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Isotopic carbon turnover in pig hoof and rib

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ABSTRACT. The objective of this study was to evaluate the behavior of carbon incorporation and turnover in hoof and ribs of pigs at different periods of development in the search for tissues that reflect longer the former diet. We used 132 commercial hybrids (barrows and females), weaned at an average age of 21 days, distributed in a completely randomized design with four treatments on different days of substitution of corn (C_4 cycle plant grain) diets with broken rice (C_3 cycle plant grain) at 21, 42, 63 and 110 days of age to change the carbon-13 isotope signal. By means of isotopic dilution curves, we observed that animals whose C_4 diet was replaced with C_3 diet at 21, 42, 63 and 110 days of age, for hoof and rib, reached a new level of isotope equilibrium. Bone samples are better choices to reflect the former diet, due to conservation of the isotopic signal for longer.

Keywords: stable isotopes; carbon-13; animal nutrition; swine.

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Introduction

The increase in per capita consumption is a challenge for the pork sector, because consumer needs and expectations contribute significantly to the choice of protein source (Moeller et al., 2010); there is an increasing awareness of the relationship between food and health and a greater interest in the origin of meat and the system for raising livestock (Grunert, 2006). Thus, the importing market has shown to be increasingly demanding in relation to the traceability of animal by-products used in animal feeding, thus, the technique of stable isotopes has great potential for practical application for the detection of these by-products through analysis of pig tissues at different developmental stages. However, few studies have been conducted in animals using foods naturally enriched in ^{13}C.

Body constituents are in a dynamic state, continuously formed and used for different purposes. This process is generally called turnover and all substances in the body, whether organic or inorganic metabolites, are subject to this process (Kennedy & Krouse, 1990), however, most studies involving turnover of cellular compounds focus on proteins. Protein synthesis is the main anabolic activity of the cell, and in the breeding of animals of zootechnical interest, protein ingredients are largely responsible for the rise in diet cost. Thus, much of the information available is based on studies carried out with animal protein metabolism.

Diets with different isotopic signatures are used to measure the turnover (Gannes, Rio, & Koch, 1998), with the change of the diet, the alteration in the isotopic composition in the tissue depends on the speed with which the constituents of the new diet will be incorporated. The choice of tissue is important, since tissues that are metabolically active in the body will have a faster turnover, such as liver, muscle and blood, and less active tissues will have lower rates, such as collagen and bone (Hobson & Clark, 1992). The growth curve is related to the speed of incorporation, because an individual who has a fast growth will have a fast turnover rate, when compared with organisms that have a slower growth. The inclusion of growth in this rate was made by Fry and Arnold (1982), who determined the growth during the increase related to biomass.

The present study evaluated the bone tissue, which is described as a specialized type of connective tissue, consisting of cells and calcified extracellular matrix, the bone matrix. Cells that make up the bone tissue are osteocytes, osteoblasts, and osteoclasts (Junqueira & Carneiro, 2004), and pig hoof is separated into two regions, one sensitive and the other insensitive. Chorion, a sensitive layer, consists of highly vascularized

connective tissue that nourishes various parts of the hoof and is located just below the insensitive layer of the hoof, consisting of hard horny tissue formed by two layers, one soft basal and the other of cornified tissue (Lopez, Sobestiansky, Coimbra, & Afonso, 1997).

In animals, destination of the absorbed food is more complex than simple metabolic combustion to obtain energy or deposition of matter in growth, mainly due to the influence of environmental factors on the biochemical status of organisms (Robinson, Harding, Brunton, & Bertolo, 2016), because any environmental factor affecting the metabolism and/or consumption of energy and other nutrients can influence the turnover of tissue components and growth. In this sense, the goal of this study was to evaluate the behavior of carbon incorporation and turnover in hoof and rib of pigs in different periods of development.

