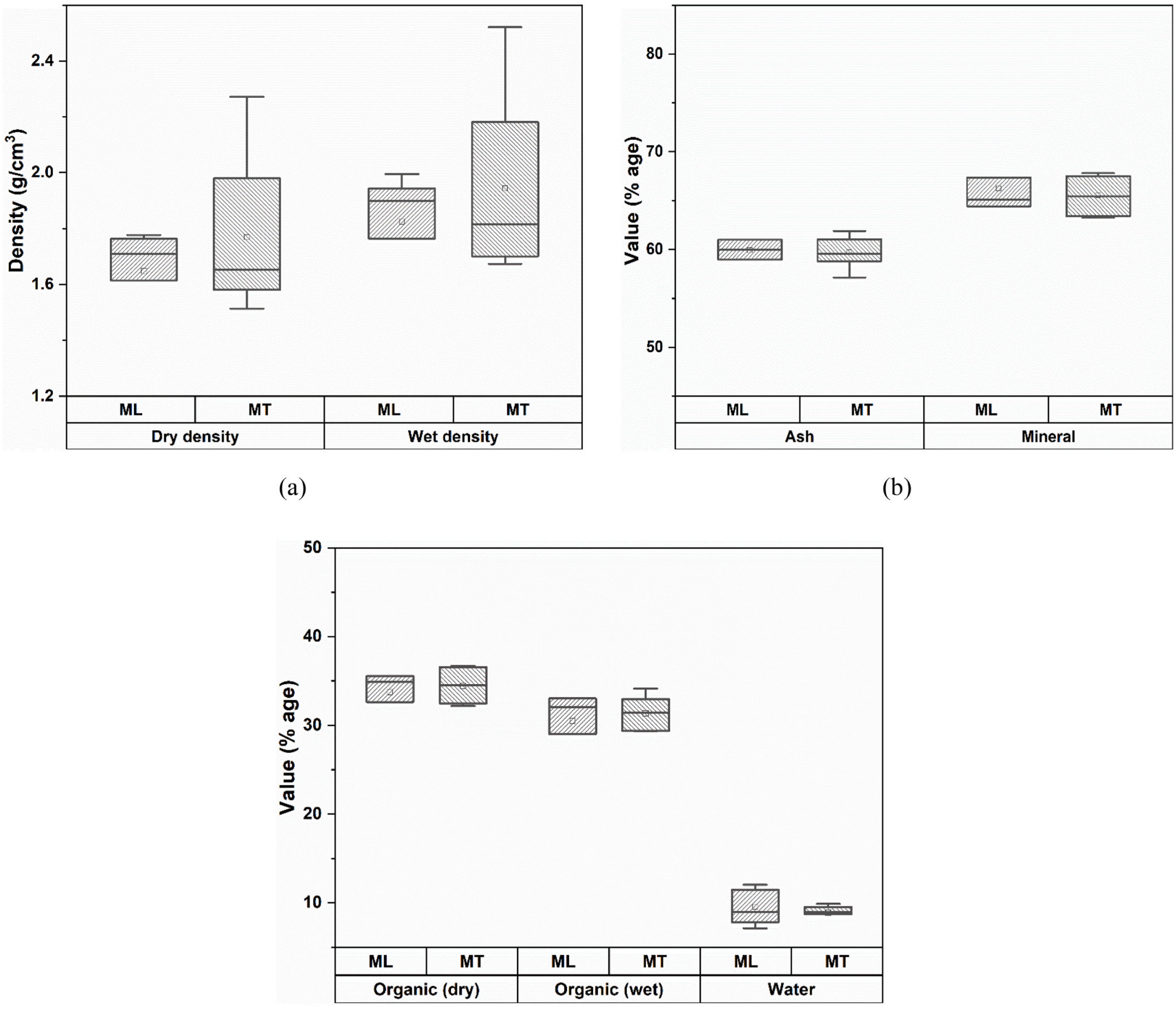
correlations with its mechanical properties [32], this wider range of bone density may result in a wider range of mechanical properties at the upper location along the longitudinal orientations. This may be helpful for the bone tissue at an upper location to effectively sustain the load transmitted along the long direction of loading.

