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Experimental archaeological study in China: implications for reconstruction of past manuring and dietary practices indicated by $\delta^{15}\text{N}$ values of *Setaria italica* and *Panicum miliaceum*

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Abstract

Understanding the crop management practices of millet agriculture is pivotal for comprehending the development of Chinese civilization. Recent studies have indicated that organic manuring plays a crucial role in ensuring sustainable millet cultivation on the Loess Plateau. However, uncertainties still exist regarding how manuring practices impact the $\delta^{15}\text{N}$ values of C_4 millets compared to C_3 cereals. Furthermore, inadequate information on crop $\delta^{15}\text{N}$ at archaeological sites has led to controversial interpretations of animal and human diets. In this study, we present new findings from an experimental archaeological research conducted in actual loess farmland in China to explore the potential range of variability in grain $\delta^{15}\text{N}$ values of millets. Our results demonstrate that animal manure significantly increases *Setaria* and *Panicum* grain $\delta^{15}\text{N}$ values, ranging from 2.7 to 9.3‰. Considering trophic enrichment effects on nitrogen isotopes, humans consuming manured millets may yield values ranging from 5.7 to 12.3‰, suggesting alternative explanations for high $\delta^{15}\text{N}$ values other than animal protein consumption. Opposite to the general hypothesis, the grain $\delta^{15}\text{N}$ values are systematically lower than those of leaves. The difference between the values of *Setaria* and *Panicum* and the process of manure influencing the grain $\delta^{15}\text{N}$ values are also discussed. Our study provides novel insights into the nitrogen stable isotopic indicator of millet manuring and will serve to set reconstructions of past manuring and diet practices in northern China on a firmer foundation.

Keywords Millet agriculture, Nitrogen stable isotopes, Animal manure, Palaeodiet, Archaeology

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Introduction

Foxtail millet (*Setaria italica*) and broomcorn millet (*Panicum miliaceum*) are domesticated crops native to prehistoric northern China [1–4]. Millet agriculture has been practiced for over 8000 years, gradually replacing hunting and gathering activities as the dominant economic activity in northern China around 6000 years ago [5, 6]. Archaeobotanical finds have revealed that millets were also cultivated in southern China, Southeast and Northeast Asia, and were common in Europe from the 2nd millennium BC along with the trans-Eurasian exchanges [7]. The spread of millet brought new species resources and even facilitated the agriculturalization of the introduced areas [8]. Therefore, studying millet agriculture provides an important approach for understanding the development of early cultures and the formation of ancient Chinese civilization [9–11], as well as prehistoric cultural interactions across Eurasia.

The rapid loss of organic matter and low nitrogen content in loess soils in northern China have remained significant constraints on the growth of millets [12–14]. The application of animal manure enhances nutrient availability for plants, and it has been viewed as an important driving factor for the expansion of millet agriculture and early cultures on the Loess Plateau of China [15, 16].

Previous field experiments conducted in western Eurasia reveal that nitrogen stable isotopes in plants serve as an essential indicator of manuring practice on C₃ crops (e.g., wheat, barley), with manuring resulting in higher δ¹⁵N values in these crops [17–22]. However,

the impact on millets of East Asia remains obscure. Although recent studies on millets have shown that manuring increases millet δ¹⁵N values (Table 1), none of them was conducted within the loess region of China [16, 23–25], and the differences in growing conditions (e.g., soil type, precipitation, temperature) limit their applicability to assess archaeological remains.

Due to the diverse subsistence economy on the Loess Plateau of China [26–28], ancient humans had access to variable types of animal manure, which has received little attention, despite their significant differences in the impact on crop yields [29, 30]. For people whose livelihood primarily relies on millet agriculture, pig dung is an easily accessible organic manure due to the close association between domestic pigs and millet farming [15, 24]. The higher δ¹⁵N values observed in charred millet grains have often been attributed to fertilization with pig manure [15, 16]. However, there is still a lack of experimental data to verify the impact of pig manure on millets. Pastoralism is characterized by cattle and sheep rearing [26, 28], resulting in the availability of their dung as animal manure for ancient people in pastoral areas when they carried out millet cultivation. Chicken is one of the “six livestock”, and chicken manure is also a high-quality fertilizer in modern agriculture [31]. Therefore, it holds great significance to explore how different animal manure influence the millet δ¹⁵N values and whether there is any difference among them, which will contribute to deeper insights into past crop management practices, utilization of

Table 1 Summary of information on manuring experiments

Location	Period	Environment	Species	Type of animal manure	Number of manuring levels	References
Rothamsted, UK	1844-present	Farmland	Bread wheat	Cattle manure	1	[32]
Rothamsted, UK	1852-present	Farmland	Two-row barley	Cattle manure	1	[32]
Askov, Denmark	1894-present	Farmland	Bread wheat, two-row barley	Cattle slurry	1	[33]
Bad Lauchstädt, Germany	1902-present	Farmland	Bread wheat, two-row barley	Cattle manure	2	[34]
Sutton Bonington, UK	2007–8	Farmland	Einkorn, emmer, spelt wheat	Cattle manure	2	[19]
Aleppo, Syria	2008–9	Farmland	Bread wheat, lentils	Sheep manure	2	[19]
Askov, Denmark	2008–9	Farmland	Emmer, spelt wheat, barley	Cattle slurry	1	[20]
Six farms, Morocco	2014	farmland	Barley	available	2	[21]
Askov, Denmark	2018	farmland	Foxtail millet, broomcorn millet	Cattle slurry	1	[23]
Oxford, UK	2018	Pots in the greenhouse	Foxtail millet, broomcorn millet, wheat	Cattle manure	3	[24]
Shandong, China	2016	Courtyard	Foxtail millet	Sheep manure	1	[25]
Shanxi, China	2020	Farmland	Foxtail millet	Pig dung	1	[16]

animal resources, and subsistence patterns of ancient people across diverse regions.

Furthermore, there has been limited discussion on *Panicum* compared to *Setaria*, along with an incomplete understanding regarding the entire process through which manuring influences millet grain $\delta^{15}\text{N}$ values, including the fermentation of animal manure and the $\delta^{15}\text{N}$ offset between different growing stages. These gaps hinder our comprehension of the mechanisms underlying manuring effects and impede the establishment of isotopic criteria for assessing manuring practices.

Regarding palaeodiet, stable carbon and nitrogen isotope analysis has played a pivotal role in reconstructing past diets and revealing social complexity in northern China (e.g., [15, 35–38]). On the one hand, stable carbon isotopes can provide an overview on the consumption of C_3 versus C_4 food in terrestrial foodwebs. Foxtail and broomcorn millets are both typical C_4 plants with higher $\delta^{13}\text{C}$ values than C_3 plants. The isotopic characteristics would be transferred through the foodwebs with isotopic fractionation. Prehistoric humans and domesticated animals exclusively consuming C_3 or C_4 plants are conventionally thought to exhibit $\delta^{13}\text{C}$ values around -20.0‰ or -6.0‰ respectively [26]. On the other hand, nitrogen isotopes often provide an estimate on the trophic levels for organisms. While the $\delta^{15}\text{N}$ values of plants are transferred up the food chain and recorded in the isotopic signatures of herbivores and human diets [19, 39, 40], plant $\delta^{15}\text{N}$ values are critical for reliable interpretation of animal and human remains. However, the lack of available information on plant $\delta^{15}\text{N}$ from archaeological sites results in assumptions based on generalized plant values, despite the considerable variation observed in plant $\delta^{15}\text{N}$ values [41–44]. This challenges conventional interpretation of $\delta^{15}\text{N}$ values of ancient animals and humans and highlights the urgent need for data on the $\delta^{15}\text{N}$ variation of millets.