Material and methods

The experiment was conducted according to the rules of the ethics committee (protocol 78/2009 - Ceua), of the Universidade Estadual Paulista (Unesp), School of Veterinary Medicine and Animal Science in the nursery, growth and finishing facilities, in the pig production area from the School of Veterinary Medicine and Animal Science (FMVZ), Botucatu Campus. We used 132 commercial hybrid piglets (barrows and females), weaned at an average age of 21 days, with initial weight of 6.87 ± 0.65 kg, randomly distributed in four treatments. In the pre-starter and starter phases, animals were housed in nursery rooms, containing suspended metal stalls with slatted floors, measuring 1.0 x 1.75 m, equipped with a nipple drinker, feeder and heaters for heating. When animals were, on average, 63 days of age, they were transferred to the growth and finishing unit, with stalls measuring 1.50 x 2.40 m containing an automated feeder, located at the front and a nipple drinker, in the back of the stall. To ensure the well-being of the animals, the environments were air-conditioned.

Experimental treatments consisted of four production phases; in treatment 1, diets were changed at 21 days of age, with evaluation from 21 to 150 days of age, with collection at 1, 3, 6, 13, 18, 25, 35, 45, 60, 80, 100 and 129 days after the change, in treatment 2, diet was changed at 42 days of age, with assessment of the change from 42 to 150 days of age and collection of samples at 1, 3, 6, 13, 18, 25, 35, 45, 60, 80, 100 and 108 days after replacement with the new diet, treatment 3 started at 63 days of age, with an assessment of 63 to 150 days of age and collection at 1, 3, 6, 13, 18, 25, 35, 45, 60, 80, and 87 after replacement with the new diet, and finally the treatment 4 started at 110 days of age, with collection of samples from 110 to 175 days of age, at 1, 3, 6, 13, 18, 25, 35, 50 and 65 days after changing the diet. Treatment four had its time extended so that there was time to isotopically exchange the studied tissues.

Before diet replacement, animals received a feed predominantly composed of C_4 plant grains (corn), having a carbon-13 isotopic signal similar to that of feed supplied to the pregnant sows that gave rise to these animals, until the replacement. From the day of the replacement, animals started to receive a diet containing broken rice (C_3 plant grains), with an isotopic value different from the diets they had been consuming previously.

The feeding program was divided into two phases in the nursery, the first from 21 to 42 days of age (pre-starter) and the second from 43 to 63 days of age (starter) and in the growth phases (64 to 105 days of age) and finishing (106 to 175 days). Feed was provided at will. All diets were formulated to meet the nutritional requirements proposed by Rostagno et al. (2005) and are listed in Table 1 and 2. Each day of collection, after electrical stunning, three animals were randomly slaughtered for tissue collection. Bone samples were taken by collecting the thirteenth right rib and the right posterior hoof was removed completely, then the samples were packed in plastic bags, identified and immediately frozen (-18°C) until isotopic analysis. In each treatment, collections were grouped in the first days due to the speed of carbon isotope dilution in the tissue during this period (Hobson & Clark, 1992).

Samples were thawed and dried in a forced ventilation oven MA 035/5 Series 9610072 at 56°C, for 48 hours. All tissue samples and experimental diets were ground in a Spex Sample freezer mill, Geno/Grinder 2010, at -196ºC. Approximately 2.0 g sample was placed in a polycarbonate flask together with three stainless steel beads (model 440, without oil/grease), then it was closed properly and immersed in liquid nitrogen. Due to the impact between the sample and the beads, the sample was sprayed. The program used for grinding the samples consisted of a 3 min. freezing and a second high-frequency spraying (15 impacts s^{-1}) for 10 min.

Table 1. Percent composition, nutritional levels and isotopic values of experimental diets in the pre-starter and starter phases of pigs.

Ingredients	Pre-Starter C_3	Pre-Starter C_4	Starter C_3	Starter C_4
Concentrate	40[1]	40[1]	16[2]	16[2]
Corn	-	40	-	54
Broken rice	40	-	54	-
Soybean meal	20	20	25	25
Sugar	-	-	5	5
Total	100	100	100	100
Calculated Nutritional Levels				
CP (%)	18.72	19.00	19.04	19.27
ME (kcal kg^{-1})	3.408	3.320	3.385	3.360
Calcium (%)	0.78	0.74	0.64	0.64
Available phosphorus (%)	0.61	0.62	0.58	0.65
Digestible lysine (%)	1.32	1.32	1.07	1.07
Digestible methionine (%)	0.48	0.48	0.37	0.37
Digestible threonine (%)	0.80	0.80	0.63	0.63
Digestible tryptophan (%)	0.19	0.19	0.20	0.20
Na (%)	0.45	0.43	0.32	0.31
Copper (ppm)	140.00	140.00	130.00	130.00
Zinc (ppm)	3.140.00	3.140.00	2.920.00	2.920.00
Mean isotopic values analyzed*				
	−26.72±1.2	−19.58±1.9	−28.11±1.1	−16.97±0.09

