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Article

Silver Accumulation and Ionomic Profile Alterations in Pigs: Evaluation of Silver Nanoparticles as Feed Supplements and Potential Human Exposure

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ABSTRACT: The antimicrobial properties of silver nanoparticles (AgNPs) in animal feed have drawn increasing attention as a potential alternative to antibiotics. However, concerns about bioaccumulation and possible effects on mineral homeostasis require a thorough evaluation. This study investigates the bioaccumulation of Ag in various tissues and ionomic alterations in pigs fed AgNPs complexed with carbohydrates (AgNPs@Carb). Silver concentrations were measured in tissues, such as the liver, kidney, spleen, heart, and cecal contents, at different time points following the withdrawal of the nanoparticle-supplemented diet. Principal component analysis (PCA) assessed the concentrations of 18 elements across 10 tissues. Results indicate that silver primarily accumulates in the liver and cecal contents, with varying clearance rates across tissues. The silver estimated daily intake (EDI) for human consumption was evaluated, revealing low values across all tissues. This suggests that potential exposure to Ag through the consumption of edible tissues from animals supplemented with AgNPs is minimal and does not pose an immediate health risk. Significant changes were also observed in the ionomic profiles, suggesting that AgNPs disrupt trace element homeostasis. These findings underscore the importance of understanding both the biodistribution of silver nanoparticles and their potential long-term impact on animal health and human consumers.

KEYWORDS: *animal feed, nanotechnology, exposure, mineral profile, AgNPs*

1. INTRODUCTION

Nanotechnology is a highly interdisciplinary field that has rapidly gained prominence, becoming one of the fastest-growing areas in science and technology worldwide.[1,2] Its applications span diverse sectors, including medicine, environmental science, cosmetics, food, energy, electronics, and engineering.[1,2] Nanoparticles (NPs), defined by the European Commission as aggregates with at least 50% of particles between 1 and 100 nm, and by ISO as particles with at least one dimension in the nanoscale range (1−100 nm), possess unique properties due to their nanoscale size and increased surface area.[2−4]

These unique characteristics have led to significant advancements but also increased human and environmental exposure, raising concerns about the safety and potential adverse effects of NPs.[1,4] This has spurred the development of nanotoxicology, a field dedicated to studying the safety and toxicological impacts of nanomaterials on biological systems.[5] The toxicity of NPs is influenced by several factors, including their size, shape, composition, surface characteristics, and the degree of aggregation, which may lead to the accumulation of NPs in sensitive organs and the induction of oxidative stress through reactive oxygen species.[1,6]

In the field of animal nutrition, antibiotics have been used for decades as growth promoters to increase productivity.[7,8] However, this practice can lead to the retention of antibiotics in animal tissues, posing a potential risk of increasing antibiotic resistance among consumers of animal products.[7] Consequently, this practice was banned in the European Union in the latter half of the 20th century, prompting the exploration of new alternatives.[7,8] The primary concerns regarding the safe use of additives in animal nutrition include ensuring effective antimicrobial action that selectively targets potential pathogens without disrupting symbiotic microbial communities, minimiz-

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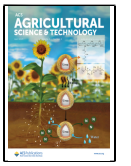

ing toxic effects on both animals and human consumers, and reducing environmental pollution risks.[8]

The rise in antimicrobial resistance has further intensified the interest in metallic NPs, such as AgNPs, as alternatives to traditional antibiotics. These NPs exhibit broad-spectrum antimicrobial activity, attributed not only to ion release but also to their small size and high surface-to-volume ratio, which allows interaction with microbial membranes.[9,10] Despite the potential benefits, concerns about the environmental release of NPs through animal excretion and their accumulation in tissues, with subsequent human consumption, necessitate thorough investigation.[11,12]

Given the broad applicability of NPs, particularly in living organisms, understanding their absorption, transport, and biotransformation is critical to mitigate potential adverse effects. This is especially relevant as NPs, such as silver nanoparticles (AgNPs), are increasingly studied for their antimicrobial properties and as potential growth promoters in animal nutrition.[13,14] However, their ability to cross biological barriers and accumulate in tissues, potentially inducing toxic effects, underscores the need for careful evaluation.[13,15]

Quantifying the total Ag concentration in animal tissues is a reliable indicator of silver nanoparticle (AgNP) accumulation. This measurement provides valuable insights into the extent to which AgNPs are absorbed, distributed, and retained in various tissues after exposure. Abad-Álvaro et al.[7] conducted an in vivo study on pigs fed a AgNP-enriched diet, revealing significant Ag accumulation in the liver and increased fecal excretion compared to the control group, with no significant Ag levels detected in muscle tissues. This suggests the potential of AgNPs as growth promoters, though environmental contamination from feces warrants further investigation. Similarly, Gallocchio et al.[16] conducted an in vivo study on chickens fed AgNPs, reporting Ag accumulation in the liver and yolk but not in muscle, kidney, or albumin. By analyzing the total Ag content, researchers can infer the degree of nanoparticle accumulation, as the Ag detected is likely derived from the administered AgNPs. Furthermore, this approach allows for the assessment of potential tissue-specific accumulation patterns, which is critical for understanding the biodistribution and possible toxicological effects of AgNPs within the organism.