This paper aims to explore the potential range of variability in grain $\delta^{15}\text{N}$ values, specifically focusing on manured millets, in order to set reconstructions of past manuring and diet practices in northern China on a firmer foundation. In this study, we employed an experimental archaeological approach by cultivating millets in actual loess farmland in the Guanzhong Basin, the core area of prehistoric millet agriculture in China. Various treatments were applied, including different types and levels of manure. Additionally, we considered the fermentation of animal manure, the $\delta^{15}\text{N}$ offset between the heading and mature period of soil and different parts of foxtail and broomcorn millet. The heading period serves as an intermediate phase during crop growth. The purpose of sampling at this stage was to systematically study the changes of stable nitrogen isotopes within the

farmland ecosystem during the growth of crops after fertilization. We expect the exploration of the complete process of manuring affecting crop stable nitrogen isotopes can provide a theoretical basis and reference for follow-up studies. Overall, these data can be used to assess past manuring and its impact on human bone collagen values, enhancing the accuracy of manuring and dietary reconstructions.

Materials and methods

Experimental materials

In this study, foxtail and broomcorn millet were cultivated separately in two large experimental areas located in the Jufeng Agricultural Specialized Cooperative in Lintong (临潼) District, Xi'an City, Shaanxi Province. A standard treatment was implemented in each experiment area to ensure similar soil fertility. The seeds used for planting were marketed as breeding grains to guarantee proper germination.

As stated in the introduction, manure from four common types of domestic animals (pig, cattle, sheep, chicken) was selected for this study. In accordance with the standard application rate of organic fertilizer in modern agriculture (15 t/ha) [45], two application rates of 9 t/ha and 21 t/ha were also set to form three manuring levels of low, medium and high. Due to the prevalent use of chemical fertilizers in modern agriculture, two common types of chemical fertilizers—urea and compound fertilizers, and an unmanured treatment were selected as controls. The compound fertilizers used here had N, P_2O_5 , and K_2O contents exceeding 45% and belonged to the potassium sulfate-type category. Urea was applied at a rate of 0.3 t/ha while compound fertilizer was 0.675 t/ha according to the recommended dosage on the package. Three replications were set for each treatment to minimize experimental errors.

Experimental design

The four types of animal manure were stacked in close proximity to each other adjacent to the experimental area. They underwent natural fermentation from May 16 to July 13, 2022, aiming to achieve complete maturity as much as possible. No strains of bacteria or other substances were added artificially to minimize disturbance.

Foxtail and broomcorn millet were separately cultivated within a large experimental area on July 14, 2022. *Panicum* was harvested on September 29, while *Setaria* was on October 13. The layout of a single experimental area is outlined in Fig. 1. Figure 2 shows the millets grown on actual farmland in Xi'an, Shaanxi Province, China, in 2022. The grains were mechanically sown while the fertilizers were manually spread after careful weighing.

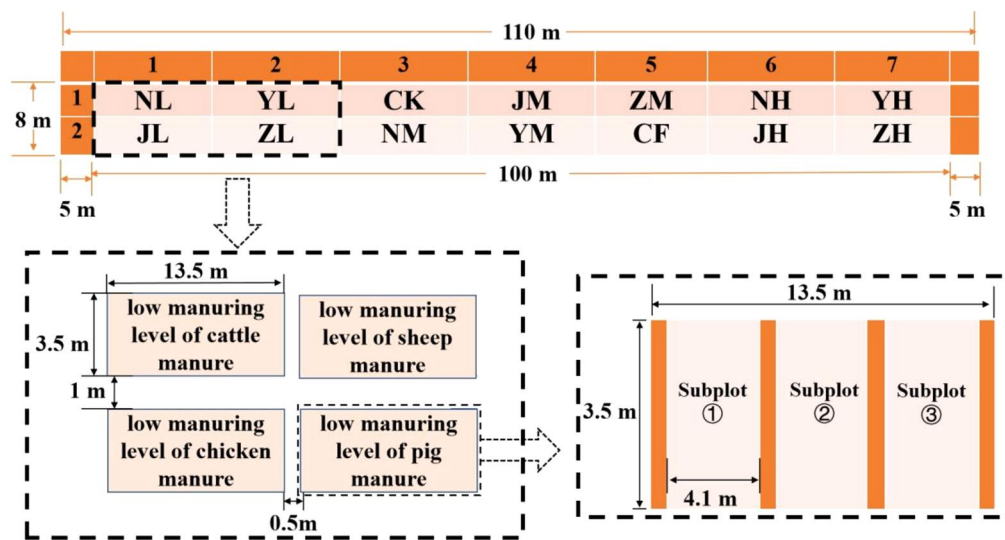


Fig. 1 The experimental layout of a single experimental area. The *Setaria* and *Panicum* plots treated with urea were set beside the *Panicum* area together



Fig. 2 The millet grown in actual farmland in Xi'an, Shaanxi Province, China, in 2022 (see Fig. 1 for details of plots involved in this study). The photos show fermentation of animal manure (upper left), the experimental area (*Setaria*, upper middle; *Panicum*, upper right), foxtail millet (lower left), and broomcorn millet (lower middle) close to harvest time. The lower right shows the harvest of broomcorn millet

Pig manure (Z), cattle manure (N), sheep manure (Y), and chicken manure (J) were applied at low (L, 9 t/ha), medium (M, 15 t/ha), and high (H, 21 t/ha) levels, with unmanured (CK), urea (U), and compound fertilizer (CF) serving as control treatments.

In total, there were fifteen treatments applied for *Setaria* and *Panicum* separately, with each plot of treatment equally divided into three subplots on behalf of three replications. Each subplot is approximately 14 m². The total experimental area is about 1332 m², with

isolation zones set up. Other planting conditions, such as irrigation regime, temperature, and soil type, were identical except for the aforementioned manuring strategies. Fertilizers were applied once as bottom fertilizers, without subsequent follow-up applications.

Sampling strategies

Three sampling sessions were conducted during the experiment, as shown in Table 2.

Table 2 The number of samples collected at different stages

Sampling stage	Date	Foxtail millet			Broomcorn millet			Fertilizer
		Grains	Leaves	Soil	Grains	Leaves	Soil	
Preplanting	July 14th	1	\	1	1	\	1	10
Heading	August 23rd	\	45	45	45	45	45	\
Mature	September 29th	\	\	\	45	45	45	\
	October 13rd	45	45	45	\	\	\	\

Before spreading the manure, soil ($n=2$), seeds for sowing ($n=2$), animal manure before and after fermentation ($n=8$), and chemical fertilizers ($n=2$) were sampled.

The second sampling was performed when crops were basically in the heading stage, while *Setaria* was still in the booting period due to its inconsistent growth progress with *Panicum*, which means there was no access to *Setaria* grains at this stage. Considering that the sampling during the heading stage is aimed at understanding the entire process of grain nitrogen isotope variation influenced by fertilization, we collected soil and plant samples at different stages, with a focus on soil and leaf data, which we believe are sufficient for achieving our objectives. The formation of testable grains occurs later in the growth cycle and varies among different crops. Therefore, grain data in the heading period is not essential. Instead, mature grain data constitutes the main and crucial part of our experiment. As a result, no additional sampling was conducted during the heading stage of *Setaria*. Plants and soil were sampled from each subplot.

The harvested material was left to dry indoors before being separated into grains and leaves. Subsequently, the grains were dehusked and cleaned. A total of 508 samples were collected for testing, including nine fertilizer samples (the urea sample could not be collected due to improper storage), 182 soil samples, 180 leaf samples, and 137 grain samples.

Elemental content and isotope analyses

Plant samples were pretreated according to the method described by Styring et al. [46]. The procedure involved the following steps: (1) Approximately 1 g of healthy leaves and grains were carefully selected from each sample and thoroughly rinsed with deionized water until all impurities adhering to the grains and leaf surfaces were removed. The cleaned leaves and grains were then transferred into small beakers. (2) The beakers containing *Setaria* and *Panicum* leaves and grains were placed in an oven at 60 °C for approximately 48 h to ensure complete drying until a constant weight was achieved. (3) The dried leaf and grain samples were finely ground into a homogeneous powder using an agate mortar/pestle and then packed into small sample vials using weighing paper.

