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Development of biofertilisers from calcined bones: production, and physicochemical and ecotoxicological analysis[1]

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ABSTRACT - The possibility of producing animal bone ash from sources other than cattle, and the determining effect of prolonged calcination, has encouraged investigation into bio-based fertilisers. The aim of this study was to develop and characterise calcined bone ash using different animal sources and heat treatments. Femurs from cattle, pigs and sheep were subjected to heat treatments of 2 or 4 hours, and the ash (CBA1, CBA2 and CBA3) was compared to a commercial bone ash of unknown processing (CCBA-Unk). The data analysis included descriptive or inferential statistics with ANOVA and Tukey's post-hoc test or linear regression. Production analysis showed that CBA1 and CBA3 had higher yields than CBA2 after 2 h, while only CBA1 still differed from CBA2 after 4h ($p < 0.05$). In the physical and chemical analyses, the ash calcined for 4 h presented both a clearer and more uniform granular morphology than did the ash calcined for 2 h and CCBA-Unk, with a high concentration of minerals, reaching twice the levels of phosphorus and calcium compared to CCBA-Unk. In the ecotoxicological analysis with *Artemia salina*, all the ashes under test were non-toxic, with 4 h CBA1 and 4 h CBA3 proving safer than the other experimental ashes, reaching four times and twice the LC_{50} of CCBA-Unk, respectively. Bone ash from cattle or sheep bones calcined for 4 h proved to be more promising than from pig bones or when calcined for 2 h for generating nutritional products for acidic soils.

Key words: Bones. Fertilisers. Heat treatment. Sustainable development.

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INTRODUCTION

Supplementing agricultural soils with fertilisers has a positive impact on human food security, with global productivity expected to meet the needs of a projected nine billion people by 2050 (González; Manavella, 2021; Kumar; Kumar; Mohapatra, 2021). In Brazil, the most widely applied agents for enriching acidic soils are potassium (38%), phosphorus (33%) and nitrogen (29%), most of which are imported from China, Russia, the United States, Morocco, Canada and Belarus (Brasil, 2021).

External dependence became evident during the Covid-19 pandemic and during global conflicts, when border isolation caused domestic shortages and increased the price of fertiliser, in addition to crop losses and food inflation (Brasil, 2021). Furthermore, non-renewable natural resources are limited, while to meet the growing mineral demand, millions of tons of fossil deposits are consumed annually (Leinweber *et al*., 2018). It is estimated that more than half of the world's phosphate and potash rock will be exhausted by the end of the next century (Balawejder *et al*., 2019).

The National Fertilizer Plan, recently created in Brazil, provides for planning the sector for the coming decades, encouraging innovative action in the use of nutrients, together with environmental conservation (Brasil, 2022). Efforts are being made to develop techniques for extracting minerals for plant nutrition by recycling by-products as part of a sustainable circular economy (Damaceno, 2017; Leinweber *et al*., 2018).

One method would be to use discarded bone by-products, which, with the appropriate thermal processing, would allow the production of calcined bone ash (CBA) containing a hyper-concentration of minerals essential for plant growth, such as phosphorus and calcium (Conde; Stachiw, 2020; Mattar; Frade-Júnior; Oliveira, 2014). Compared to fertilisers obtained from mining companies, CBA has a total phosphorus content of more than 40% and fewer contaminants than rock phosphates, which have a maximum of 17% phosphorus and contain impurities such as fluorine and aluminium (Saeid *et al*., 2014; Santos, 2012; Souza, 2001).

CBA applied to agriculture is predominantly bovine in origin, despite the manufacturing process being heterogeneous or difficult to reproduce (Balawejder *et al*., 2019; Mattar; Frade-Júnior; Oliveira, 2014; Miyahara; Gouvêa; Toffoli, 2007). Producing CBA from cattle carcasses for agricultural use is an affordable alternative for rural producers due to the low technological investment coupled with environmental sustainability, which minimises the disposal of waste from generating these natural fertilisers (Damaceno *et al*., 2018).

The number of animals slaughtered in Brazil for meat consumption is significant, with 46,508,459 pigs, 25,108,360 cattle and 36,649 sheep slaughtered in 2023, generating a high amount of unusable animal waste, in the order of millions of tons (Brazil, 2024). In this respect, the use of CBA could help to mitigate the intense demand for fertilisers as a substitute for mineral deposits, in addition to preserving the environment (Damaceno, 2017).

The lack of evidence on bioprospecting for other animal sources and on standardised processing and evaluation protocols to ensure the safety and effectiveness of fertilisers leads to the following hypothesis: it is possible to generate quality CBA from animal bone by-products other than those of bovine origin, as well as considering that a longer calcination time may be decisive for better productive performance. Therefore, the aim of this study was to develop and characterise CBA from cattle, pigs and sheep under different heat treatments.

MATERIAL AND METHODS

Bioethical aspects

The raw materials from cattle, pigs and sheep used to generate the CBA came from commercial sources and from carcasses that had already been slaughtered for human consumption. As the microcrustacean *Artemia salina* is not a chordate, its handling and use require no bioethical evaluation.

Since this study did not involve Brazilian biodiversity or any form of procedure on live animals that might cause pain or suffering, there was no requirement for ethical-legal registration in the systems that regulate these areas, such as the National System for the Management of Genetic Heritage (SISGEN) of the Ministry of the Environment, in accordance with Law no 13,123/2015, or the Ethics Committee on the Use of Animals - National Council for Animal Experimentation (CEUA-CONCEA) of the Ministry of Science, Technology and Innovation, in accordance with Law no 11,794/2008.

Obtaining and sanitising the bone material

The fresh, refrigerated bone residue was donated by a butcher in Sobral, Ceará, Brazil (latitude: -40.351978853093456, longitude: -3.685406890226075). Cattle, pig and sheep femurs were collected separately, cut into 10 cm pieces and processed within 24 hours after slaughter, in accordance with Normative Instruction 34/2008 of the Ministry of Agriculture, Livestock and Food Supply. Any visible organic matter on the surface of the bones was removed manually. The bones were then immersed in 1% aqueous sodium hypochlorite for 30 minutes, washed in

running water for 10 minutes, dried at room temperature and forwarded for the appropriate heat treatment.