[1]Commercial product for pre-starter phase providing per kg product: calcium, 14.20 g; ether extract, 63.80 g; crude fiber, 16.70 g; phosphorus, 7,300.00 mg; lysine, 2.00 mg; mineral matter, 78.30 g; methionine, 0.97 mg; crude protein, 172.50 g; moisture, 56.90 g; folic acid, 2.25 mg; cobalt, 2.50 mg; copper, 250.00 mg, choline, 2,487.35 mg, colistin, 100.00 mg; iron, 65.27 mg; fluorine, 20.80 mg; phytase, 1,200.00 ftu; iodine, 3.75 mg; manganese, 107.00 mg; niacin, 75.00 mg; calcium pantothenate, 35.06 mg; selenium, 1.13 mg; threonine, 14.50 g; tryptophan, 2,400.00 mg; vitamin A, 22,500.00 IU; vitamin B1, 5.06 mg; vitamin B12, 75.00 mcg; vitamin B2, 15.00 mg; vitamin B6, 7.50 mg; vitamin D3, 5,630.00 IU; vitamin E, 56.25 IU; vitamin H, 0.30 mg; vitamin K, 5.63 mg; zinc, 8,050.00 mg. [2]Commercial product for starter phase I providing per kg product: calcium, 37.70 g; ether extract, 99.60 g; crude fiber, 12.70 g; phosphorus, 15.50 g; lysine, 24.30 g; mineral matter, 139.10 g; methionine, 9,700.00 mg; crude protein, 180.20 g; moisture, 51.50 g; folic acid, 6.00 mg; cobalt, 6.50 mg; copper, 690.00 mg; choline, 2,516.74 mg; colistin, 250.00 mg; iron, 145.75 mg; phytase, 3,000.00 ftu, iodine, 9.75 mg; manganese, 274.00 mg; niacin, 200.00 mg; calcium pantothenate, 93.50 mg, selenium, 3.00 mg; threonine, 12.10 g; vitamin A, 60,000.00 IU; vitamin B1, 13.50 mg; vitamin B12, 200.00 mcg; vitamin B2, 40.00 mg; vitamin B6, 20.00 mg; vitamin D3, 15,000.00 IU; vitamin E, 150.00 IU; vitamin H, 0.80 mg; vitamin K, 15.00 mg; zinc, 16.00 g. *Average isotopic values expressed in $\delta^{13}C$ relative to the 'Pee Dee Belemnite' (PDB) standard in ‰.

Table 2. Percent composition, nutritional levels and isotopic values of experimental diets in the growth and finishing phases of pigs.

Ingredients	Growth C_3	Growth C_4	Finishing C_3
Núcleo	3[1]	3[1]	3[2]
Corn	-	70	-
Broken rice	70	-	73
Soybean meal	27	27	24
Total	100	100	100
Nutritional Levels			
CP (%)	18.18	19.39	16.64
ME (kcal kg^{-1})	3.289	3.267	3.296
Calcium (%)	0.709	0.75	0.735
Available phosphorus (%)	0.43	0.53	0.39
Digestible lysine (%)	0.85	0.85	0.77
Digestible methionine (%)	0.28	0.28	0.26
Digestible threonine (%)	0.57	0.57	0.53
Digestible tryptophan(%)	0.22	0.22	0.20
Mean isotopic values analyzed*			
	-28.79±0.80	-17.3±0.98	-28.5±0.75