(4) Tin containers were used to weigh 3 to 5 mg of each sample, which was prepared for subsequent analysis.

Soil samples were pretreated using the method of Liu et al. [47], which included the following steps. (1) 5 g of soil from each sample was extracted, fully dried in an oven at 60 °C, and subsequently milled until it could pass through a sieve with a mesh size of 200 mesh. (2) The milled soil samples were soaked in a dilute hydrochloric acid solution (0.5 mol/L) for at least 24 h, followed by washing with deionized water until neutral pH was reached. After drying again, they underwent further milling to obtain powdered form. (3) Approximately 20 mg of soil powder was weighed out and placed into tin capsules for measurement.

Animal manure pretreatment referred to the method described by Codron et al. [48]. Firstly, 5 g of fecal samples were carefully selected and mechanically cleaned before being placed into small beakers. Subsequently, the beakers were transferred to a 50 °C oven for drying. Afterward, the dried samples were ground into powder and approximately 3–5 mg of sample powder was packed into a tin capsule for analysis using high-temperature dry combustion to measure total N and ¹⁵N levels. Chemical fertilizers were directly ground and packed as well.

Sample testing was performed at the Key Laboratory of Vertebrate Evolution and Human Origins of the Chinese Academy of Sciences, Institute of Vertebrate Palaeontology and Palaeoanthropology, Chinese Academy of Sciences, using an IsoPrime 100 IRMS (Elementar, UK) coupled with an ElementarVario (Elementar, UK) with nitrogen stable isotope ratios expressed as δ per mil (‰) relative to the internationally defined standards for nitrogen (Ambient Inhalable Reservoir, AIR). The standards of the tests were IAEA-600 and IAEA-N-2 calibrated nitrogen cylinder gas with Te measurement errors less than $\pm 0.2\%$.

Results

All the data of 508 samples, including fertilizers, soils, leaves and grains covering the three pre-sowing, heading, and maturity periods, are shown in Fig. 3 and Table 3. Each fertilizer sample was tested twice to obtain its $\delta^{15}\text{N}$ values to minimize errors.

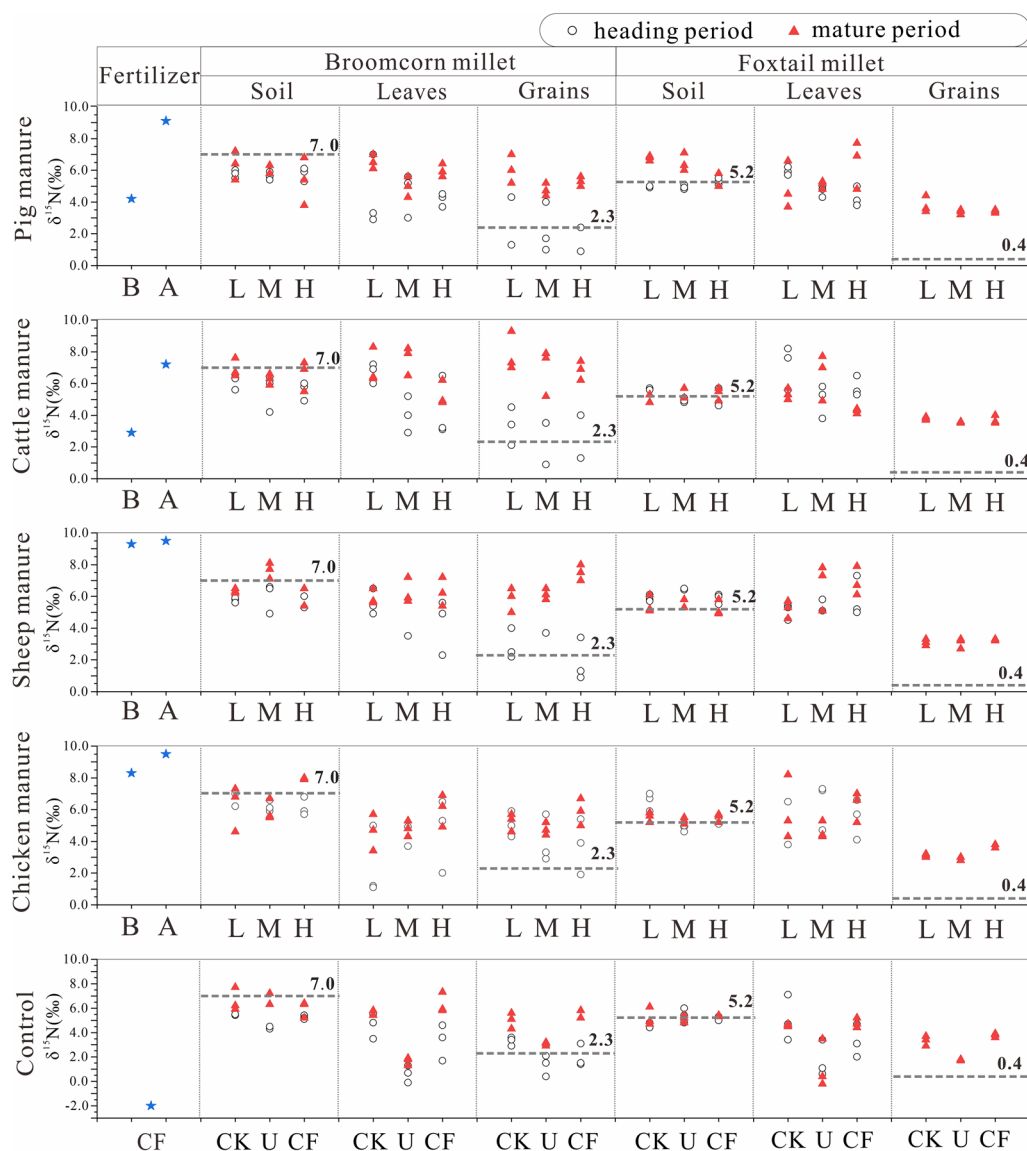


Fig. 3 The nitrogen isotope values of 508 samples in this experiment. (B and A under the fertilizer box refer to the samples before and after fermentation. L, M, and H correspond to low, medium, and high manuring levels, respectively, while CK, U, and CF correspond to unmanured, urea, and compound fertilizer treatments, respectively. The horizontal dashed line in the soil box indicates the original soil $\delta^{15}\text{N}$ value before being manured (5.2 ‰ for *Setaria* and 7.0 ‰ for *Panicum*), and the horizontal dashed line in the grain box indicates the $\delta^{15}\text{N}$ value of the seeds used for sowing (0.4 ‰ for *Setaria* and 2.3 ‰ for *Panicum*). Each of the horizontal dashed lines and points in the figure corresponds to the data of one sample.)

Variation in grain $\delta^{15}\text{N}$ values

Compared with soil and leaves, millet grains are a key plant part identifiable to species and widely preserved in charred form. As a result, grain $\delta^{15}\text{N}$ values receive more attention from us.

The results for grains are shown in Fig. 3 and Table 3. The $\delta^{15}\text{N}$ values of original seeds for sowing were 0.4 ‰ for *Setaria* and 2.3 ‰ for *Panicum*.

When treated with animal manure, the mature grain $\delta^{15}\text{N}$ values for *Setaria* ranged from 2.7 to 4.4 ‰ (mean 3.4 ± 0.3 ‰, $n=36$). For *Panicum*, the grain $\delta^{15}\text{N}$ values in the heading period ranged from 0.9 to 5.9 ‰ ($n=36$), while in the mature period, they ranged from 4.4 to 9.3 ‰ (mean 6.1 ± 1.2 ‰, $n=36$).