Application of the heat treatments

The bones were calcined on a barbecue made of cast aluminium (Parlumin, Brazil), using charcoal as the energy source. Ethyl alcohol (70%) was added to the charcoal, which was constantly changed to maintain incandescence during the burning process.

Once the charcoal was completely heated, the bones were placed on the embers and calcined separately, according to origin (cattle, pig or sheep). Two heat treatments were applied for each origin (T1 = 2 h and T2 = 4 h) with five replications for each treatment.

During calcination, the temperature stability was monitored every 30 minutes using a digital thermometer coupled to a K-type porcelain thermocouple sensor (Lemaqs Automação, Brazil) with a measuring capacity of up to 1,300 °C.

After cooling at room temperature following calcination, the bones had a lighter, more friable appearance. They were then ground in a wooden mortar to a flour-like consistency and passed through a domestic sieve with a 1-mm mesh to ensure a minimum of homogeneity in the final CBA prototypes.

Analysis of bone ash production

After processing, ten samples of CBA were obtained for each animal origin, giving a total of 30 samples. The yield of each group was analysed by weighing the cattle, pig or sheep material after manually deboning around 1 kg of bone residue, and then grinding it after calcination until the ash was in powder form. The weights were determined using a digital scale, recording the absolute values of the initial and final weights from which the relative value or percentage (%) use of each product was calculated.

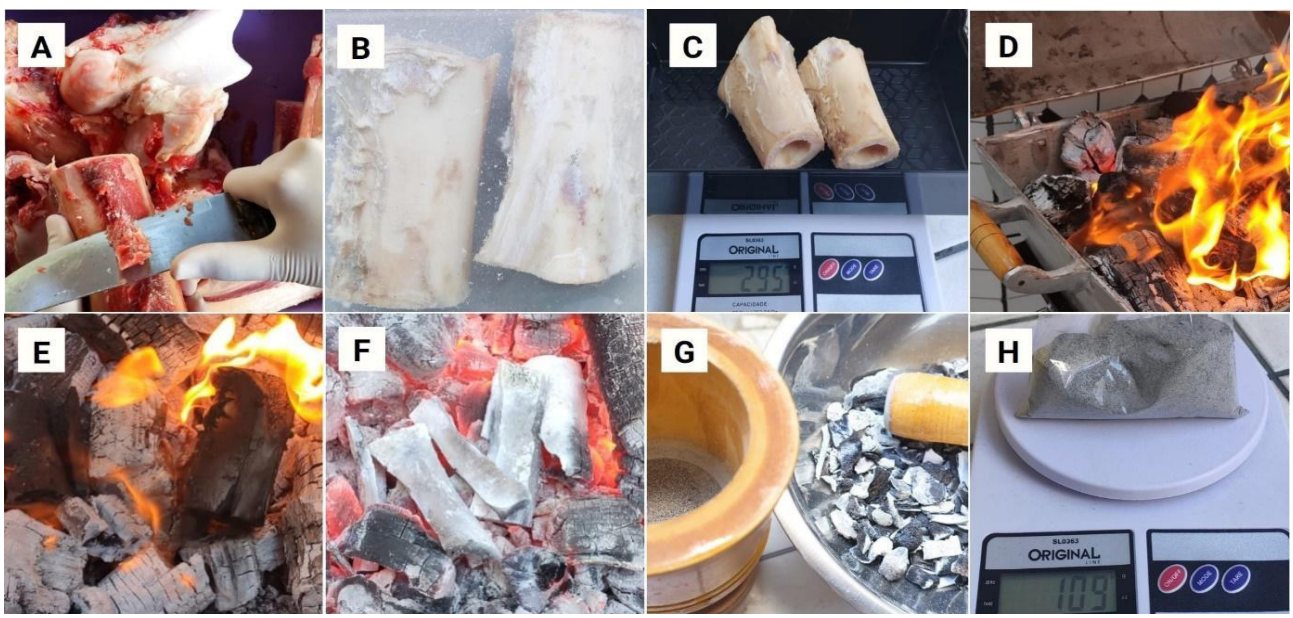
Physicochemical analysis of the structure and composition

The morphological aspects of the different ashes were compared for colour, homogeneity and particle shape. A macroscopic analysis was carried out using actual size photographs, and a microscopic analysis using photomicrographs in an ECZ-BLACK stereomicroscope (Biofocus, Brazil) with 240x magnification.

In accordance with the official analytical methods for fertilisers described in Brasil (2017), a quantitative determination was made of the macronutrients, micronutrients and contaminants in the calcined bone ash CBA1, CBA2 and CBA3 and in CCBA-Unk, a commercial sample used as a reference standard, whose calcination time was unknown.

Different tests were conducted to detect the elements considered essential for plant development, including the macronutrients phosphorus, calcium, nitrogen, magnesium, sulphur and potassium, and the micronutrients iron, copper, zinc, manganese and boron. At the same time, the analysis also aimed to identify the presence of elements, such as aluminium, that are potentially contaminating in processing the ash and toxic to plants.

Figure 1 - Production of the calcined bone ash. A: Removal of organic matter. B: Sanitisation in sodium hypochlorite. C: Initial weighing of the raw bone. D: Burning on a barbecue. E-F: Colour change during calcination. G: Grinding and sieving. H: Final weighing of the ash



To detect the total nitrogen, a Kjeldahl nitrogen digester block (Labtec, Foss Analytical, Brazil) was used, together with an SL-74 nitrogen distiller (Solab, Brazil) to carry out sulphuric digestion employing the Raney alloy macromethod.

For total phosphorus, acid extraction was performed using 6.0 mol/L HCl, followed by precipitation of the orthophosphate ion in the form of quinoline phosphomolybdate, which was filtered, dried and weighed. The phosphorus pentoxide content was determined using the Quimociac gravimetric method.