The objective of this study was to evaluate the distribution and accumulation of silver in various tissues of pigs whose diets were supplemented with a nanocomplex containing silver nanoparticles (AgNPs@Carb). Given that metallic nanoparticles can interact with the absorption of other elements and potentially disturb the mineral profile of animal tissues,[17−19] the study also aimed to investigate changes in 18 elements across 10 different tissues. Additionally, the analysis sought to provide insights into the biodistribution of silver in relation to the withdrawal time of the supplement, contributing to a better understanding of the potential risks and benefits associated with the use of AgNPs in animal feed.

2. MATERIALS AND METHODS

2.1. AgNPs@Carb Synthesis. The colloidal solutions were provided by Brazilian Nano Feed, Ltd., Santo André, SP, Brazil. The suspensions were obtained via chemical reduction of $AgNO_3$ using the polyol method,[20] with polyvinylpyrrolidone (PVP) as a stabilizer. In brief, PVP was mixed in an aqueous solution with propylene glycol (the reductant) at 90 °C under magnetic stirring. $AgNO_3$ was then slowly added dropwise to each mixture, and the formation of nanoparticles was indicated by the transformation of the transparent solution into a yellowish-green colloid. The reaction was stopped by quenching with deionized water at room temperature. The colloidal suspension was added to a carbohydrate to facilitate the targeted delivery of nanoparticles to the intestine. The nanostructured additive was produced through the sorption of AgNPs onto the carbohydrate surface, which occurred during the drying process. The diet additive was named AgNPs@Carb.

2.2. X-ray Diffraction (XRD). Powder X-ray diffraction data were collected in a diffraction angle range between 30° and 90° at ambient temperature using a Bruker D8 diffractometer with Cu Kα1,2-radiation (λ = 0.154018 nm, 1600 W) source. The detector used was a LynxEye 1D linear silicone strip that acquires a diffraction pattern in 1/200 of the time required by using a conventional point detector of the same quality.

2.3. Transmission Electron Microscopy (TEM). Images of the synthesized AgNPs@Carb were obtained with a TEM (Carl Zeiss 120 TEM, Zeiss International, Oberkochen, Germany) operating at 80 kV. One drop of the aqueous suspension of synthesized AgNPs was deposited on a 200-mesh hole carbon film supported on a copper grid and dried at room temperature. All images were treated with iTEM software. The particle size and distribution were determined by ImageJ 1.53t software (Wayne Rasband, National Institutes of Health, USA) and Origin 10.1.0.178 software (OriginLab Corporation, Northampton, MA, USA).

2.4. Animals, Diet, and Experimental Design. The procedures adopted in this research were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science at the University of São Paulo, under protocol #1037240523 and are in compliance with the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines GL9[21] and GL48[22] and Brazilian Technical Instruction #26 - Technical Regulation for the Manufacturing, Quality Control, Marketing, and Use of Antimicrobial Products for Veterinary Use.[23] The use of animals in the study was carried out in accordance with Brazilian Law No. 11,794 (Arouca Law),[24] which establishes procedures for the use of animals in scientific experimentation.

A total of 30 pigs obtained from a commercial farm (±110 days old; weighing between 55 and 65 kg) were used, consisting of 15 castrated barrows and 15 gilts. The pigs were housed in individual masonry pens separated by metal grid partitions. Each pen was equipped with a nipple-drinker and an individual semiautomatic stainless-steel feeder.

Two groups of animals were formed. One group consisted of 24 animals receiving the diet with the additive (AgNPs@Carb), and the other group consisted of six animals not receiving the additive, forming the control group. The experimental diets used were formulated to meet or exceed the nutritional requirements for the animal category, according to Rostagno et al.[25] All of the feed used in the experiment was corn- and soybean-based. Each diet was supplemented or not with AgNPs@Carb (which contains 20 g of silver per kilogram of the nanocomplex) at a concentration of 0.25 g of AgNPs@Carb per feed kilogram (or 5 mg of silver per feed kilogram). The feed was provided *ad libitum*, and supply was controlled and measured, as well as feed waste. The ingredient and chemical compositions of the experimental feeds are presented in Table 1.

The animals received the diet containing AgNPs@Carb for 24 days, and the control group received the diet without AgNPs@Carb for 3 days. Throughout the experimental period, the animals were slaughtered on days 3 (control group), 24 (zero days of withdrawal period for AgNPs@Carb), 25 (1 day of withdrawal period for AgNPs@Carb from the diet), 26 (2 days of withdrawal period for AgNPs@Carb), and 27 (3 days of withdrawal period for AgNPs@Carb) of the experiment, for the collection of samples from the liver, kidneys, spleen + lymph nodes, skin + fat, lungs, heart, muscle, small intestine, and, in the case of females, uterus + ovary. The initial weight

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Table 1. Composition of the Diets Utilized during This Study

ingredient	concentration (g/kg)
corn	679
soybean meal 45%	300
vitamin-mineral mix[a]	40
sodium chloride	50
limestone	30
dicalcium phosphate	80
kaolin[b]	10