Variations in $\delta^{15}\text{N}$ values of mature grains after fertilization with different animal manure and manuring

Table 3 Summary of the nitrogen isotope values of soil and plants

Treatment	Species	Type	Heading period				Mature period			
			Range (‰)			Mean value (‰)	Range (‰)			Mean value (‰)
			Low	Medium	High		Low	Medium	High	
Original values	Setaria	Soil	5.2 (n= 1)							
		Seed	0.4 (n= 1)							
	Panicum	Soil	7.0 (n= 1)							
		Seed	2.3 (n= 1)							
Pig manure	Setaria	Soil	4.9–5.0	4.8–5.1	5.1–5.5	5.1±0.2 (n=9)	6.6–6.9	6.0–7.1	5.0–5.8	6.3±0.7 (n=9)
		Leaf	5.7–6.2	4.3–5.0	3.8–5.0	5.0±0.8 (n=9)	3.7–6.6	4.8–5.3	4.8–7.7	5.5±1.3 (n=9)
		Grain	\	\	\	\	3.4–4.4	3.2–3.5	3.3–3.5	3.5±0.3 (n=9)
	Panicum	Soil	5.5–6.0	5.4–5.9	5.3–6.1	5.7±0.3 (n=9)	5.4–7.2	5.8–6.3	3.8–6.8	5.9±1.0 (n=9)
		Leaf	2.9–7.0	3.0–5.6	3.7–4.5	4.4±1.4 (n=9)	6.1–7.0	4.3–5.6	5.6–6.4	5.8±0.8 (n=9)
		Grain	1.3–4.3	1.0–4.0	0.9–2.4	2.1±1.3 (n=9)	5.2–7.0	4.4–5.2	5.0–5.6	5.4±0.8 (n=9)
Cattle manure	Setaria	Soil	5.6–5.7	4.8–5.1	4.6–5.7	5.2±0.4 (n=9)	4.8–5.3	5.1–5.7	4.9–5.7	5.3±0.3 (n=9)
		Leaf	5.6–8.2	3.8–5.8	5.3–6.5	6.0±1.3 (n=9)	5.0–5.7	4.9–7.7	4.1–4.4	5.4±1.2 (n=9)
		Grain	\	\	\	\	3.7–3.9	3.5–3.6	3.5–4.0	3.7±0.2 (n=9)
	Panicum	Soil	5.6–6.4	4.2–6.4	4.9–6.0	5.7±0.7 (n=9)	6.5–7.6	5.9–6.6	5.5–7.3	6.6±0.7 (n=9)
		Leaf	6.0–7.2	2.9–5.2	3.1–6.5	5.0±1.7 (n=9)	6.3–8.3	6.5–8.2	4.8–6.2	6.6±1.3 (n=9)
		Grain	2.1–4.5	0.9–3.5	1.3–4.0	3.0±1.3 (n=9)	7.0–9.3	5.2–7.9	6.2–7.4	7.2±1.1 (n=9)
Sheep manure	Setaria	soil	5.7–6.1	6.4–6.5	5.5–6.1	6.1±0.3 (n=9)	5.1–6.1	5.3–5.8	4.9–5.8	5.4±0.4 (n=9)
		Leaf	4.5–5.4	5.1–5.8	5.0–7.3	5.5±0.8 (n=9)	4.6–5.7	5.1–7.8	6.1–7.9	6.3±1.2 (n=9)
		Grain	\	\	\	\	2.9–3.3	2.7–3.3	3.2–3.3	3.1±0.2 (n=9)
	Panicum	Soil	5.6–5.9	4.9–6.6	5.3–6.0	5.8±0.5 (n=9)	6.2–6.5	7.1–8.1	5.4–6.5	6.7±0.8 (n=9)
		Leaf	4.9–6.5	3.5–3.5	2.3–5.6	4.7±1.4 (n=9)	5.6–6.5	5.7–7.2	5.4–7.2	6.2±0.7 (n=9)
		Grain	2.2–4.0	3.7–3.7	0.9–3.4	2.6±1.2 (n=9)	5.0–6.5	5.8–6.5	7.0–8.0	6.5±0.9 (n=9)
Chicken manure	Setaria	Soil	5.9–7.0	4.6–5.0	5.1–5.5	5.5±0.8 (n=9)	5.2–5.8	5.1–5.5	5.2–5.7	5.4±0.3 (n=9)
		Leaf	3.8–6.5	4.7–7.3	4.1–6.6	5.7±1.4 (n=9)	4.3–8.2	4.3–5.3	5.2–7.0	5.6±1.4 (n=9)
		Grain	\			\	3.1–3.4	2.8–3.1	3.6–3.8	3.3±0.4 (n=9)
	Panicum	Soil	6.2–7.0	5.9–6.6	5.7–6.8	6.3±0.4 (n=9)	4.6–7.3	5.5–6.7	7.9–8.0	6.7±1.2 (n=9)
		Leaf	1.1–5.0	3.7–4.9	2.0–6.5	3.8±2.0 (n=9)	3.4–5.7	4.3–5.3	4.9–6.9	5.1±1.0 (n=9)
		Grain	4.3–5.9	2.9–5.7	1.9–5.4	4.3±1.4 (n=9)	4.6–5.7	4.4–5.2	5.0–6.7	5.3±0.7 (n=9)
Unmanured	Setaria	Soil	4.4–4.8				4.7–6.1			
		Leaf	3.4–7.1				4.5–4.7			
		Grain	\				2.9–3.7			
	Panicum	Soil	5.4–5.5				5.9–7.7			
		Leaf	3.5–5.5				5.4–5.8			
		Grain	2.9–3.6				4.3–5.6			
Urea	Setaria	Soil	4.8–6.0				4.8–5.4			
		Leaf	0.6–3.4				-0.2–3.5			
		Grain	\				1.7–1.8			
	Panicum	Soil	4.3–4.5				6.0–6.8			
		Leaf	– 0.1 to 1.3				1.3–1.9			
		Grain	0.4–2.1				2.9–3.2			
Compound fertilizer	Setaria	Soil	5.0–5.3				5.3–5.4			
		Leaf	2.0–4.7				4.4–5.2			
		Grain	\				3.6–3.9			
	Panicum	Soil	5.1–5.4				5.2–6.4			
		Leaf	1.7–4.6				5.8–7.3			
		Grain	1.4–3.1				5.2–5.8			

levels must also be expected. Specifically, the $\delta^{15}\text{N}$ values of *Panicum* grains treated with cattle manure ranged from 5.2 to 9.3‰ (mean $7.2 \pm 1.1\text{‰}$, $n=9$), while *Setaria* grains treated with sheep manure exhibited a range of 2.7‰ to 3.3‰ (mean $3.1 \pm 0.2\text{‰}$, $n=9$). Among the four types of animal manure, the NL treated *Panicum* grains had the highest value, whereas *Setaria* grains on YM plots yielded the lowest one. Regarding different levels of animal manure application, grains from the low level of pig and cattle manure (ZL and NL) were associated with the highest $\delta^{15}\text{N}$ values than the medium and high level, whereas the high level of sheep and chicken manure (YH and JH) showed the highest values than the low and medium level. However, it is worth noting that there are no clear distinctions among the four types of animal manure due to their largely overlapping ranges. The results also showed no lower or higher $\delta^{15}\text{N}$ values with higher manuring levels.

For grains on the control plots, the $\delta^{15}\text{N}$ values of mature grain ranged from 1.7 to 3.9‰ for *Setaria* and 2.9 to 5.8‰ for *Panicum*. And the grain values under urea treatment were the lowest regardless of *Setaria* or *Panicum*.

Data on fertilizers

Table 4 summarizes the results for four kinds of animal manure before and after fermentation.

The $\delta^{15}\text{N}$ values of animal manure ranged from 2.9 to 9.3‰ ($n=4$) before fermentation and 7.2 to 9.5‰ ($n=4$) after fermentation, while the TN (total nitrogen content) values of animal manure ranged from 1.66 to 3.08% before fermentation and 1.56% to 2.27% after fermentation. Additionally, compound fertilizer exhibited the lowest $\delta^{15}\text{N}$ values (mean $-2.0 \pm 0.1\text{‰}$, $n=2$) and the highest TN values (18.4%).