For total potassium, the potassium oxide content was determined following strong acid extraction with HCl, eliminating the organic matter. The analysis was carried out by flame atomic absorption spectrometry using the Pye Unicam SP9 (Pye Unicam, United Kingdom) and the Kipp & Zonen BD12 recorder (Kipp & Zonen, Netherlands).

The secondary macronutrients calcium and magnesium were also determined by acid extraction followed by flame atomic absorption spectrometry. Sulphur was determined using the barium sulphate gravimetric method.

The micronutrients iron, copper, zinc and manganese were likewise analysed by acid extraction and detected using flame atomic absorption spectrometry. In the case of the micronutrient boron, acid extraction was carried out and the azomethine-H spectrophotometric method was used as the chromophore reagent, with readings taken on a visible light spectrophotometer (FEMTO, Brazil) at a wavelength of 420 nm.

To determine the total aluminium, the iCAP 6000 inductively coupled plasma optical emission spectrometer (ICP-OES, Thermo Fisher Scientific, USA) was used at a plasma power of 1300 W and wavelength of 396 nm. In addition, the specific solubility of the phosphorus in the form of phosphorus pentoxide was tested in neutral ammonium citrate with water, in 2% citric acid (1:100), and in water. The results were interpreted using the Quimociac gravimetric method.

The quantitative data for most of the compositions were obtained in g/L and expressed in g/100mL or as a percentage (%). For aluminium, the results were expressed in mg/kg. The final results showed the arithmetic mean ± standard deviation for all the tests carried out on the samples in triplicate.

The spectra for the Fourier-transform infrared spectroscopy (FTIR) analysis of CBA1, CBA2, CBA3 and CCBA and samples of raw or non-calcined fragmented cattle, pig or sheep bone (FB1, FB2 and FB3, respectively) were obtained using an Alpha-P spectrometer (Brucker Optics, USA) in attenuated total reflectance mode in the 4000 to 400 cm^{-1} range at a resolution of 0.8 cm^{-1} with 40 scans.

Ecotoxicity bioassay with *Artemia salina*

The ecotoxicity potential of the CBA in relation to aquatic ecosystems was investigated by bioassay using nauplii of *Artemia salina* following the standard protocol of ISO Technical Specification 20787:2017.

Artificial seawater with a pH between 8.0 and 9.0 was prepared by mixing 30 g/L NaCl with mineral water. Cysts of *Artemia salina* were then added and left for 48 hours at 25 °C. Incubation was carried out in an aquarium under constant aeration by a submersible pump, after which all the hatched, mobile and phototropic larvae were captured. Ten nauplii were added by micropipette to each 1-mL well in 24-well test plates containing artificial seawater solution with no sample (negative control); concentrations of 10, 100 or 1000 µg/mL of CBA1, CBA2, CBA3 or CCBA (tests); or potassium dichromate (toxic, positive control). These were then incubated for a further 24 hours at 25 °C. The test for each experimental condition was carried out in quadruplicate. To interpret the results, the total and dead larvae were counted using a stereomicroscope, applying the Abbott formula, where:

$$\%\ deaths = \frac{test - control}{100 - control} \times 100 \quad (1)$$

The lethal concentration for 50% of the population (LC_{50}), calculated in relation to the exposure period, was derived from the best curve obtained in the linear regression analysis.

Data analysis

The parametric numerical data for ash yield and the toxicity bioassay were analysed using the Past v4.16c software (University of Oslo, Norway). The Jarque-Bera test for normality was applied, followed by analysis of variance (ANOVA) and Tukey's post-hoc test. A confidence level of 95% was adopted, considering differences to be statistically significant when $p < 0.05$. The results were expressed in graphs that were generated using the Prism v5.0 software (GraphPad Inc., USA), or presented in tabular format.

Nonparametric data, such as nominal results of morphology or the presence of spectra in the physicochemical analyses, as well as numerical parametric data on the elemental composition, were interpreted by simple descriptive analysis, comparing the experimental groups with their respective controls.

RESULTS AND DISCUSSION

Bone ash yield

The average temperature throughout the experiments remained stable when comparing the bone

ash with the 2h heat treatment (CBA1: 438.93 ± 170.19 ºC; CBA2: 486.68 ± 152.19 ºC; CBA3: 442.08 ± 162.21 ºC) or the 4 h treatment (CBA1: 498.13 ± 137.44 ºC; CBA2: 492.44 ± 153.88 ºC; CBA3: 506.07 ± 162.40 ºC). These values reached a temperature range that explains the occurrence of the calcination process of the bone residue and decomposition of the organic portion, especially over a longer period. The average calcination temperature seen in this study is in line with the values reported in other studies (Carús; Bento; Bragança, 2013; Hammood; Hassan; Alkhafagy, 2017; Miyahara; Gouvêa; Toffoli, 2007; Vegh; Marquez-Grant; Schulting, 2022).

One factor for the increased standard deviation in temperature might be the production of CBA on a barbecue and not in a more-controlled mechanised environment such as a muffle furnace, which would make the calcination more thermostable (Carús; Bento; Bragança, 2013; Hammood; Hassan; Alkhafagy, 2017; Miyahara; Gouvêa; Toffoli, 2007). On the other hand, the choice of an effective calcination protocol, independent of more-limited laboratory techniques that would make processing more expensive, is in line with with the central idea of generating a sustainable CBA that is more accessible to the realities of the agricultural market (Balawejder *et al*., 2019; Damaceno *et al*., 2018), thereby justifying the methodological design.

The loss of unbreakable particles larger than 1 mm when sieving the CBA was minimal and similar for the six experimental conditions, ranging from 0.5% to 0.7% of the initial weight of each group. The final yield showed that the cattle bones had the best performance, of 35.9% for the 2 h heat treatment and 36.25% for the 4 h treatment. This were followed by the sheep bones, with yields of 35.7% for 2 h and 32.89% for 4 h. The poorest performance was obtained with the pig bones, where yields were 28.97% for 2 h and 26.08% for 4 h, showing a significant difference for CBA2 in relation to CBA1 and CBA3 at 2 h and only CBA1 at 4 h (Figure 2).