[a]Per kg composition: folic acid (minimum) 126.00 mg; pantothenic acid (minimum) 3000.00 mg; biotin (minimum) 36.00 mg; calcium (maximum) 280.00 g; calcium (minimum) 200.00 g; copper (minimum) 4500.00 mg; choline (minimum) 67.00 g; iron (minimum) 14,000.00 mg; iodine (minimum) 210.00 mg; manganese (minimum) 8400.00 mg; niacin (minimum) 5250.00 mg; selenium (minimum) 62.90 mg; vitamin A (minimum) 1,100,000.00 IU; vitamin B1 (minimum) 450.00 mg; vitamin B12 (minimum) 5400.00 μg; vitamin B2 (minimum) 1170.00 mg; vitamin B6 (minimum) 630.00 mg; vitamin D3 (minimum) 350,000.00 IU; vitamin E (minimum) 5,000.00 IU; vitamin K (minimum) 540.00 mg; zinc (minimum) 16,800.00 mg; phytase (minimum) 125,000.00 ftu. [b]Substituted by AgNPs@Carb in the supplemented diets.

and weight at slaughter were measured, as well as the daily feed intake.

2.5. Sample Preparation. Frozen samples were thoroughly lyophilized for 72 h (lyophilizer Liotop l101, Liobras, Brazil). The moisture content of the samples was determined, and the results expressed in this study are relative to the fresh mass of the tissues. Then, 200 mg of the dried sample was accurately weighed into 50 mL polypropylene centrifuge tubes, and 3.0 mL of HNO_3 (65% m/m, Synth, Brazil), previously purified in a distillation system below boiling point (DST-1000, Savillex, USA), was added. A predigestion of the samples was performed for 48 h at room temperature. The tubes were then heated (95 °C) in a graphite-covered digester block (EasyDigest, Analab, France) for 4 h. After they were cooled, the samples were diluted to 30 mL of ultrapure water (Master System All, Gehaka, Brazil). The resulting solutions were stored under refrigeration to determine Ag, As, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, and Zn by mass spectrometry with inductively coupled plasma (ICP-MS).

2.6. Determination of Ag Content and Other Elements in Swine Tissues by ICP-MS. Elemental analysis was performed using an inductively coupled plasma mass spectrometer (ICP-MS Agilent 7900, Japan). All instrumental conditions have been described by Paniz et al.[26] Before the measurements, the instrument was calibrated with curves ranging from 1 to 200 $\mu g\ L^{-1}$ for Ag, As, Cd, Co, Cr, Cu, Li, Mn, Ni, P, Pb, Se, and Zn from an ICP-MS calibration standard solutions (Inorganic Ventures, USA) and from 100 to 5000 $\mu g\ L^{-1}$ for Ca, Fe, K, Mg, Na, and P from a multielement standard solution (PerkinElmer, USA). Standard solutions were prepared in 2.5% (v/v) nitric acid. In addition to the calibration curve, an internal standard (Rh solution, 25 $\mu g\ L^{-1}$) prepared from a stock solution of 1000 mg L^{-1} (PerkinElmer, USA) was used to control changes in the signal. A collision cell (He > 99.999%) was used during the determinations to avoid interferences. Procedural blanks were performed following the same protocol. Bovine liver 1577c NIST SRM (National Institute of Standards and Technology, USA) was used as the QC for digestions. In short, the results obtained from SRM analysis did not present significant differences (Student's t test, 90% confidence level) of the certified reference concentrations.

2.7. Estimated Daily Intake (EDI). The estimated daily intake (EDI) refers to the amount of a specific substance that an individual consumes or is exposed to daily, often expressed in micrograms or milligrams per kilogram of body weight. This estimation is crucial in determining the potential for adverse health effects due to long-term exposure to both chemical and biological agents. The EDI of Ag from the consumption of the analyzed tissues was calculated using the following eq 1:[27]

$$\text{EDI} = \frac{\text{Concentration of silver} \times \text{Dietary ingestion}}{\text{Body weight}} \quad (1)$$

The EDI is expressed in μg per day per body weight (70 kg for adults and 24 kg for children)[28] and depends on the average daily pork intake, estimated at 0.049 kg per day of fresh weight of pork meat in Brazil.[29]

2.8. Statistical Analyses. We used principal component analysis (PCA) to analyze the global variance of the measured ionomic profiles. PCA was performed on Statistica software version 14.0.015 licensed to USP-University of Sao Paulo (Statistica, TIBCO Software Inc., Tulsa, USA). The data were visualized as PCA score and loading plot. Each coordinate on the score plot represented an individual sample, and each coordinate on the loading plot represented one type of tissue related to the element concentration among different experimentation groups. PCA is considered an unsupervised learning method that is capable to uncover hidden relationships between samples and variables. For the analysis across days and diets (control and diets with nanoparticles), we used the data from all control and tissue samples. The data set consisted of a matrix with 240 cases (tissues) and 18 variables (elements). Data were normalized and rotated (normalized varimax), grouping parameters. Only eigenvalues >1 were considered in this study. Group loading values >0.70 were used with high or strong correlation.