Variation in soil $\delta^{15}\text{N}$ values

As shown in Fig. 3 and Table 3, the original soil $\delta^{15}\text{N}$ values before manuring were 5.2 ‰ for *Setaria* and 7.0‰ for *Panicum*.

In the *Setaria* area, the soil $\delta^{15}\text{N}$ values ranged from 4.4 to 7.0‰ ($n=45$) in the heading period and from 4.7 to 7.1‰ ($n=45$) in the mature period. Similarly, in

the *Panicum* area, the values ranged from 4.2 to 7.0‰ ($n=45$) in the heading period and from 3.8 to 8.1‰ ($n=45$) in the mature period.

Variations in soil $\delta^{15}\text{N}$ values after fertilization with different types of animal manure and manuring levels are also presented in Fig. 3 and Table 3. It should be noted that differences in soil $\delta^{15}\text{N}$ values among different treatments generally do not align with those observed in grain values. For instance, the highest manured soil $\delta^{15}\text{N}$ values occur on YM plots whereas the lowest values are on ZH plots in the mature period of *Setaria*. Furthermore, the interval widths of soil $\delta^{15}\text{N}$ variation under cattle manure treatment were 0.9‰ (for *Setaria*) and 2.1‰ (for *Panicum*), which were relatively low compared with other manure types. The widths of soil $\delta^{15}\text{N}$ variation under pig manure treatment were 2.1‰ (for *Setaria*) and 3.4‰ (for *Panicum*), both reaching higher levels.

Variation in leaf $\delta^{15}\text{N}$ values

The leaf data are shown in Fig. 3 and Table 3. In the heading period of *Setaria*, the leaf $\delta^{15}\text{N}$ values ranged from 0.6 to 8.2‰ ($n=45$) while in the mature period, a wider range of values was observed, from -0.2 to 8.2‰ ($n=45$). Similarly, for *Panicum*, the leaf $\delta^{15}\text{N}$ values were -0.1 to 7.2‰ ($n=45$) in the heading period and 1.3 to 8.3‰ ($n=45$) in the mature period.

For leaves treated with different types of animal manure and manuring levels, the values of mature leaves range from 3.7 to 8.2‰ for *Setaria* and from 1.3 to 8.3‰ for *Panicum*, respectively, both of which are wider than those of grains. It might be noteworthy that the leaf under sheep manure treatment exhibited the minimum $\delta^{15}\text{N}$ values of 4.6‰ (for *Setaria*) and 5.4‰ (for *Panicum*), both lower than the minimum values observed with other types of manure. Moreover, these values above also displayed a narrower range of variation, specifically 3.3‰ (for *Setaria*) and 1.8‰ (for *Panicum*), which were also the lowest among the four types. Mature leaves on JH and NM plots were associated with highest $\delta^{15}\text{N}$ values on average than those at the other two manuring levels, showing an overall irregular variation pattern.

Table 4 The nitrogen isotope values and the total nitrogen content of fertilizers

Type	Before fermentation (B)		After fermentation (A)		$\Delta_{(A-B)}$	
	$\delta^{15}\text{N}$ (‰)	TN (%)	$\delta^{15}\text{N}$ (‰)	TN (%)	$\Delta^{15}\text{N}_{(A-B)}$ (‰)	$\Delta\text{TN}_{(A-B)}$ (%)
Pig manure	4.2 ± 0.1 ($n=2$)	3.08	9.2 ± 0.2 ($n=2$)	1.77	5.0	-1.31
Cattle manure	2.9 ± 0.1 ($n=2$)	2.49	7.2 ± 0.2 ($n=2$)	2.24	4.3	-0.25
Sheep manure	9.3 ± 0.6 ($n=2$)	1.66	9.5 ± 0.2 ($n=2$)	2.27%	0.2	0.61
Chicken manure	8.3 ± 0.7 ($n=2$)	2.35	9.5 ± 0.4 ($n=2$)	1.56%	1.2	-0.79

Discussion

Grain $\delta^{15}\text{N}$ values and implications for past manuring and diets

Previous modern experiments conducted in western Eurasia have demonstrated that nitrogen stable isotope constitutes an effective indicator of past manuring for C_3 crops [17–19]. While for C_4 millets, the data from the farmland experiment in China reported here suggest that animal manure application also significantly influenced the *Setaria* and *Panicum* grain values. Compared to the seeds for sowing, manuring resulted in an increase of 2.3–4.0‰ and 2.1–7.0‰ in grain $\delta^{15}\text{N}$ values of *Setaria* and *Panicum* respectively. From the data in Fig. 4 and Table 3, the variation ranges of grain $\delta^{15}\text{N}$ values are largely overlapping and cannot be clearly distinguished when they are treated with different types of animal manure or manuring levels. Furthermore, it is always unlikely to ascertain precise information regarding both the amount and type of animal manure used by prehistoric farmers due to the diversity of animal husbandry on the Loess Plateau. Thus, the overall scope of grain $\delta^{15}\text{N}$ values grown on manured plots ranging from 2.7 to 9.3‰ may serve as a more practical reference for evaluating past manuring practices. It should be noted that a recent manuring experiment on millet in the greenhouse at the University of Oxford found manured millets with high $\delta^{15}\text{N}$ values (8.2–18.2‰, mean 13.1 ± 2.3 ‰, $n=43$), surpassing those typically observed among modern millets [24]. The disparity could potentially be attributed to differences arising from contrasting growth environments compared to Chinese millets cultivated on actual farmland.

The impact of manure on grain $\delta^{15}\text{N}$ values comes with implications for reconstructing past dietary practices. The trophic enrichment of nitrogen isotope ratio is conventionally estimated to be 3–5‰ [44], based on what they eat [49]. For herbivores, including humans with plant-based diets, the $\delta^{15}\text{N}$ values deriving from the analysis of bone collagen would range from 3 to 7‰, while for diets primarily consisting of animal products, the values would be higher than 9‰. Values for a mixed diet would lie between 7 and 9‰ [50, 51].

To achieve more precise identification of C_4 consumption in humans and animals, it is necessary to combine carbon and nitrogen. Table 5 presents the $\delta^{15}\text{N}$ and the $\delta^{13}\text{C}$ data collected from several archaeological sites in northern China. The $\delta^{13}\text{C}$ values of human bone collagen were predominantly around -6.0 ‰, indicating a diet dominated by millet-related resources when combined with archaeobotanical results. Values of associated domestic animals are critical for the accurate interpretation of human values. As shown in Table 5, pig $\delta^{13}\text{C}$ values are basically equal to human values, suggesting

their similar millet-based diets and pigs are also possible resource of C_4 food for humans. Although the $\delta^{15}\text{N}$ values of humans are slightly higher than those of pigs, the difference is far from the trophic enrichment of 3‰. The results suggest that plant-based C_4 foods were staple foods for people, instead of animal protein from pigs.

Human $\delta^{15}\text{N}$ values have traditionally been interpreted as indicative of a diet primarily based on animal protein when exceeding 9‰ (Table 5). However, our study reveals that manured millet grain $\delta^{15}\text{N}$ values can range from 2.7 to 9.3‰. If we assume that humans were the primary consumers of millet grains and with trophic enrichment of 3‰, the values of humans primarily consuming grains would be expected to reach 5.7–12.3‰. Thus, fairly high values of ancient humans could result from millet consumption, rather than the attribution of animal protein. Since the middle and late Yangshao period, the prosperity of Yangshao culture was accompanied by the intensification and expansion of millet agriculture [15], whereas the unbalanced development of agriculture across different temporal and spatial contexts offers diverse interpretations for the $\delta^{15}\text{N}$ values of ancient people. For instance, current evidence suggests that animal husbandry gradually became the predominant livelihood in central and southern Inner Mongolia around 3000 BP [52]. For subsequent sites such as the Nalintaohai [53], Fuluta [54], Xindianzi [55] cemetery, the high human $\delta^{15}\text{N}$ values may be attributed to the consumption of animal protein derived from meat or dairy products from domesticated animals. However, the Guanzhong region underwent a transition towards agricultural production dominance around 6000BP, characterized by the flourishing development of dryland agriculture represented by millets [56, 57]. Consequently, the high $\delta^{15}\text{N}$ values observed in human remains from sites since then, such as the Jianyu [58], Guangming, Jichang and Guandao cemeteries [59], are more plausibly explained by the consumption of manured millets rather than animal protein. Similar considerations are also applicable to regions like Henan and Shanxi which transitioned into agricultural societies since the middle and late Neolithic period [60, 61], exemplified by sites like Neiyangyuan [62] and Bali-gang site [60].