Fig. 3(b) and (c) show the comparison plot of other composition parameters for specimens taken from upper, middle, and lower diaphysis in a longitudinal direction. For all these composition parameters at three different diaphysis locations in a longitudinal direction, there found no significant difference between the mean values. Therefore, the distribution of these parameters was also found to be homogeneous along the longitudinal direction of the bone diaphysis with a wider range for the upper diaphysis location.

The comparative plots of bone density and composition parameters for the transverse orientation are shown respectively in Fig. 4(a), (b), and 4(c). In this case, also no significant difference was observed between the bone density and any of the composition parameters. For transverse orientation, the wider range of density values was obtained for the middle diaphysis location which may result in wider variation in mechanical properties such as strength and stiffness of bone at this location as per the correlations of later properties with the bone density reported in the literature [32]. For the percentage of minerals, the wider range was obtained for the upper location, whereas, for other parameters the wider range was noticed for both the middle (%organic) and upper locations (%ash and %water) as compared to the other locations.

3.2. *Variation in composition parameters for two orientations of bone diaphysis*

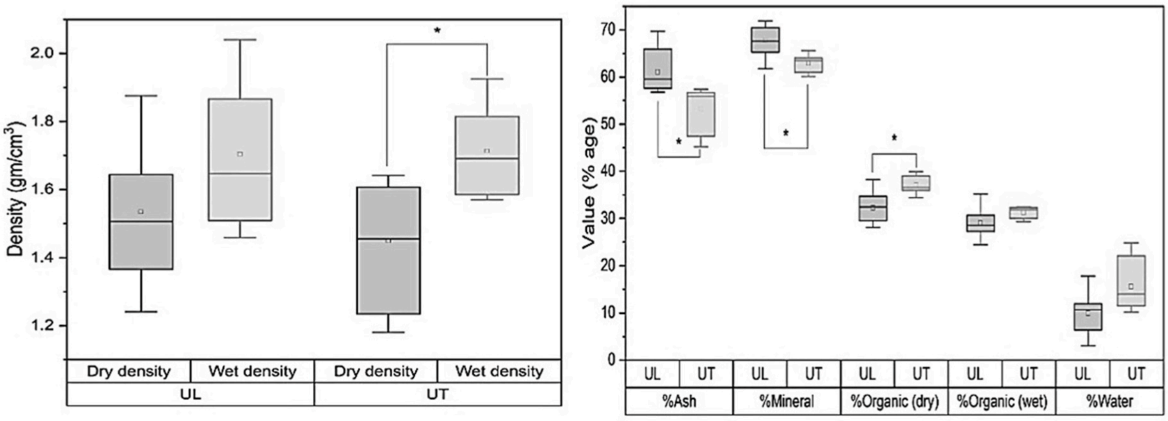
A comparative study has been conducted to analyze the variation in composition parameters due to orientation. For this, the composition parameters of specimens extracted from the same location but with two different orientations (longitudinal and transverse) have been plotted and conducted *t*-test to check the significant difference in the mean values of the corresponding parameters at the confidence interval of 95 % ($p < 0.05$).



(a) (b)