Each of the yields under analysis was higher than those found in the literature, which reports an average percentage yield of up to 24.4% for samples of cattle bone (Mattar; Frade-Júnior; Oliveira, 2014). The lack of information regarding the biological origin of the samples, together with the lack of a standard time and temperature for the calcination process, might explain the poorer performance and variation in the final CBA yields of other studies (Balawejder *et al*., 2019; Carús; Bento; Bragança, 2013; Damaceno *et al*., 2018; Hammood; Hassan; Alkhafagy, 2017; Miyahara; Gouvêa; Toffoli, 2007).

Morphology and composition of the bone ash

The 2 h heat treatment produced CBA of a greyish colour that were more difficult to grind, with more heterogeneous granules, small to large in size, with shapes ranging from polyhedral to amorphous, and with smooth to irregular surfaces, while the 4 h treatment resulted in products that were more friable and easily ground, whitish, with granules that were more homogeneous, of moderate size, amorphous and with irregular surfaces. The CCBA differed from the experimental ash, having a browner colour and large number of polyhedral granules (Figure 3).

According to Boni *et al*. (2020), the calcination time of the bone residue may explain the CBA with lighter tones, which can be used as an indicative parameter of the best mineral quality as the residue contains the highest percentage of phosphorus and calcium, while insufficient calcination may leave the bone ash more yellowish to brown in colour, as seen in the CCBA.

Figure 2 - Analysis of ash yield after 2 h - T1 (A) or after 4 h of calcination - T2 (B). CBA1: cattle, CBA2: pig and CBA3: sheep. Mean ± standard deviation, analysed using one-way ANOVA and Tukey's post-hoc test. Significant differences: * p = 0.0461 and ** p = 0.0022

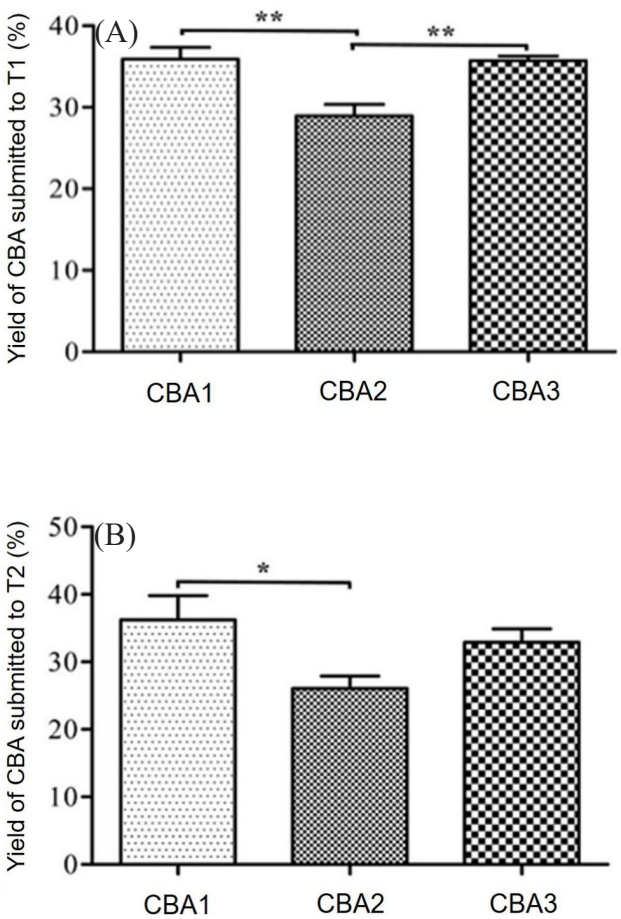
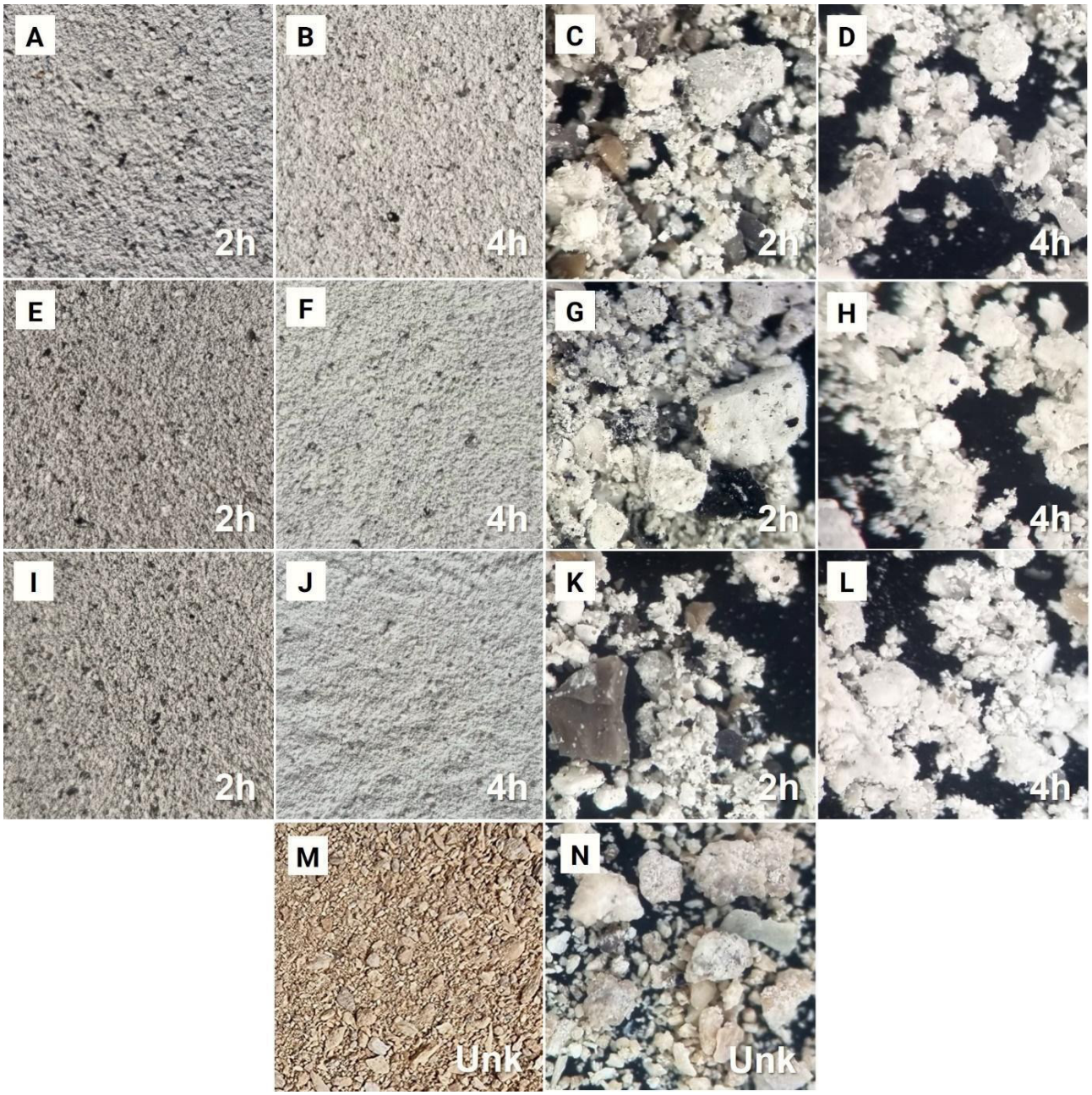