3. RESULTS AND DISCUSSION

3.1. AgNPs@Carb Characterization. The present study developed silver nanoparticles (AgNPs) incorporated into a complex carbohydrate matrix (AgNPs@Carb), which serves as a protective carrier for the nanoparticles. Several studies have previously explored the benefits of carbohydrate-coated or functionalized Ag nanoparticles.[30,31] Complex carbohydrates are predominantly digested in the lower sections of the gastrointestinal (GI) tract,[32] making the incorporation of AgNPs into a carbohydrate matrix a strategic approach for protecting the nanoparticles as they pass through the upper GI tract. This also improves targeted delivery to the intestines, reducing systemic absorption. As a result, AgNPs can exert their antimicrobial activity directly on gut bacteria, enhancing their effectiveness as a potential antimicrobial growth promoter.

The synthesis of AgNPs was confirmed by X-ray diffraction (Figure 1). The diffraction peaks of 38.2, 44.2, 64.5, 77.1, and 81.6° are in accordance with the Inorganic Crystal Structure Database (ICSD) for Ag^0 formation.

After the AgNPs were incorporated into the carbohydrate matrix, they were characterized by transmission electron microscopy (TEM). Figure 2A,B shows a representative micrograph of AgNPs@Carb and the particle diameter distribution, respectively. They have a spherical shape, with an average diameter of 5.9 ± 9.4 nm. The present work focused on developing nanoparticles below 20 nm, as it is well-stated in the literature that the absorption and penetration of AgNPs are inversely proportional to their size.[33−35] The goal was achieved, even though the nanoparticles were polydisperse due to incorporation into the carbohydrate matrix.

3.2. Silver Accumulation in Tissues and Excretion. Silver concentration in tissues was determined immediately when the AgNPs@Carb were withdrawn from the diet (day 24), 1 day after withdrawal (day 25), 2 days after withdrawal (day 26), and 3 days after withdrawal (day 27). From Figure 3, it is possible to note that the highest Ag concentrations in

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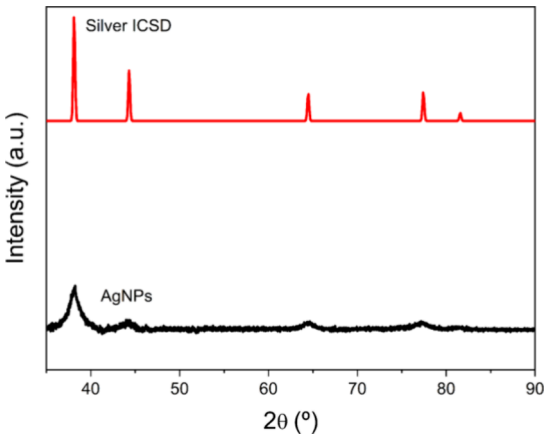

Figure 1. Silver ICSD (red line) and AgNP diffraction peaks (black line).

tissues occurred on day 24, when the animals were continuously receiving the AgNPs@Carb. Particularly, the cecal content had the highest Ag concentration (4.361 ± 0.696 mg/kg). By estimating the amount of feces produced (Supporting Information), it was possible to calculate the total amount of Ag excreted, which was 6.28 mg. Considering that the animals were consuming approximately 13.03 mg of Ag per day, we can state that on day 24, the animals excreted ∼48% of the Ag daily dose. However, on day 25, the Ag levels in the cecal content were not statistically different ($p < 0.05$) from the control, meaning that the rest of the Ag was retained in the tissues or excreted by other pathways. Three days after the Ag was withdrawn from the diet (day 27), muscle, skin + fat, kidneys, uterus + ovary, lungs, and small intestine had returned to the initial levels similar to the control group ($p > 0.05$). On the other hand, the heart, spleen + lymphnodes, and liver did not ($p < 0.05$). These results show that Ag tends to be cleared over time, but some tissues might require a longer time to be cleared.

Table S1 (Supporting Information) illustrates several studies on the bioaccumulation of AgNPs in vivo. Comparing these studies is challenging due to significant methodological heterogeneity. For example, some studies report the administered concentration of AgNPs relative to body weight, while others use feed weight as a reference. Additionally, some studies do not clarify the total amount of Ag contained in the nanoparticles, making it difficult to estimate the total Ag intake by the animals. Furthermore, the analytical procedures vary considerably. Some studies measure Ag content in dried tissues and others in wet tissues, using different techniques such as coupled plasma mass spectrometry (ICP-MS), single-particle ICP-MS (SP-ICP-MS), inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma optical emission spectrometry (ICP-OES), neutron activation analysis, and atomic absorption spectroscopy (AAS).

The choice of a specific technique is complex, as each has unique principles, advantages, and limitations. All the techniques presented in Table S1 (Supporting Information) can detect total element concentration, with variations regarding detection limits, matrix effects, multielement analysis, and cost.[33,34] Among them, only SP-ICP-MS has the ability to detect nanoparticles. However, this technique can only detect nanoparticles bigger than 10 nm, which can underestimate the number of nanoparticles, and specific methods to extract the nanoparticles for different types of matrixes are required, being the reason why it was not chosen for this study.[36,37] Moreover, soluble ions can react with different elements and form salt particles, with can be mistaken with the original nanoparticles.[37] The choice of technique depends on factors such as the element of interest, concentration range, sample matrix, and accuracy, but these choices need to be clarified.