[1]Commercial product for growth phase providing per Kg product: folic acid, 20.00 mg; calcium, 150.00 g; cobalt, 33 mg; copper, 3333.00 mg; iron, 3369.00 mg; phosphorus, 20,000 g; iodine; 50.00 mg; manganese, 1333.00 mg; niacin, 666.00 mg; selenium, 10.00 mg; vitamin A, 200,000.00 IU; vitamin B1, 45 mg; vitamin B2, 133 mg; vitamin B6, 66 mg; vitamin D3, 50,000.00 IU, vitamin E, 500.00 IU; vitamin H, 2.66mg; vitamin K, 50.00 mg; zinc, 3333.00 mg. [2]Commercial product for finishing phase providing per Kg product: folic acid, 20.00 mg; calcium, 140.00 g; cobalt, 35 mg; copper, 3333.00 mg; iron, 3500.00 mg; phosphorus, 20,000 g; iodine; 52.00 mg; manganese, 1400.00 mg; niacin, 670.00 mg; selenium, 10.00 mg; vitamin A, 201,000.00 IU; vitamin B1, 45 mg; vitamin B2, 134 mg; vitamin B6, 67 mg; vitamin D3, 50,250.00 IU, vitamin E, 502.00 IU; vitamin H, 2.68 mg; vitamin K, 50.00 mg; zinc, 3500.00 mg. *Average isotopic values expressed in $\delta^{13}C$ relative to the 'Pee Dee Belemnite' (PDB) standard in ‰.

After grinding, as the lipid fraction can cause isotopic fractionation of up to 5‰ in carbon-13 values (Piasentier, Valusso, Camin, & Versini, 2003), samples were degreased in ethyl ether, at a temperature of 65ºC, using a Soxhlet apparatus (TE-044) for four hours. After this period, samples were suspended for one hour so that only the reconditioned ether washed them. Subsequently, samples were removed from the apparatus and dried in a forced ventilation oven for one hour, for ether evaporation.

For isotopic analysis, samples were packed and weighed (50 to 70 µg), in tin capsules and introduced, through an automated sampler, into the Carbon, Hydrogen, Oxygen, Nitrogen and Sulfur analyzer, Flash 2000 model EA, Thermo Scientific, in which, in the presence of oxygen (O_2) and copper oxide (CuO), they were quantitatively burned for CO_2 and other gases (N_2, O_2 and H_2O). The formed gas was separated in a gas

chromatographic column and analyzed in the isotope ratio mass spectrometer (Delta V Advantage) from the Center for Environmental Stable Isotopes, from the Biosciences Institute of Unesp, Botucatu Campus.

The results were expressed in delta per thousand of the isotope ratio in relation to the international standard Pee dee Belemnite (PDB) for carbon, according to Equation 1:

$$\delta^{13}C(sample, standard) = \left[\left(\frac{Rsample}{Rstandard}\right) - 1\right] x\ 10^{3} \quad (1)$$

where:

$\delta^{13}C$ (sample, standard) = enrichment of the $^{13}C/^{12}C$ of the sample in relation to the standard and R = isotope ratio ($^{13}C/^{12}C$) of the sample and the standard.

To evaluate the speed of carbon turnover in animal tissues after a certain time interval, we used the exponential time function expressed by Equation 2, of the Origin® 6.0 Professional software (Microcal Software, 1999):

$$\delta^{13}C(t) = \delta^{13}C(f) + [\delta^{13}C(i) - \delta^{13}C(f)]e^{-kt} \quad (2)$$

where:

$\delta^{13}C(t)$ = isotope enrichment of the tissue at any time (t); $\delta^{13}C$ (f) = isotope enrichment of the tissue at the equilibrium level, or final condition; $\delta^{13}C$ (i) = isotope enrichment of the tissue, in the initial condition; k = turnover constant in time units^{-1} and t = time (in days) since the replacement of the feed (Ducatti, Carrijo, Pezzato, & Mancera, 2002).

Carbon half-life for the tissues involved in the treatments was calculated by Equation 3:

$$T = ln\ 2/k \quad (3)$$

where:

T = half-life, unit: time (days); ln = Neperian logarithm; k = turnover constant, unit: day^{-1}, suggesting an idea of 'speed' in the process of changing stable isotopes in tissues (Ducatti et al., 2002).

The time required for 95% of the initial atoms to be replaced by the final atoms ($t_{95\%}$) can be determined by Equation 4:

$$t_{95\%} = (-1/k)\ ln(1 - F) \quad (4)$$

in which, F (exchanged atoms) equal to 95% was considered as a stabilized system.

Values of isotope fractionation of tissues at the end of the experimental period in relation to the diet were calculated according to DeNiro and Epstein (1978) by Equation 5:

$$\Delta = \delta tissue - \delta diet \quad (5)$$

where:

Δ = fractionation factor between tissue and diet, in part per thousand (‰);

δtissue = relative enrichment of the tissue in question, in part per thousand (‰);

δdiet = average relative enrichment of the respective diet, in part per thousand (‰).