Figure 3 - Macroscopic (actual size) and microscopic (240x magnification) photographs of bone ash calcined with heat treatments of 2 h and 4 h from cattle (CBA1, A-D), pigs (CBA2, E-H) and sheep (CBA3, I-L), and commercial bone ash (CCBA, M-N) with an unknown processing time (Unk)



The results of the analysis of the mineral composition of the CBA in the two heat treatments compared to the CCBA are shown in Table 1.

The different heat treatments affected the elemental constitution of the CBA, which proved to be more significant after 4 h, as these had the highest percentage of necessary nutrients.

CBA1, CBA2 and CBA3 after both the 2 h and 4 h heat-treatments showed higher values than the minimum mandatory requirements for a mineral fertiliser, with a calcium content greater than 16% and phosphorus, in the compound form of phosphorus pentoxide (P_2O_5), greater than 20%, or 16% when soluble in 2% citric acid, approaching or exceeding twice the values found for both

Table 1 - Mineral composition of the experimental and commercial bone ash

Analyte	P	Ca	N	Mg	S	K	Fe	Cu	Zn	Mn	B	Al
Value	%	%	%	%	%	%	%	%	%	%	%	mg/Kg
CBA1 – 2 h	34.65 ± 0.80	30.28 ± 0.61	2.54 ± 0.05	0.46 ± 0.01	0.23 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	7.97 ± 0.16
CBA2 – 2 h	34.94 ± 0.70	32.43 ± 0.65	2.44 ± 0.04	0.43 ± 0.01	0.25 ± 0.01	0.14 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	22.08 ± 0.44
CBA3 – 2 h	35.48 ± 0.72	33.15 ± 0.66	1.01 ± 0.02	0.50 ± 0.02	0.24 ± 0.01	0.14 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	38.47 ± 0.78
CBA1 - 4 h	41.75 ± 0.84	36.20 ± 0.73	0.64 ± 0.01	0.56 ± 0.03	0.40 ± 0.01	0.16 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	104.01 ± 2.21
CBA2 – 4 h	41.24 ± 0.83	36.70 ± 0.74	0.40 ± 0.01	0.56 ±± 0.02	0.42 ± 0.02	0.20 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	119.50 ± 2.55
CBA3 – 4 h	41.59 ± 0.91	38.70 ± 0.78	0.37 ± 0.01	0.53 ± 0.02	0.37 ± 0.01	0.15 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	122.50 ± 2.65
CCBA - Unk	16.87 ± 0.43	18.50 ± 0.38	1.57 ± 0.03	3.70 ± 0.09	0.25 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.00	1132.50 ± 23.71

Calcined bone ash from cattle (CBA1), pigs (CBA2) and sheep (CBA3), and a commercial bone ash (CCBA)

elements in the CCBA, which had less than the required total phosphorus content and a borderline calcium content (Brasil, 2017). Similar experimental studies of CBA involving calcination on a brick barbecue by Damaceno *et al*. (2018) obtained 35.7% P_2O_5 and 43.76% CaO, while Mattar, Frade-Júnior and Oliveira (2014) achieved a phosphorus content of 35.81% and total calcium of 33.07%. Avelar *et al*. (2009), characterising the phosphorus sources available to rural producers, also reported commercial CBA with a phosphorus concentration of 14.4%, which is below the minimum limit according to Brasil (2017). These findings underline the need for consumers to verify the necessary requirements for mineral fertilisers in the technical specifications of each product. According to Rengel, Cakmak and White (2022) and Taiz *et al*. (2017), phosphorus and calcium play an essential role in plant physiology, where phosphorus comprises sugars, nucleic acids, coenzymes and phospholipids, and acts in reactions involving ATP, while calcium is required by enzymes in the hydrolysis of ATP and phospholipids, plays a part in metabolic regulation, and comprises the median lamella of cell walls.

The nitrogen concentration decreased with the longer heat treatment, and remained high in the CCBA compared to the 4 h CBA. This phenomenon may be related to an organic fraction that is still present in the CBA and that is reduced by a longer exposure to temperature (Figueiredo; Gamelas; Martins, 2012). In fertilisers, the presence of nitrogen is beneficial because a large quantity of the mineral is needed by plants to form chlorophyll, amino acids and nucleic acids, and its restriction limits plant growth (Rengel; Cakmak; White, 2022; Taiz *et al*., 2017).