Certain trends inferred from comparisons of isotope data also warrant reevaluation. Taking the Qingliangsi site in Shanxi as an example, the human $\delta^{15}\text{N}$ values increased from 8.1 ± 1.1 ‰ ($n=13$) during the Yangshao period to 8.8 ± 0.9 ‰ ($n=14$) during the Longshan period. The elevation likely reflects continuous development of agriculture since the Yangshao period, with manuring practices contributing to higher crop $\delta^{15}\text{N}$ values and subsequently affecting human $\delta^{15}\text{N}$ values. The previous interpretation that attributed this general rise solely to

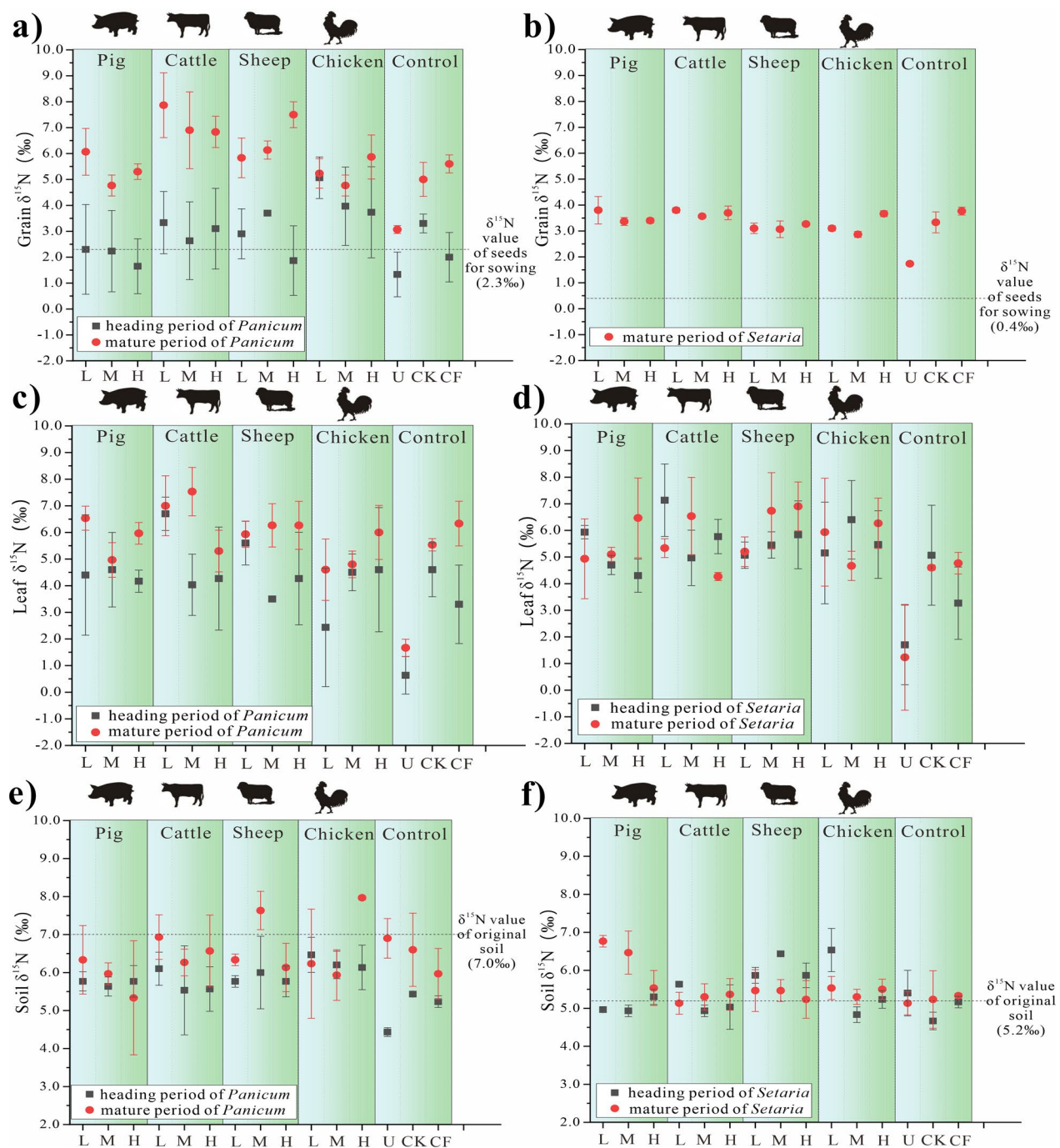


Fig. 4 Variation in $\delta^{15}\text{N}$ values of grain, leaf and soil during the growth of millet. The left pictures show data of *Panicum* grain (a), leaf (c) and soil (e), while the right pictures show data of *Setaria* grain (b), leaf (d) and soil (f), respectively. Bars indicate mean values ± 1 standard error (n=3)

increased meat consumption compared to the Yangshao period may be problematic, particularly during the Longshan period characterized by evident social differentiation and stratification.

According to zooarchaeological evidence, animal husbandry was widespread and intensified since the middle

and late Neolithic in northern China [63, 64], suggesting it was not implausible to collect more than 0.9 kg/m² (9 t/ha) of animal manure each year. The discussion above also indicates that it is crucial to establish an isotope foodweb including plants and animals when scientifically interpreting isotopic data from humans

Table 5 summary of $\delta^{15}\text{N}$ values from bone collagen of humans, domestic pig in some archaeological sites in northern China

Site	Period	Human $\delta^{13}\text{C}$	Human $\delta^{15}\text{N}$ Range	Human $\delta^{15}\text{N}$ Average	n	Pig $\delta^{13}\text{C}$	Pig $\delta^{15}\text{N}$	n	Reference
Qingliangsi Shanxi	4050-1700BC	-8.1 ± 0.9	6.2–10.6	8.5 ± 1.1	27	\	\	\	[67]
Miaozigou Inner Mongolia	3500-3000BC	-7.2 ± 0.2	9.0–9.5	9.2 ± 0.2	9	\	\	\	[66]
Shenggedaliang Shaanxi	1875-1665BC	-8.5 ± 1.8	6.2–11.8	8.8 ± 1.4	28	-8.3 ± 0.8	7.7 ± 0.8	7	[26]
Baligang Henan	1600-1046BC	-11.7 ± 1.0	9.2–10.9	10.2 ± 0.9	3	\	\	\	[60]
Hengshui Shanxi	1046-771BC	-8.3 ± 1.1	7.2–11.9	9.0 ± 1.0	82	\	\	\	[68]
Neiyangyuan Shanxi	770-476BC	-8.6 ± 1.6	7.9–11.7	9.6 ± 1.0	23	\	\	\	[62]
Chongpingyuan Shaanxi	770-403BC	-7.9 ± 0.5	7.9–10.4	8.8 ± 0.6	15	-8.1 ± 0.5	7.5 ± 0.5	2	[27]
Taosibei Shanxi	770-221BC	-7.9 ± 0.4	\	9.6 ± 0.9	66	\	\	\	[69]
Jianyu Shaanxi	468-221BC	-8.8 ± 0.6	9.4–11.4	10.3 ± 0.5	26	\	\	\	[58]
Fuluta Inner Mongolia	221BC-8AD	-8.5 ± 0.4	8.3–10.3	9.2 ± 0.5	29	-8.6 ± 1.1	7.7 ± 1.1	3	[54]
Xindianzi Inner Mongolia	476-453BC	-11.5 ± 0.9	9.2–12.0	10.3 ± 0.8	20	\	\	\	[55]
Xiaonanzhuang Shanxi	550-403BC	-8.0 ± 0.4	9.0–11.7	10.5 ± 0.9	16	\	\	\	[70]
Nalintaohai Inner Mongolia	8-220AD	-10.0 ± 0.8	11.9–14.8	13.3 ± 1.2	7	\	\	\	[53]
Guangmin, Guandao and Jichang Shaanxi	202BC-220AD	-11.3 ± 1.1	7.1–12.5	9.5 ± 1.1	40	\	\	\	[59]