Results and discussion

The $\delta^{13}C$ isotopic values of the two tissues analyzed (hoof and rib) demonstrate the process of replacing carbon in these tissues (Figure 1 and 2).

The graphs illustrate that, over time, values of $\delta^{13}C$ of the two tissues evaluated moved towards the isotopic signal of the diets containing broken rice. Isotopic dilution curves of the analyzed samples (Figure 1 and 2) demonstrated that the pigs that received a C_3 diet from 21, 63 and 110 days of age, in the hoof and rib, reached the level of isotopic equilibrium, however for the animals whose C_4 diet was replaced with diet C_3 at 42 days, this effect was more evident.

Through the use of isotopic terminology to the general equation of the model, the percentage contribution of food sources for piglets from the post-weaning period on the composition of the two tissues analyzed was estimated. Table 3 and 4 list the exponential equations as a function of time and the coefficients of determination for the variables analyzed in the different treatments. Values of these equations for the treatments referring to the animals that had their corn-based diets replaced with broken rice-based diets at 21, 42 and 63 days of age, for the two tissues, were very close, however the values of the last treatment (animals that received a C_3 diet after 110 days of age) differed from the others. These equations proved the isotopic dilution of carbon-13 in the tissues throughout the experimental period.

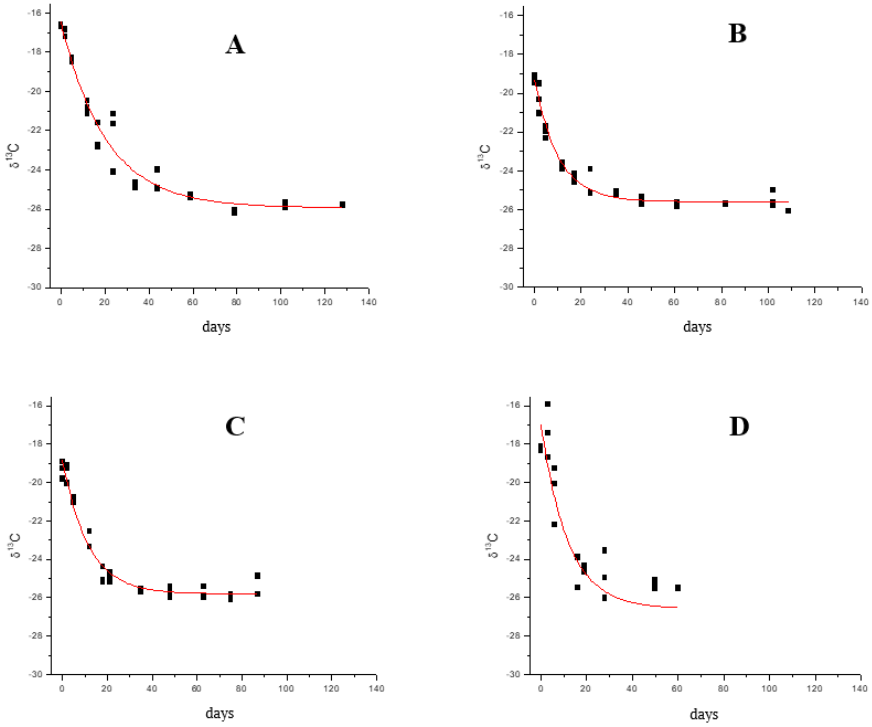

Figure 1. Isotopic dilution curves of carbon in hoof of pigs that had the diet based on corn (C_4) replaced with a diet based on broken rice (C_3) at 21 (A), 42 (B), 63 (C) and 110 (D) days of age, depending on evaluation intervals.