The magnesium concentration in each of the experimental CBA was far lower than in the CCBA; however, the level of 1.13% reported by Miyahara, Gouvêia, and Toffoli (2017) in CBA obtained after 1 h of calcination in a muffle furnace was also lower than that in the CCBA. In any case, the element is essential for photosynthesis, forming chlorophyll, mediating energy processes and favouring zinc absorption (Rengel; Cakmak; White, 2022; Taiz *et al*., 2017).

The sulphur concentration increased slightly with the calcination time, being higher in the 4 h CBA than in the 2 h CBA or the CCBA. The potassium concentration showed a slight increase between the 2 h and 4 h CBA1 and 2 h and 4 h CBA2, with no changes for CBA3, and with each of the CBA well above CCBA. According to Rengel, Cakmak and White (2022) and Taiz *et al*. (2017), sulphur is a component of amino acids, coenzymes and vitamins, and potassium regulates cellular osmotic potential, activating enzymes involved in photosynthesis and cellular respiration. When these nutrients are scarce, chlorosis occurs, a deficiency in chlorophyll production that reduces growth, more specifically in new leaves due to the lack of sulphur and in old leaves due to the lack of potassium, in addition to apical leaf necrosis, thin and weak stems, and very short internodes (Rengel; Cakmak; White, 2022; Taiz *et al*., 2017).

Although the 2 h CBA showed a small presence of the other nutrients, the 4 h CBA and the CCBA showed trace elements of the other compounds, with concentrations below 100 µg/g or 0.01% (Brasil, 2017). Despite being micronutrients that are needed by plants in low concentrations, it is clear that the role of iron, copper, zinc, manganese and boron in fertilisers may involve different pathways for enzyme modulation, photosynthesis, mitosis, cell differentiation and stress response, promoting or at least not limiting the growth and productivity of vegetable crops (Kumar; Kumar; Mohapatra, 2021; Rengel; Cakmak; White, 2022; Taiz *et al*., 2017). These results reinforce the role of CBA as a source of nutrients rich in a variety of minerals for crop development (Conde; Stachiw, 2020).

Despite the same 2 h heat treatment, the presence of aluminium was lower in CBA1 than in CBA2 and CBA3, while the three animal sources were almost equal under the 4 h treatment, showing an increase in content; however each of the experimental conditions was well below the value found in the CCBA. Since all the CBA were obtained from femurs, one hypothesis to explain the phenomenon might be interference from

the animal origin, as proposed by Tang, Parsons and Perl (1999), who, using highly accurate and sensitive analytical methods to detect aluminium in cattle, goat and human bones, found variations within and between species depending on age, bone part and origin.

The tendency for the aluminium concentration to increase with the longer high-temperature calcination time may also be due to the prolonged exposure of the CBA to coal ash, including by-products from the barbecue itself when reaching the melting point of aluminium, at 660 ºC, which is within the calcination temperature range used to prepare the samples (Gupta; Meenu; Peshin, 2019; Tang; Parsons; Perl, 1999).

In the CBA, aluminium was expressed as a trace element, a minimum level indicating no contamination during processing, which is a better alternative than the higher concentration of aluminium in the CCBA, albeit current regulations set no exact threshold for the metal (Brasil, 2017). Despite aluminium being a potential plant growth stressor, CBA is still more beneficial than fertilisers from phosphate rock due to the smaller amount of unwanted contaminant residue (Santos, 2012).

Table 2 shows the results for phosphorus solubility, which corresponds to the level of phosphorous in the products available for assimilation by plants.

Both the experimental CBA and the CCBA showed very low solubility in water. In neutral ammonium citrate and water, the 2 h CBA and the CCBA showed a slight increase in solubility compared to the 4 h CBA. The solubility in citric acid showed that most of the fertilisers tested were suitable for fertilising acidic soils, with the 4 h CBA being particularly noteworthy, especially CBA1 followed by CBA3, both of which were superior to the CCBA. The dissociation of hydroxyapatite from CBA releases calcium into the soil and hydroxyls that modulate the increase in pH, making it useful for phosphate fertilisation and correcting soil acidity (Ferreira; Ferreira; Cavali, 2020). However, CBA2 did not meet the minimum requirement of 16% solubility described in Brasil (2017). On the other hand, Oliveira *et al*. (2018) state that CBA with low solubility may be particularly advantageous in acidic soils rich in iron and aluminium oxides/hydroxides, irreversibly preventing the capture of available phosphorus, meaning the borderline solubility of CBA2 is not in any way undesirable.

Figure 4 shows the FTIR spectra of the raw and calcined cattle, pig and sheep bone ash compared to the CCBA.

The fragmented raw cattle bones (FB1) were yellowish in colour, showed bands suggestive of absorbed water molecules (3276 cm^{-1}) and organic matter compatible with collagen (2918 cm^{-1} and 1651 cm^{-1}), carbonates (1408 cm^{-1}) and phosphates (1010 cm^{-1} and 559 cm^{-1}). CBA1 after 2 h or 4 h calcination was greyish or whitish in colour and showed fewer bands compatible with collagen (2350 cm^{-1}), carbonates (1456 cm^{-1}) or phosphates (1025 cm^{-1}, 564cm^{-1} or 562cm^{-1}), which can be explained by the removal of water and organic matter by the two heat treatments.

The fragmented raw pig bones (FB2) were yellowish in colour, presented bands suggestive of absorbed water molecules (3283 cm^{-1}) and organic matter compatible with collagen (2918 cm^{-1} and 1641 cm^{-1}), carbonates (1408 cm^{-1}) and phosphates (1010 cm^{-1} and 557 cm^{-1}). CBA2 after 2 h or 4 h calcination was greyish or whitish in colour and showed fewer bands compatible with collagen (2360 cm^{-1} or 2350 cm^{-1}), carbonates (1456 cm^{-1}) or phosphates (1026 cm^{-1} or 1024 cm^{-1}, 564 cm^{-1} or 563 cm^{-1}), which can be explained by the removal of water and organic matter by the two heat treatments.