For instance, in Table S1 (Supplementary materials), the bioaccumulation in the liver of piglets fed with 20 mg Ag kg^{-1} of feed for 14 days[7] was 4800 ± 1400 $\mu g\ kg^{-1}$, values considerably higher compared to those observed in our study. This suggests that the liver has a high capacity for Ag accumulation in both studies. In rats,[37] after 28 days of exposure, Ag concentrations in the small intestine reached 984.6 ± 219.3 $\mu g\ kg^{-1}$, and in the liver, 223 ± 36.3 $\mu g\ kg^{-1}$, which are also significantly higher than those observed in pigs, possibly due to differences in the administered dose, species metabolism, and exposure duration. Mice exposed to 10 mg of AgNPs per body weight for 3 days[38] showed liver Ag

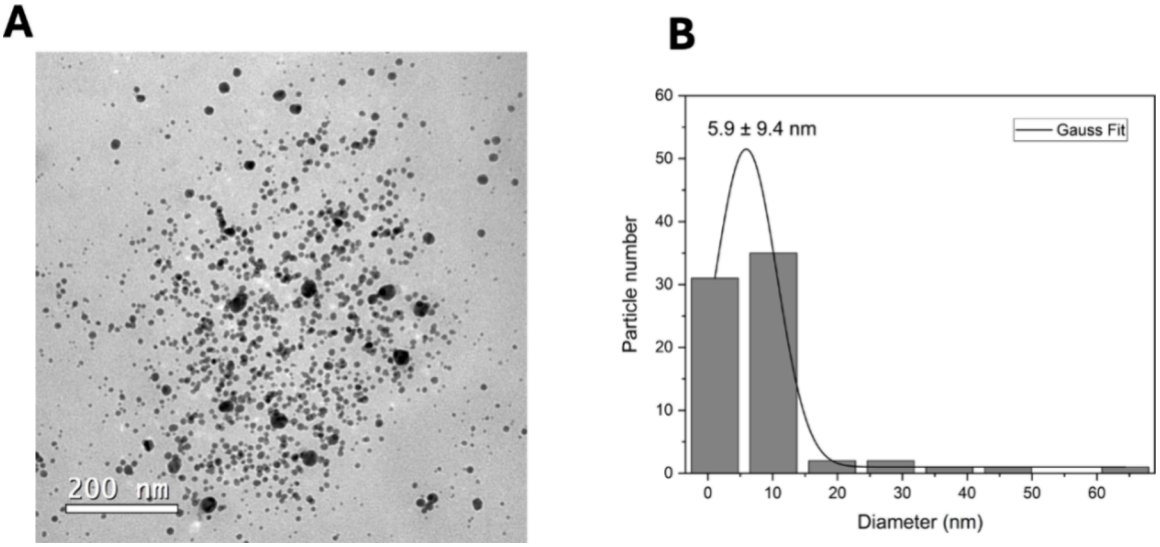

Figure 2. Representative micrograph of AgNPs@Carb (A) and particle diameter distribution (B).

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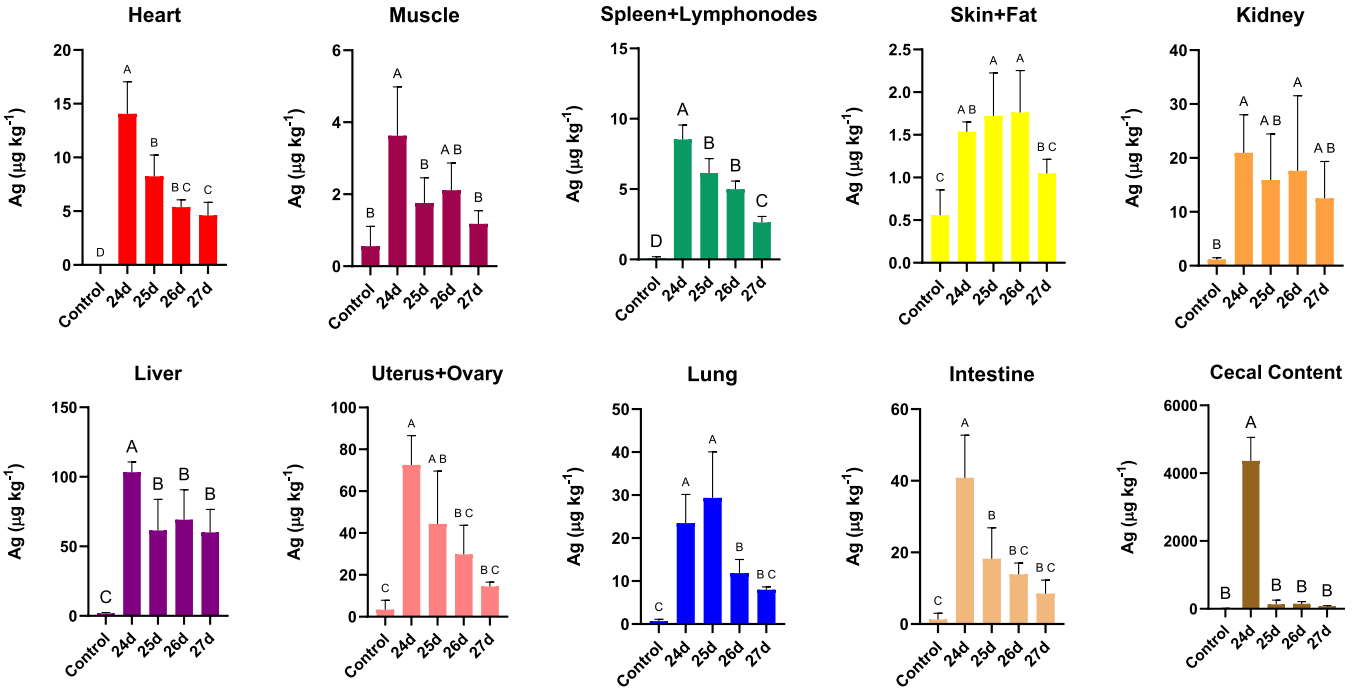