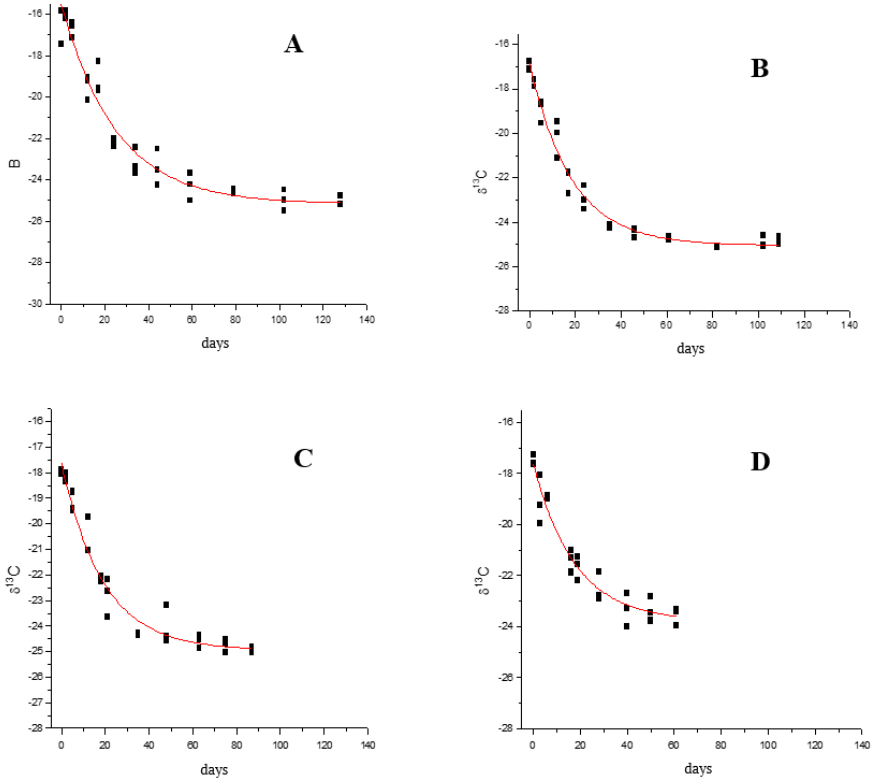

Figure 2. Isotopic dilution curves of carbon in rib of pigs that had the diet based on corn (C_4) replaced with a diet based on broken rice (C_3) at 21 (A), 42 (B), 63 (C) and 110 (D) days of age, depending on evaluation intervals.

Values of half-life values are listed in Table 5 and the isotopic fractionation factors calculated for the two tissues are presented in Table 6. The rib presented higher half-life values in relation to the hoof, probably because it is a bone tissue with a higher turnover, that is, lower metabolism speed and carbon-13 incorporation than the hoof. Values of half-life of the hoof may be related to the change in the type of floor after leaving the nursery (approximately 63 days of age), with consequent greater wear that occurs when the

animals are kept on the cement floor, especially when they have higher body weight. The animals that received C_3 diet after 21 days of age showed carbon half-life values in hoof and rib of 14.2 and 17.3 days, respectively. The delay in replacing about 50% carbon atoms in these two tissues is probably due to the low intake of food by the piglets, right after weaning.

Santos et al. (2019) evaluated the mucosa turnover of post-weaned piglets, and found that the incorporation of the isotopic signal from the C_3 diet only begins after the end of the critical period assigned to weaning. This critical phase is characterized by reductions in food intake and growth rate, and an increase in the occurrence of diarrhea in piglets (Rhouma, Fairbrother, Beaudry, & Letellier, 2017).

Although the animals that received the C_3 diet from 21 and 63 days of age also reached the isotopic equilibrium, probably, those that received this diet at 42 days showed this equilibrium more clearly through the half-life values, 7.1 and 12.7, for hoof and rib, respectively, probably because piglets left the critical period related to weaning. In addition, the change in diet is reflected in the isotopic composition of the tissues, and refers to the period of development and growth of new tissue, and not only to the metabolic turnover present in the tissues.

According to Tieszen, Boutton, Tesdahl, and Slade (1983), tissues and components of the body that have a high level of metabolic activity, or a high amount of fat, as well as blood and liver of guinea pigs, showed a fast turnover rate in relation to less active or fatty tissues, for example, collagen and hair.

Table 3. Exponential equations for isotopic dilutions, in pig hoof, depending on the period of substitution of the diet based on corn (C_4) with a diet based on broken rice at 21, 42, 63 and 110 days of age, with their respective coefficients of determination (R^2).