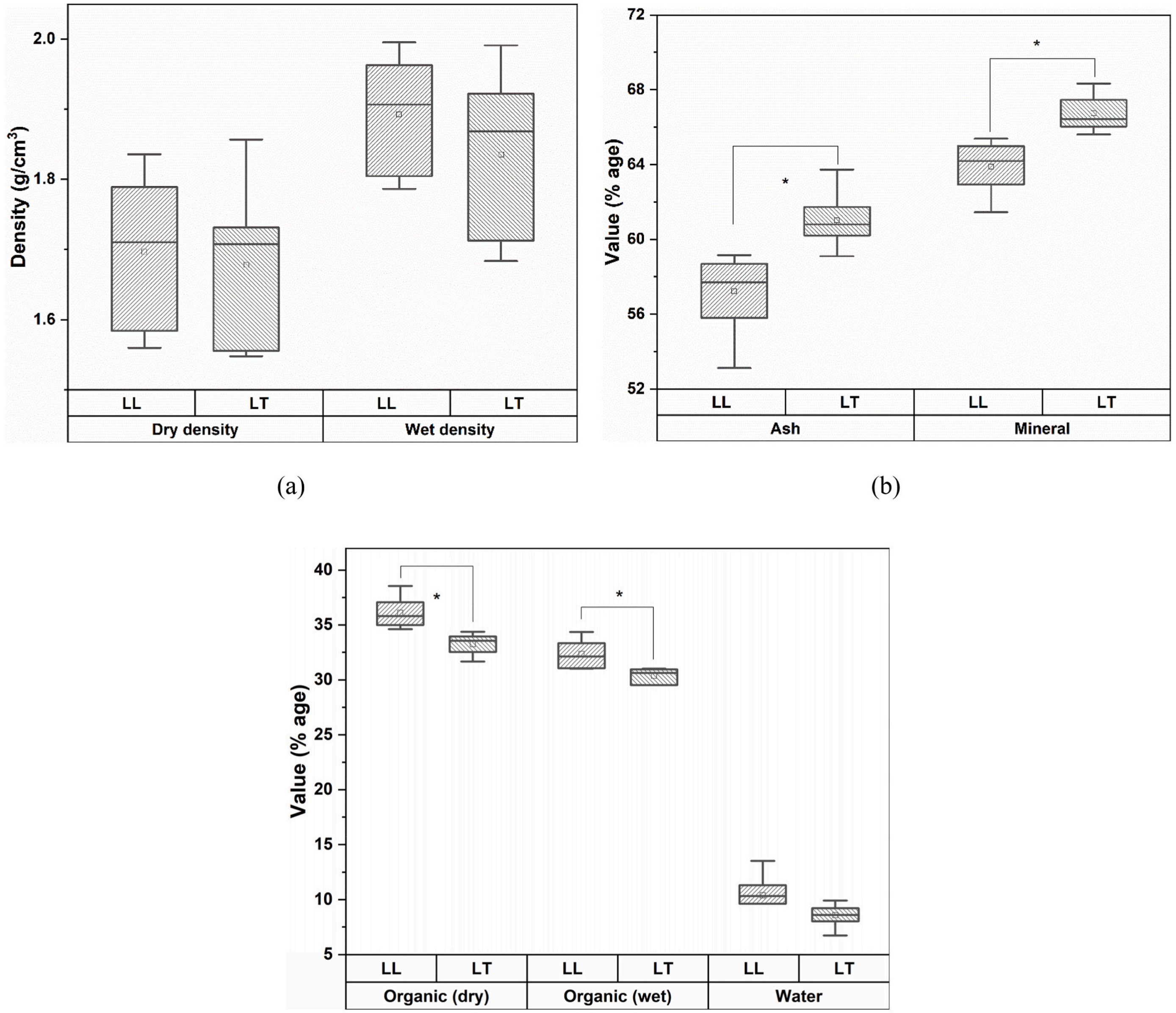
(c)

Fig. 5. Comparison of apparent density and composition parameters for UL and UT.



(a) (b)

Fig. 6. Comparison of apparent density and composition parameters for ML and MT.



(a)

(b)

(c)

Fig. 7. Comparison of apparent density and composition parameters for LL and LT.