Figure 3. Silver concentration in different tissues immediately after AgNPs@Carb were withdrawn from the diet (day 24), 1 day after withdrawal (day 25), 2 days after withdrawal (day 26), and 3 days after withdrawal (day 27). Column bar represents concentration means (n = 5). Different letters above the bars indicate differences in the Ag concentration among the periods according to Tukey's test ($p < 0.05$). Whiskers correspond to the standard error for each bar.

concentrations of 16.87 ± 11.43 μg kg^{-1}, which is considerably lower compared to pigs and piglets. For instance, Lee et al.[39] studied the biopersistance of AgNPs in rat tissues. They administered 100 and 500 mg of AgNPs per body weight per day, with diameters of 10 and 25 nm, for 28 days, and then monitored the Ag levels in tissues over 180 days. They found that the size of NPs did not affect Ag tissue distribution, and Ag was also cleared over time. The liver, spleen, ovaries, and kidneys showed a degree of clearance of accumulated Ag during the 4-month recovery period. Contrastingly, Ag concentrations in the testes and brain did not decrease to control levels after the 4-month recovery period, indicating that silver clearance is difficult across biological barriers. This highlights the importance of considering both the duration of exposure and the physiological differences between species when evaluating the impact of AgNPs. However, in our study, the brain was excluded due to logistical constraints and prioritization of tissues most relevant to food safety. Additionally, in Brazil, brain tissue is not commonly consumed by humans or utilized in pork products intended for consumption, further reducing its relevance to the aim of this study. There is a notable lack of studies investigating the internalization of nanoparticles within tissues and the subsequent Ag ion release, which are critical for understanding the full biological impact of AgNPs. This gap in research makes it difficult to fully comprehend the mechanisms of bioaccumulation and potential toxicity. Therefore, it is crucial to establish standardized methodologies for assessing the bioaccumulation of AgNPs. Standardization would enhance comparability and reliability across studies, ultimately advancing our understanding of the biological impacts of AgNPs

3.3. Impact on Ionomic Profile and PCA. The administration of AgNPs@Carb not only affects Ag accumulation but also influences the homeostasis of various elements in the animal's tissues (Figure S1). The elemental analysis (excluding silver) revealed significant changes in the concentrations of several key minerals and trace elements across different tissues, comparing the concentrations among the days after AgNPs@Carb were withdrawn from the diet, suggesting potential interactions between AgNPs and these elements.

The liver, as a central organ for detoxification and mineral storage,[40] displayed noticeable changes in the concentrations of Na, Ca, Ni, Cu, and Zn. The increase in Cu and Zn and the decrease in Ca and Na concentrations, observed in the liver, may indicate a disturbance in redox homeostasis due to oxidative stress induced by AgNPs. It is known that AgNPs can generate reactive oxygen species (ROS), leading to oxidative damage,[6] which often results in the upregulation of Cu storage as a protective mechanism. Sawosz et al.[19] also noted such effects when Cu nanoparticles were administered to chickens, where increased oxidative stress led to changes in mineral metabolism. Jankowski et al.[18] observed similar disruptions in mineral levels (Cu and Zn), where Mn_2O_3 nanoparticles interfered with nutrient absorption and kidney function in turkeys.

The kidney, a critical organ for excretion and mineral balance,[41] exhibited a decrease in Co and Ni levels after withdrawing the AgNPs@Carb supplementation, indicating that the same metalloenzymes that help the detoxification due to Ag exposure also regulates Co and Ni detoxification. It is important to highlight that nutrients such as K, P, Mg, and Na were not affected in the kidney, suggesting that AgNPs@Carb exposure may not affect electrolyte balance and renal function, and its alteration could indicate stress responses or disturbed metabolic regulation.