n number of samples

[65]. Moreover, if we assume that pigs primarily consumed millet by-products, the difference in $\delta^{15}\text{N}$ values between domestic pigs and humans may be result from the discrepancy in $\delta^{15}\text{N}$ values between millet grains and by-products, though their relationship remains unclear compared to C_3 cereals and requires more modern experiments to provide evidence.

Furthermore, *Setaria* and *Panicum* are both C_4 plants, but they follow two different biochemical photosynthetic types [71]. The $\delta^{15}\text{N}$ comparison between *Setaria* and *Panicum* has been rarely addressed. Our results show higher $\delta^{15}\text{N}$ values of *Panicum* ($4.4\text{--}9.3\text{‰}$, mean $6.1 \pm 1.2\text{‰}$, $n = 36$) than *Setaria* ($2.7\text{--}4.4\text{‰}$, mean $3.4 \pm 0.3\text{‰}$, $n = 36$) and the response of *Panicum* grain $\delta^{15}\text{N}$ variation is more pronounced than that of *Setaria*, which probably suggests the potential of stable nitrogen isotope analysis of millets for distinguishing *Setaria* and *Panicum* at the species level. Moreover, the discrepancy may also influence the accuracy of dietary reconstruction, as human consumers of *Panicum* grains

may tend to exhibit higher $\delta^{15}\text{N}$ values, implying the importance to combine with archaeobotanical results.

The relationship in $\delta^{15}\text{N}$ values between grain and leaf

In accordance with previous studies on modern C_3 crops, the grain $\delta^{15}\text{N}$ values are higher than those of other parts (e.g., leave, straw, rachis) [17–19]. For C_4 millets, leaf $\delta^{15}\text{N}$ values are generally thought to be lower than those observed in grain. This relative relationship has led to the interpretation that pigs were fed millet leaves or other byproducts and grains were mostly consumed by humans at prehistoric sites, which explains the slightly higher human $\delta^{15}\text{N}$ values than those of pigs [15, 16, 27]. However, the results from this experiment suggest that the $\delta^{15}\text{N}$ values in grain tend to be lower than those in leaf, with an average difference of approximately 0.9‰ (Fig. 5). This finding holds significant implications for palaeodietary work, where the values of grains consumed by humans are often assumed to be higher. Previous interpretations for the mean $\delta^{15}\text{N}$ offset

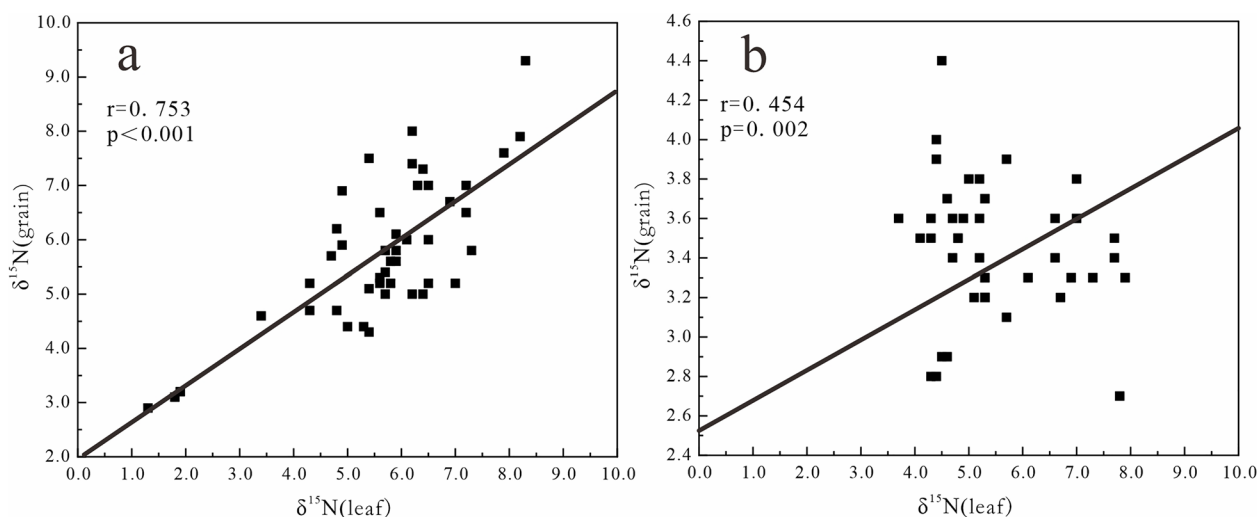


Fig. 5 The relationship between $\delta^{15}\text{N}$ values of grain and leaf for *Panicum* (a) and *Setaria* (b). The r -value reports the Pearson product-moment correlation coefficient and P -value at a significance level of 0.05

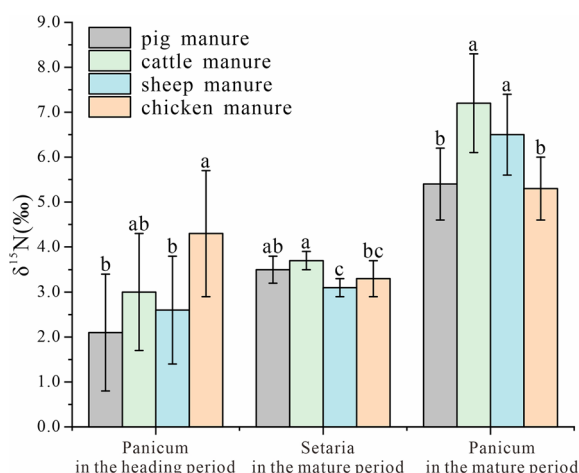


Fig. 6 $\delta^{15}\text{N}$ values of grains treated with different types of animal manure. Bars indicate mean values ± 1 standard error ($n=9$). Within millet varieties in one period, letters denote statistical significance at $P<0.05$ with the LSD test

between archaeological grains, domestic animals and humans based on this hypothesis [72, 73] may require reconsideration.

The response of grain $\delta^{15}\text{N}$ values to different fertilizers and manuring levels

Previous studies have primarily focused on investigating the impact of a single type of animal manure on $\delta^{15}\text{N}$ values in modern crops (Table 1). In this study, we further discussed the implications of manuring with different types of animal manure on millet grain values. Figures 4

and 6 show that manure type significantly influenced millet grain $\delta^{15}\text{N}$ values. For example, mature grain $\delta^{15}\text{N}$ values are the highest on average after fertilization with cattle manure. Conversely, grains fertilized with pig and chicken manure exhibited lower but comparable levels to each other, showing no significant difference. Notably, the minimum values of grains under cattle manure treatment were 3.5‰ (for *Setaria*) and 5.2‰ (for *Panicum*), while the maximum values were 4.0‰ (for *Setaria*) and 9.3‰ (for *Panicum*). Compared to other types of manure, these values are all relatively high, suggesting that grains treated with cattle manure may have a greater potential for achieving higher $\delta^{15}\text{N}$ values. On the contrary, both minimum and maximum grain $\delta^{15}\text{N}$ values under chicken manure treatment were comparatively lower than those associated with other types of manures used in this study, potentially indicating a weaker ability of chicken manure to increase grain $\delta^{15}\text{N}$ levels.