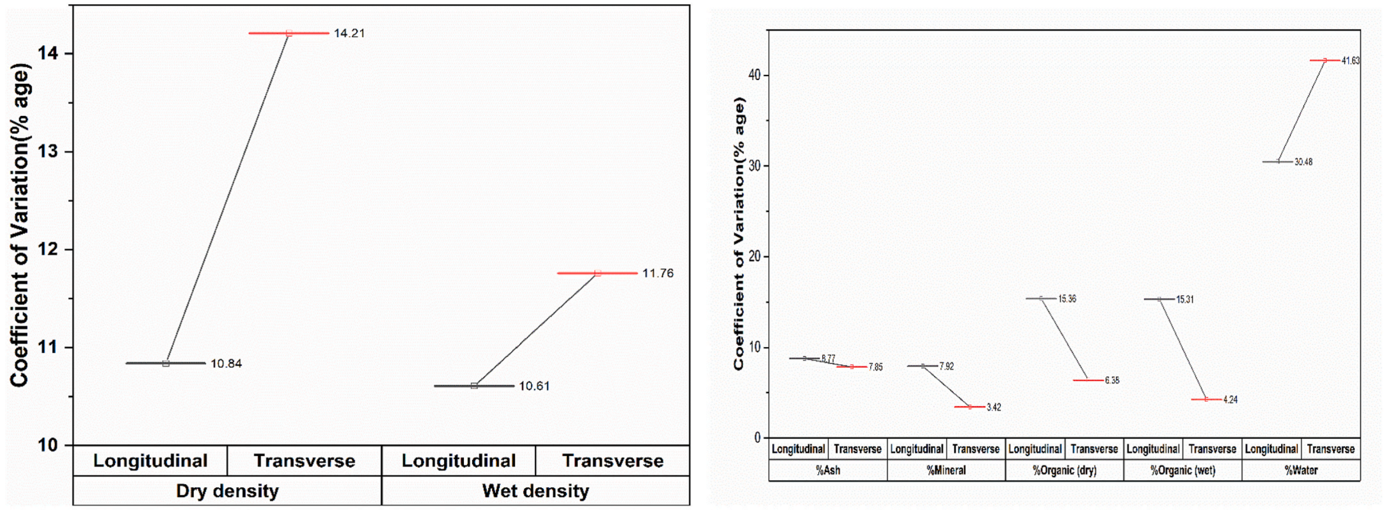
i. UL vs UT

While comparing the dry density and wet density of the Upper diaphysis longitudinal and transverse sample, no significant difference was found in the values as shown in Fig. 5(a). Corresponding comparison for other composition parameters is shown in Fig. 5(b) and (c). A significant difference was found in the values of % Ash content, % mineral, and %organic (dry), however, no significant difference was found in the values of % organic (wet) and % water. The % ash and mineral content was observed to be higher for longitudinal samples as compared to the transverse samples, however for %organic (dry) this trend was noted to be opposite. The higher mineral content along longitudinal direction may be responsible for higher stiffness in this direction for upper diaphysis location as mineral contact has positive correlation with the strength and stiffness values [32,33]. On the other hand, higher organic content in the transverse direction may result in formation of micro-cracks during mechanical loading with enhanced post yield strain values at this diaphysis location [34].

iii. ML vs MT

While comparing the variation in values of composition parameters for middle diaphysis in two different orientations i.e., longitudinal and transverse as shown in Fig. 6(a), (b) and 6(c). No significant difference in the mean values of any of the composition parameters is observed. This shows that at middle location bone density and composition parameters are almost homogeneously distributed along both the directions. However, the microstructural features of bone such as orientation of collagen fibers, bone lamellae and type of bone (osteonal, lamellar or woven) may result in variation in mechanical properties of bone along two directions at the middle location.

v. LL vs LT



(a) (b)

Fig. 8. Comparison of %CV of apparent density and compositional parameters for two orientations.

Table 2
Values of % CV for apparent density and composition parameters of bone.

Location and Orientation of Diaphysis	Wet density	Dry density	%Mineral	%Organic (dry)	%Ash	%Organic (wet)	%Water
Upper Diaphysis Longitudinal	12.84	13.54	5.15	10.83	8.01	11.45	47.29
Upper Diaphysis Transverse	7.77	12.47	3.01	5.11	9.16	4.07	36.34
Middle Diaphysis Longitudinal	11.97	11.18	12.45	24.46	12.18	24.16	20.11
Middle Diaphysis Transverse	15.70	15.08	2.73	5.20	2.61	5.61	10.25
Lower Diaphysis Longitudinal	4.08	5.98	2.21	3.91	3.71	3.82	20.51
Lower Diaphysis Transverse	6.13	6.57	1.40	2.82	2.39	2.08	11.90

In the case of lower diaphysis location also no significant difference was found in the values of wet density and dry density for the two orientations as shown in Fig. 7(a). However, significant differences were found in the values of % ash, % mineral, % organic (dry) and % organic (wet); %water doesn't find any significant difference as shown in Fig. 7 (b) and (c). This shows that at lower location bone minerals and organic contact are in homogeneously distributed along the longitudinal and transverse orientations. This may consequently result in inhomogeneous distribution of mechanical properties of bone along these two orientations at lower location of bone diaphysis.