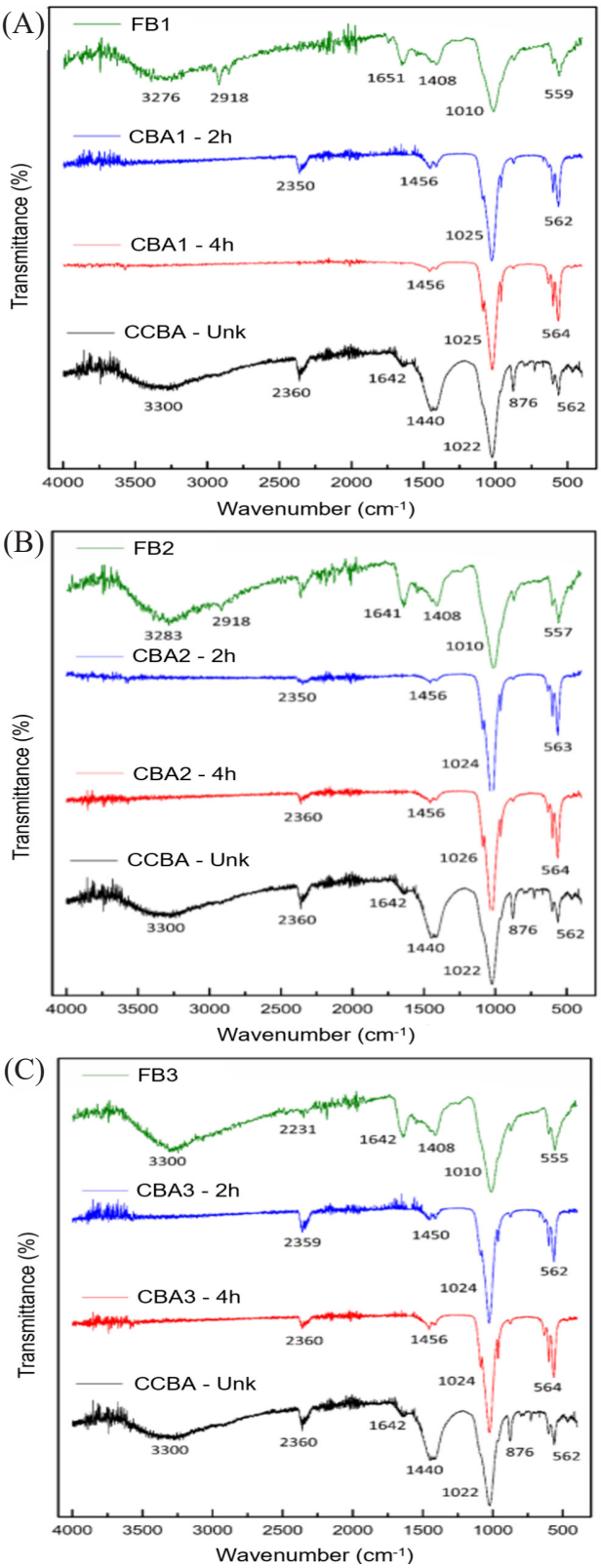
The fragmented raw sheep bones (FB3) were yellowish in colour and had bands suggestive of

Table 2 - Solubility of phosphorus pentoxide present in the experimental and commercial bone ash

Analyte	P2O5 soluble in neutral ammonium citrate + water	P2O5 soluble in 2% citric acid (1:100)	P2O5 soluble in water
Value	%	%	%
CBA1 – 2 h	12.50 ± 0.30	18.08 ± 0.37	0.15 ± 0.01
CBA2 – 2 h	11.87 ± 0.24	13.40 ± 0.28	0.22 ± 0.02
CBA3 – 2 h	10.08 ± 0.21	15.95 ± 0.32	0.22 ± 0.01
CBA1 – 4 h	9.22 ± 0.19	23.43 ± 0.48	0.21 ± 0.01
CBA2 – 4 h	8.21 ± 0.18	15.53 ± 0.35	0.21 ± 0.02
CBA3 – 4 h	8.23 ± 0.16	16.45 ± 0.33	0.16 ± 0.01
CCBA - Unk	13.48 ± 0.27	16.17 ± 0.32	0.11 ± 0.01

P_2O_5: Phosphorus pentoxide. Calcined bone ash from cattle (CBA1), pigs (CBA2) and sheep (CBA3), and a commercial bone ash (CCBA)

Figure 4 - Infrared spectroscopy comparison of bone ash after 2 h or 4 h calcination from cattle (A, CBA1), pigs (B, CBA2) and sheep (C, CBA3) with their respective fragmented bone (FB) and a commercial bone ash (CCBA) of unknown processing time



absorbed water molecules (3300 cm^{-1}) and organic matter compatible with collagen (2231 cm^{-1} and 1642 cm^{-1}), carbonates (1408 cm^{-1}) and phosphates (1010 cm^{-1} and 555 cm^{-1}). CBA3 after 2 h or 4 h calcination was greyish or whitish in colour and showed fewer bands compatible with collagen (2360 cm^{-1} or 2359 cm^{-1}), carbonates (1456 cm^{-1} or 1450 cm^{-1}) or phosphates (1024 cm^{-1}, 564 cm^{-1} or 562 cm^{-1}), which again can be explained by the removal of water and organic matter by the two heat treatments.

The CCBA had a more organo-mineral profile, with bands suggestive of absorbed water molecules (3300 cm^{-1}) and organic matter compatible with collagen (2360 cm^{-1} and 1642 cm^{-1}) or associated with hydroxyl groups (876 cm^{-1}), as well as mineralised areas identified as carbonates (1440 cm^{-1}) and phosphates (1022 cm^{-1} and 562 cm^{-1}), similar to the CBA.

In general, each of the CBA produced in this study, regardless of animal origin, had a similar composition that was characteristic and specific to calcined bone ash. This corroborates studies carried out with cattle or goat bones, where thermal processing generated hydroxyapatite containing carbonate and phosphate groups (Figueiredo; Gamelas; Martins, 2012; Hammood; Hassan; Alkhafagy, 2017; Santos, 2012), which was also found in ash from pig bones (Vegh; Marquez-Grant; Schulting, 2022). There are no reports of CBA of sheep origin in the literature.