Replacement age	Equations	R^2
21	$\delta^{13}C = -25.93 + 9.45e^{-0.0487t}$	0.966
42	$\delta^{13}C = -25.59 + 6.26e^{-0.0974t}$	0.973
63	$\delta^{13}C = -25.80 + 6.98e^{-0.0884t}$	0.961
110	$\delta^{13}C = -26.58 + 9.60e^{-0.0848t}$	0.801

Table 4. Exponential equations for isotopic dilutions, in pig rib, depending on the period of substitution of the diet based on corn (C_4) with a diet based on broken rice at 21, 42, 63 and 110 days of age, with their respective coefficients of determination (R^2).

Replacement age	Equations	R^2
21	$\delta^{13}C = -25.17 + 9.66e^{-0.0401t}$	0.956
42	$\delta^{13}C = -25.04 + 8.16e^{-0.0548t}$	0.979
63	$\delta^{13}C = -25.95 + 7.30e^{-0.0527t}$	0.968
110	$\delta^{13}C = -23.80 + 6.17e^{-0.0565t}$	0.944

Table 5. Calculated half-life values (days) of the hoof and ribs of piglets that had the diet based on corn (C_4) replaced with a diet based on broken rice (C_3) at 21, 42, 63 and 110 days of age.

Replacement age	Hoof		Rib	
	50%	95%	50%	95%
21	14.2	62	17.3	75
42	7.1	31	12.7	55
63	7.8	34	13.1	57
110	8.2	35	12.3	53

Table 6. Isotopic fractionation factor (Δ) between tissue and diet* of the piglets that had the diet based on corn (C_4) replaced with a diet based on broken rice (C_3) at 21, 42, 63 and 110 days of age (Δ = δtissue − δdiet).

Replacement age	Hoof	Rib
21	2.84	3.59
42	3.49	4.16
63	3.00	4.08
110	3.14	5.08

*The relative enrichment of the diets was calculated from the average of the feed consumed before the replacement of corn with broken rice to obtain the isotopic value of the diet consumed initially and after the replacement, until the end of each experimental period, to find the isotopic value of the final diet.

Values of the isotopic fractionation (Table 6) demonstrated that probably the animals that received C_3 diet at 21, 42 and 63 days of age, reached the level of equilibrium referring to this new diet. These results are in agreement with DeNiro and Epstein (1978), when they affirm that the animal is what it consumes ± 1 ‰ for carbon isotopes.

The rib presented higher values of isotopic fractionation in relation to the hoof, this demonstrates that the isotopic ratios of the tissues can vary within an individual fed a constant diet, because the isotopes

fractionate differently between the diet and the various tissues (Tieszen et al., 1983). The results of this research agree with Howland et al. (2003), who used diets with different amino acid compositions for pigs and found that the fractionation factors in animal bone collagen varied from 0.5 to 6.1 ‰. Like Nardoto, Godoy, Ferraz, Ometto, and Martinelli (2006) who determined the values of $\delta^{13}C$ and $\delta^{15}N$ in different pig tissues, such as hair, nail, liver, muscle, fat and cartilage, according to the animal diet, with the aim of documenting the existing fractionation between the tissues and the diet and observed that tissues such as fat, liver and muscle were on average 2.1 ‰ lighter than the diet, while hair and nail did not show ^{13}C enrichment in relation to the diet.

Other factors can influence isotopic fractionation such as differences in food intake (Mirón, Herrera, Ramírez, & Hobson, 2006), age (Jenkins, Partridge, Stephenson, Farley, & Robbins, 2001), metabolic rate (MacAvoy, Arneson, & Bassett, 2006) and reproductive status (Kurle & Worthy, 2002). Moreover, different external environmental pressures or nutritional content of feed can lead to differences in the assimilation and storage of nutrients between different types of tissues.

It was possible to verify that with the progress of samplings, there was an increase in the value of isotopic fractionation factor in the tissues, however, there was enough time for the two tissues to reach the level of isotopic equilibrium in animals that received C_3 diet at 21, 42, 63 and 110 days of age. The rib proved to be the best option to be used in the traceability process, as it reflects changes in the pig diet for a longer period, with great potential for practical application in order to find animal by-products in the pig diet.

Conclusion

Sample of the rib bone is the best option to reflect the former diet than the hoof of pigs, due to the preservation of the isotopic signal for longer.

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