3.3. Comparisons of coefficient of variation for two orientation of bone diaphysis

The percentage of coefficient of variation (CV) values were determined to analyze and compare the inhomogeneity in corresponding bone density and composition parameters along the two different orientations of bone diaphysis. Fig. 8 (a) and 8(b) show the comparison of the % CV for longitudinal and transverse orientations, whereas, the values of % CV for different diaphysis locations and orientations are reported in Table 2. The %CV for bone density was observed to be higher along the transverse orientation as compared to the longitudinal orientation. This shows that in terms of apparent density bone material is more heterogeneous along the transverse orientation. However, for other composition parameters (except for %water) the %CV was found to be higher for the longitudinal orientation of bone diaphysis, as shown in Fig. 8 (b), resulting in more heterogeneity in bone material along the longitudinal orientation as compared to the transverse one in terms of the mineral and organic content of cortical bone. The %CV for %water was noted to be higher for the longitudinal orientation as compared to the transverse orientation of bone samples. As %water content is positively correlated to the bone porosity, this indicates higher variation in the porosity level of bone material along the longitudinal orientation. The distribution of vascular network along the longitudinal orientation may attribute to this observation in the current finding.

As reported in Table 2, for both the upper and lower diaphysis locations the %CV for %water was observed to be higher as compared to % CV for bone density and other composition parameters in both the longitudinal and transverse orientations. Therefore, bone porosity, resulting from %water, contributes more to bone heterogeneity as compared to distribution of bone density, mineral and organic content at these two locations of bone diaphysis along both the orientations. This is interesting as these two ends make joints with other parts of the skeleton and are responsible to uniformly transmit load through the junction points. The minimum values of %CV for the later diaphysis locations were found for the %mineral. This shows that mineral content is more homogeneously distributed at upper and lower diaphysis locations of bone diaphysis along both the longitudinal and transverse orientations. For middle diaphysis, the %CV was found to be maximum in the case of %organic (dry) along the longitudinal orientation and for the bone density along the transverse orientation. This indicates that for middle location organic content contributed significantly to bone material heterogeneity along the longitudinal orientation, whereas, at this location the contribution of bone density to material heterogeneity was higher along the transverse orientation. The minimum values of %CV were observed for the density and mineral content values in case of the middle diaphysis longitudinal and transverse orientations respectively. This signifies that for middle diaphysis location the bone material is more homogeneous in terms of bone density along the longitudinal orientation as compared to the other composition parameters determined in this study, whereas, for transverse orientation at this location, mineral content is more homogeneously distributed as also noticed for the upper and lower diaphysis locations discussed earlier.