The spleen, involved in immune function and filtering of blood,[42] showed changes in the concentrations of Fe

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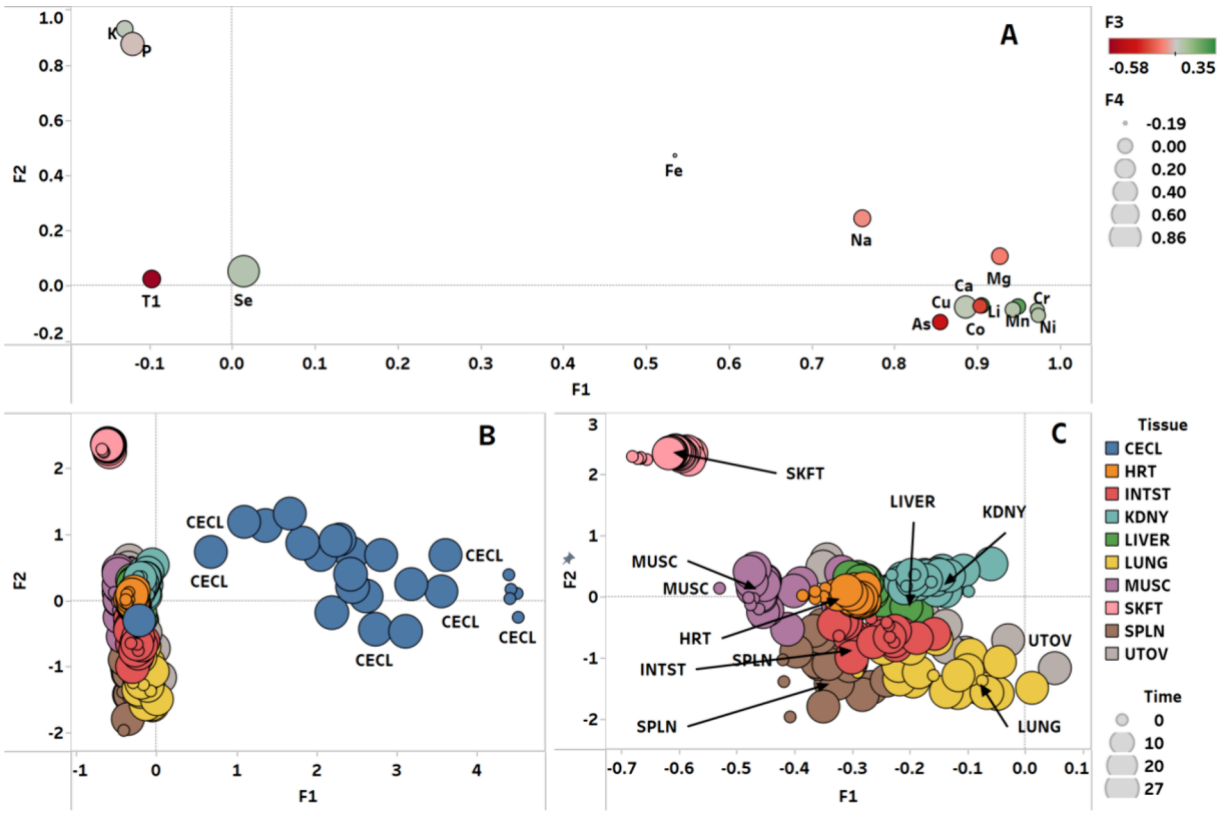

Figure 4. PCA factors (F1xF2) by loading/variables (A) and by cases considering different tissues as colors and treatment time as size (B and C).

(increase), Ni and Pb (decrease). Iron is an essential micronutrient that plays vital roles in the development of the immune system, and this nutrient imbalance may disrupt redox homeostasis and may affect host innate and adaptive immunity.[43] However, Pb and Ni, which are undesirable, are apparently under the action of the same detoxification mechanisms as Ag. The observed changes could suggest that the administration of AgNPs influences immune-related processes and element metabolism in immune-related tissues. AgNPs have been shown to interact with immune cells, as reported by Hadrup and Lam.[44]

The heart exhibited significant fluctuations in Co, Fe, K, Mn, Na, Ni, and Se concentrations, which suggests that AgNPs@Carb exposure may be altering cellular ion balance, which could affect cardiac physiology. Another study suggested that AgNPs affect cardiac physiology in function of the concentration, with effects that involve nitric oxide generation and oxidative stress in rats.[45]

Muscle tissue, as a major site for protein storage and metabolism, showed changes in the concentrations of several elements (Co, Cu, Fe, K, Mg, Mn, Na, P, Pb, and Zn). Immediately after AgNPs@Carb were withdrawn from the diet (day 24), Co, Cu, Fe, Mn, and Zn presented the highest levels, which is a good indicator for a beneficial effect of the additive, since these elements are critical for oxygen transport and enzymatic activity in muscle function.[46] However, changes in skin + fat tissue, which displayed slightly altered levels of Co, Mg, Mn, and P, could be related to the role of adipose tissue in storing and buffering elements during nanoparticle exposure.

The small intestine and cecal content are key sites for nutrient absorption and excretion, respectively.[47] In these tissues, there was a more pronounced variation in the concentrations of micronutrients compared to macronutrients, where the effects of AgNPs@Carb were minimal or negligible. This suggests that AgNPs@Carb primarily disrupts the homeostasis of trace elements, with relatively little impact on the levels of macronutrients.

The principal component analysis (PCA) (Figure 4) identified two key factors (F1 and F2) that explained most of the variance in ionomic profiles. Factor 1 (F1) strongly associated with elements such as As, Ca, Co, Cr, Cu, Li, Mg, and Ni and showed moderate associations with Na and Fe, indicating that these elements may be influenced by similar biological or metabolic processes in the tissues studied (Figure 4A). Factor 2 (F2) presented a strong correlation between K and P, with a weaker association with Fe, suggesting that these elements may be regulated by distinct physiological pathways (Figure 4A).