The composition of the raw bone fragments was expected, with the presence of absorbed water molecules, collagen, carbonates and phosphates identified in bands described in the literature as part of the reference spectrum for this type of material (Figueiredo; Gamelas; Martins, 2012; Gouvêa *et al*., 2008). However, the CCBA showed bands that indicated a higher organic content, which is not recommended for this type of mineral product, suggesting that the calcination process may have been inadequate (Conde; Stachiw, 2020; Gouvêa *et al*., 2008; Mattar; Frade-Júnior; Oliveira, 2014; Vegh; Marquez-Grant; Schulting, 2022). Furthermore, the lack of traceable information on the calcination of this standard commercial product underlines the need for greater emphasis on the standardisation of heat treatments for CBA.

Environmental safety of the bone ash

A comparative analysis of the experimental CBA and CCBA showed no statistically significant difference in mortality caused by the exposure of *Artemia salina* to the different ashes under test; there were however differences between all the samples for potassium dichromate, which showed high mortality at each of the concentrations being tested. The linear regression of the data followed an increasing linear pattern whose positive slope showed that the slight increase in the percentage of artemia deaths was directly proportional to the concentration of the CBA

Table 3 - Toxicity in *Artemia salina* calculated from mortality, linear regression and lethal concentrations (LC_{50}) of experimental bone ash, commercial bone ash and the control

Heat treatment	Material	Mean mortality (%)			Linear regression (R^2)	Linear regression (Equation)	LC_{50} (µg/mL)
		10 µg/mL	100 µg/mL	1,000 µg/mL			
2 hours	CBA1	1.40 ± 1.05	5.27 ± 4.37	10.34 ± 11.38	0.15	Y = 2.81806 + 0.00761X	6,194.43
	CBA2	0.36 ± 0.42	4.32 ± 2.58	7.62 ± 1.35	0.57	Y = 1.92653 + 0.00581X	8,267.71
	CBA3	0.89 ± 1.24	7.23 ± 5.00	10.73 ± 9.17	0.16	Y = 0.34167 + 0.0104X	4,771.09
4 hours	CBA1	0.00 ± 0.00	0.11 ± 0.16	0.66 ± 0.99	0.16	Y = 0.01389 + 0.00064X	78,098.00
	CBA2	0.00 ± 0.00	0.12 ± 0.18	3.90 ± 3.87	0.38	Y = - 0.155 + 0.00404X	12,402.23
	CBA3	1.61 ± 1.61	3.83 ± 4.28	3.98 ± 3.43	0.03	Y = 2.58056 + 0.00149X	31,819.46
Unk.	CCBA	0.04 ± 0.06	1.85 ± 1.85	3.46 ± 2.40	0.30	Y = 0.75625 + 0.00278X	17,713.58
Absent	PD	5.65 ± 2.18	56.04 ± 6.88	94.02 ± 0.54	0.74	Y = 25.936 + 0.07018X	342.89

Calcined bone ash from cattle (CBA1), pigs (CBA2) and sheep (CBA3), and a commercial bone ash (CCBA). Potassium dichromate (PD): positive control for toxicity

under test. The values for the coefficients of determination (R^2) for CBA1 and CBA3 were low, meaning that the regression model explains little of the variation around the mean in the response data (between 3% and 16%). However, higher R^2 values for CBA2, of 57% after 2 h and 38% after 4 h, and for potassium dichromate of 74%, more robustly explain the increase in concentration as a predictor of mortality, with the model showing a better fit to the data. The LC_{50} was greater than 1000 µg/mL under each of the experimental conditions, confirming their lack of toxicity, with the values for CBA1, CBA2 and CBA3 being closer after 2 h than after 4 h, when the intergroup variation was more evident. Intragroup variation was also evident between 2 h and 4 h, with the value for LC_{50} increasing 12.6 times for CBA1, 1.5 times for CBA2, and 6.7 times for CBA3, based on the data in Table 3.

The absence of toxicity in the bioproducts is confirmed by the classic test by Meyer *et al*. (1982), with the death of more than 50% of *Artemia salina* under conditions of high toxicity at low concentrations, such as $LC_{50} \leq 30$ µg/mL, to decreasing toxicity at higher concentrations, with an extract considered non-toxic from $LC_{50} \geq 1000$ µg/mL onwards, which corroborates our results. The beneficial effect of the longer calcination time on biological safety in the *Artemia* model, which substantially increases the LC_{50} for CBA1 and CBA3 after 4 h, or of more-stable behaviour regardless of the calcination time, as seen in CBA2, where the LC_{50} was far lower than the other CBA and less than CCBA, is not discussed in the literature, and challenges our understanding of this frontier of science. As expected, potassium dichromate meets the requirement for a positive control for toxicity, with an LC_{50} far below that of the CBA, showing that at minimum concentrations it is still highly lethal to *Artemia salina*, being known for its toxicity to aquatic organisms (Meyer *et al*., 1982).

In a study with microcrustaceans carried out by Gonçalves (2019), hydroxyapatite powder at a concentration of 0.05 mg of sample per 5 mL of saline solution caused 50% mortality in *Artemia salina* after 24 hours, showing a lack of acute toxicity and differing from our results. The large variation in toxicity between ceramic mineral compounds based on calcium phosphate confirms the need for careful and continued testing of biological systems, taking into account the variety of experimental conditions and methodological approaches (Gonçalves, 2019). Although, according to Gonçalves (2019), the test with *Artemia salina* is an initial, inexpensive and reproducible method for addressing the question of acute biological safety that is both fast and safe, there should be more in-depth applied research into fertiliser products in terms of potential phytotoxic effects and longer periods of time.

CONCLUSIONS

Cattle and sheep bone ash calcined for four hours proved to be more promising than bone ash from pigs or bone ash calcined for two hours for generating nutritional products for acidic soils. Future studies on the effectiveness of these products on crops should be carried out to determine the ideal amount for use with each plant category.

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