The variation probably results from factors such as the isotopic composition and the mineralization rate of the fertilizers [74–77], implying the potential to differentiate the manuring effect of various types of manure. However, as shown in Figs. 4 and 6, the differences in grain $\delta^{15}\text{N}$ values are inconsistent within different species and periods, and whether the physiological characteristics of crops or other factors contributed to this still need to be further explored.

Different crops have been cultivated with continuous fertilizers applied in the experimental area for many years. The grain $\delta^{15}\text{N}$ values on CK, CF and manured plots did not differ significantly (Fig. 3), probably due to the farmland history of cultivation and fertilization. The

grains grown on urea plots displayed lower values, consistent with the depletion of ^{15}N associated with urea [78].

Furthermore, although three manuring levels were set for each type of animal manure, no distinct relationship between grain $\delta^{15}\text{N}$ values and manuring levels was observed. For instance, grains fertilized with pig and cattle manure exhibited the highest $\delta^{15}\text{N}$ values at low manuring level, while sheep and chicken manure resulted in highest values at high manuring level (Fig. 4). As for the upper and lower limits of grain $\delta^{15}\text{N}$ values after fertilization, it can be observed that the minimum and maximum values of grain on ZL plots are higher than those on ZM and ZH plots. Similar trends were observed under cattle manure treatment, whereas both the upper and lower limits of grain values under sheep and chicken manure treatments at high manuring levels were higher than those at low or medium levels. Nevertheless, the

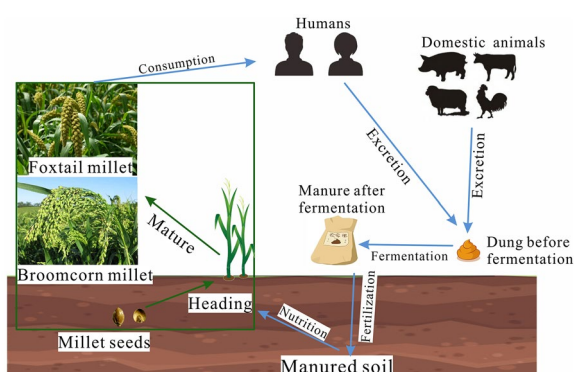


Fig. 7 The process of manuring affecting grain $\delta^{15}\text{N}$ values and human diets

grain values after fertilization with animal manure show no discernible correlation trend and the ranges largely overlap as the amount of fertilizer applied increases. This may be ascribed to the fact that one year of cultivation is too short to show a significant relationship, highlighting the need for further studies.

The process of manure influencing the grain $\delta^{15}\text{N}$ values

We present a preliminary depiction of the impact of manuring on grain $\delta^{15}\text{N}$ values and human diets, encompassing the process from animal manure fermentation to human consumption of grains (Fig. 7). Our aim is to provide a reference for further investigations into the mechanism behind nitrogen stable isotope response to fertilization in millet, as well as establish isotopic criteria.

In accordance with previous studies, animal manure exhibits marked enrichment in ^{15}N , which results in the rise of the grain $\delta^{15}\text{N}$ values by manuring [19, 79–81]. We found that fermentation increased the $\delta^{15}\text{N}$ values of animal manure by 0.2–5.0‰ while reducing its N content by 0.23–1.31% (Table 4 and Fig. 8), probably due to biochemical reactions such as denitrification and ammonia volatilization during fermentation [82]. These factors contribute to the high $\delta^{15}\text{N}$ values of animal manure.

After applying animal manure to the soil, the offset in soil $\delta^{15}\text{N}$ between the heading and mature period was 0.3‰. In contrast, Kanstrup et al. [20] reported significant soil $\delta^{15}\text{N}$ offset (1.9‰) in Askov long-term arable soils. This implies that one year of animal manure addition may be inadequate to significantly influence the huge soil N pools, and the distinct N isotopic imprint on soil is potential for indicating past long-term manuring. Compared to soil, both grains and leaves displayed lower

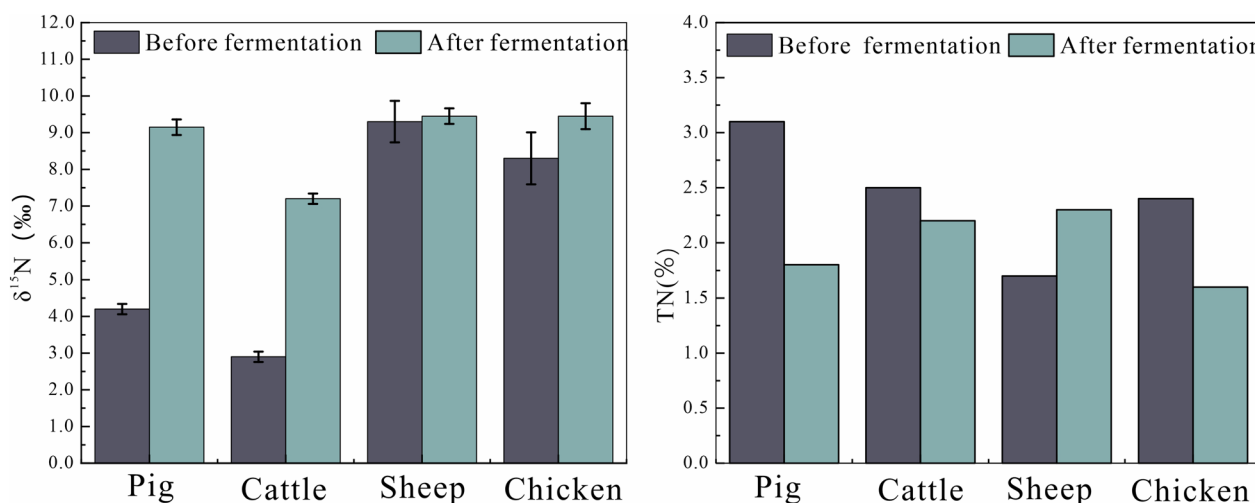


Fig. 8 The $\delta^{15}\text{N}$ values and total nitrogen content of animal manure before and after fermentation. Bars indicate mean values ± 1 standard error ($n = 2$)

values, conforming to the preferential uptake of ^{14}N from soil by plants [83]. As manured crops grew, the offset in grain $\delta^{15}\text{N}$ between the heading and mature period was 3.1‰ (for *Panicum*), probably resulting from the gradual nitrogen accumulation [84]. Finally, this process contributes to the high $\delta^{15}\text{N}$ values in humans consuming manured grains.

Conclusions

Our field experiment conducted in the core area of prehistoric millet agriculture in China has confirmed the significant impact of manuring on grain $\delta^{15}\text{N}$ values of foxtail and broomcorn millets, with manured grains exhibiting a range of $\delta^{15}\text{N}$ values from 2.7 to 9.3‰. The variation provides an alternative interpretation for the high $\delta^{15}\text{N}$ values of humans in specific archaeological sites in northern China when reliable measurements of plant $\delta^{15}\text{N}$ values are unavailable. The grain $\delta^{15}\text{N}$ values are systematically lower than those observed in millet leaves, indicating that reconsideration is required for the $\delta^{15}\text{N}$ offset between archaeological grains, domestic animals and humans. These findings underscore that determining the $\delta^{15}\text{N}$ values of archaeobotanical remains is critical for accurately evaluating past manuring and dietary practices.

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Author contributions

XS designed the research. ZZP performed the experiments. HYOY sampled and analyzed the samples. HYOY and JCL made contributions to the acquisition of data. HYOY and XS analyzed the data, and were both the major contributors in writing the manuscript. YWH, XQL and JCL made substantial contributions to the revision. All authors read the final manuscript. HYOY and XS approved the final manuscript.

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Availability of data and materials

All data supporting this study's findings are presented in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing of interests.

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