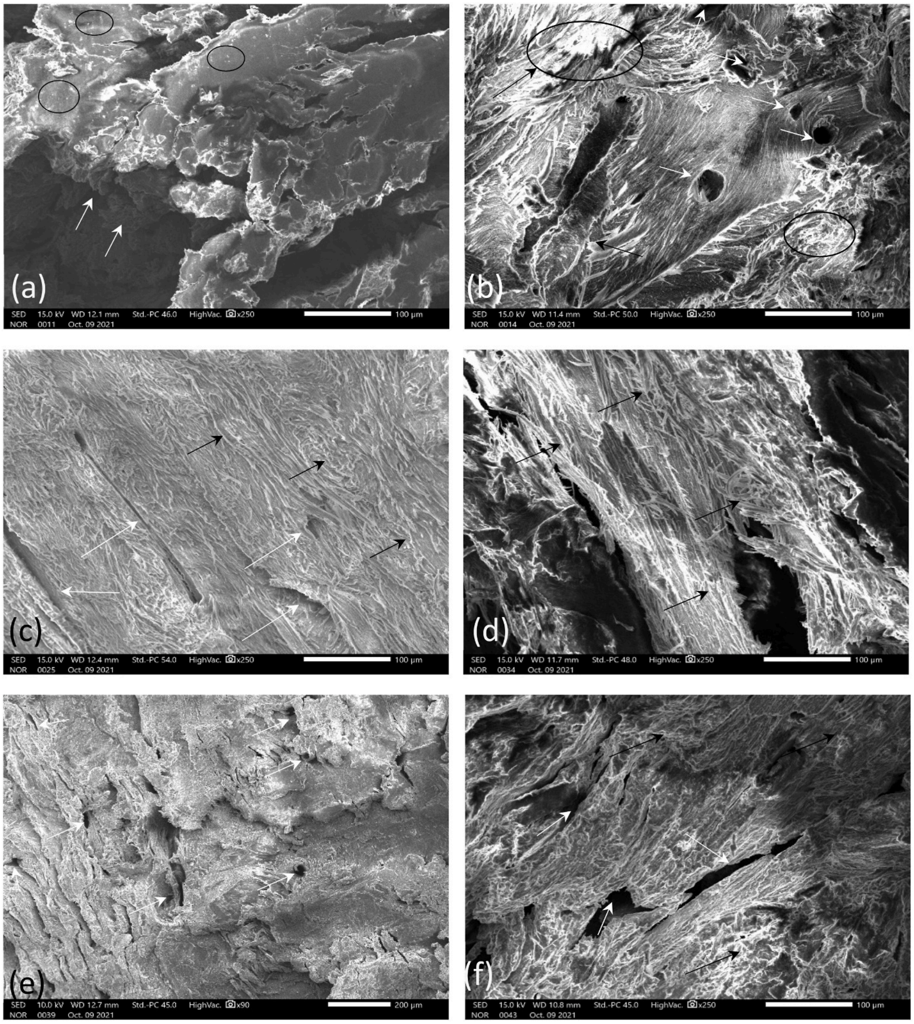


Fig. 9. Microstructural features of bone obtained from upper (a, b), middle (c,d) and lower (e, f) diaphysis locations.

3.4. SEM analysis of bone microstructure

The SEM images of samples obtained from upper, middle and lower diaphysis locations are shown in Fig. 9 (a) to 9 (f) respectively. The images 10 (a), (c) and (e) are for samples obtained for the longitudinal direction, whereas, images 10 (b), (d) and (f) are for the transverse orientations.

The microstructure of bone was found to be lamellar in nature with lamellae mostly oriented along the longitudinal direction of bone diaphysis. The extrafibrillar minerals distributed inside the bone material are shown with in the black circles in Fig. 9 (a). The canalicular/vascular networks, cavities and pores are represented with the help of white arrows in different images. The later features contribute to the % water in bone as water can reside inside these cavities in bone material. The black arrows in different images represents the mineralized (including intrafibrillar minerals) fibers in cortical bone which are assembled in bundles to form the bone lamellae as shown in images 10 (b), (d) and (f). The intrafibrillar and extrafibrillar minerals together contribute to the %minerals whereas collagen fibres contribute to the % organic content of bone.

4. Conclusions

The apparent bone density and composition parameters (mineral, organic and water content) are measured for specimens extracted from three different locations and along two orientations of the bovine femoral bone diaphysis. The comparison of later parameters was performed based on the locations and orientations of the specimens with the help of statistical paired *t*-test ($p < 0.05$). The percentage of coefficient of variation was calculated to examine the variation in bone density and composition parameters for different types of specimens. The heterogeneity in bone material was examined and discussed in terms of the measured values of the later apparent parameters of the cortical bone diaphysis. However, structural organization of these components was not considered for the presented study. There was no significant different observed in the mean values of these components for three diaphysis locations in case of both the longitudinal and transverse orientations. For upper location higher mineral content was observed along the longitudinal orientation, whereas, in transverse orientation organic content was found to be higher as compared to the other locations. At the middle diaphysis location, both density and measured composition parameters were found to be homogeneously distributed along both the directions. In case of lower diaphysis location mineral and organic contents were observed to be in homogeneously distributed. The %CV values were further used to identify the maximum and minimum contributions of resulting density and composition parameters to bone heterogeneity. Bone microstructure was found to be lamellar in nature with different microstructural features contributing to the various composition parameters of bone studies in this manuscript.

CRediT authorship contribution statement

Sachin Kalsi: Writing – original draft, Software, Investigation, Data curation. **Jagjit Singh:** Validation, Project administration, Formal analysis, Data curation. **N.K. Sharma:** Writing – review & editing, Supervision, Resources, Investigation. **Raman Kumar:** Writing – original draft, Supervision, Methodology, Funding acquisition. **Ali Khatibi:** Writing – original draft, Supervision, Project administration, Investigation. **Ankit Kedia:** Writing – review & editing, Validation, Resources, Investigation, Data curation. **Vikasdeep Singh Mann:** Writing – original draft, Supervision, Resources, Investigation. **Abhijit Bhowmik:** Writing – review & editing, Validation, Project administration, Investigation, Conceptualization. **A. Johnson Santhosh:** Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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