The orthogonal relationship between F1 and F2 (Figure 4B) highlights independent mechanisms governing the distribution of these elemental groups. Interestingly, temporal changes in element concentrations were observed, particularly for F1-associated elements, which increased over time in certain tissues such as the heart, liver, muscle, and skin + fat, while showing a decrease in the cecal content. In contrast, no clear time-related trend was observed for F2-associated elements in tissues such as the kidney, lung, and small intestine, implying complex and tissue-specific interactions affecting element concentrations during the study period.

The PCA findings suggest that AgNPS@Carb supplementation may influence both the bioaccumulation of specific elements and their metabolic regulation in various tissues. This underscores the need for further investigation into the long-term effects of AgNPs on mineral homeostasis and overall animal health.

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Table 2. Estimated Daily Intake (EDI) of Different Tissues on Day 24 (Immediately after AgNPs@Carb Withdrawal from Pigs' Diet), Day 25 (1 Day after Withdrawal), Day 26 (2 Days after Withdrawal), and Day 27 (3 Days after Withdrawal) for 70 kg Adult and 24 kg Children

EDI (μg kg^{-1} day^{-1}) - adults									
days	heart	muscle	spleen + lymph node	skin + fat	kidney	liver	uterus + ovary	lung	small intestine
24	0.010	0.003	0.006	0.001	0.015	0.072	0.051	0.016	0.029
25	0.006	0.001	0.004	0.001	0.011	0.043	0.031	0.021	0.013
26	0.004	0.001	0.003	0.001	0.012	0.048	0.021	0.008	0.010
27	0.003	0.001	0.002	0.001	0.009	0.042	0.010	0.006	0.006
EDI (μg kg^{-1} day^{-1}) - children									
days	heart	muscle	spleen + lymph node	skin + fat	kidney	liver	uterus + ovary	lung	small intestine
24	0.029	0.007	0.017	0.003	0.043	0.211	0.148	0.048	0.083
25	0.017	0.004	0.013	0.004	0.033	0.125	0.091	0.060	0.037
26	0.011	0.001	0.010	0.004	0.036	0.141	0.061	0.024	0.028
27	0.009	0.001	0.005	0.002	0.002	0.123	0.029	0.016	0.017

3.4. Estimated Daily Intake of Silver by Humans. As previously discussed, the use of AgNPs as feed additives can lead to bioaccumulation in animals, which may eventually be ingested by humans through meat consumption. Therefore, it is critical to assess human exposure to Ag when meat is consumed from animals fed with AgNPs. Table 2 presents the Estimated Daily Ingestion (EDI) of Ag for various tissues over the days following the withdrawal of AgNPs@Carb from pigs' diets. The data show a clear trend of decreasing EDI values across all tissues as the withdrawal period increases. This reduction can be attributed to the animals having more time to excrete the Ag accumulated.

The discussion surrounding Ag regulatory limits in various contexts, such as drinking water, food, and daily exposure, reveals a significant disparity between countries and regulatory agencies. In Brazil, specific limits for Ag concentration in food have yet to be established, leaving a critical gap in the regulation of AgNP exposure via diet. For instance, the National Environment Council (CONAMA) sets a limit of 10 μg L^{-1} for total silver in drinking water (Resolution No. 357/05),[48] which is more restrictive compared to the 50 μg L^{-1} allowed by the United States Environmental Protection Agency (EPA).[49] The United States has also established a Tolerable Daily Intake (TDI) for Ag of 5 μg day^{-1} kg^{-1}, based on 70 documented cases of argyria, a condition where silver accumulates in the body, and a no observed adverse effect level (NOAEL) of 14 μg day^{-1} kg^{-1}.[28]

The 2021 directive from the European Food Safety Authority (EFSA)[50] outlines the requirements for submitting and approving new food additives, including nanomaterials, with a focus on chemical characterization, manufacturing processes, and toxicological data. The goal of this regulation is to ensure the safety of additives and protect consumer health. EFSA's 2024 technical report (EN-8826)[51] discusses the use of New Approach Methodologies (NAMs) in nanoparticle risk assessment, highlighting challenges such as stability and dosimetry, as well as the difficulty in estimating daily intake due to the lack of robust human exposure data.

A study examined the oral toxicity of Ag ions, AgNPs, and colloidal Ag in humans and animals.[44] The authors identified that chronic exposure to silver can cause argyria and emphasized the difficulty in defining a safe level due to the variability in organism responses. The authors suggested a TDI for AgNPs of 2.5 μg day^{-1} kg^{-1}, noting that this value provides a significant margin of safety.

In this study (Table 2), tissues with the highest EDI for adults on day 24 (immediately after AgNPs@Carb withdrawal) are the liver and uterus/ovary, with values of 0.072 and 0.051 μg kg^{-1} day^{-1}, respectively. These represent 1.44 and 1.02% of the TDI for Ag, which is set at 5 μg kg^{-1} day^{-1} by the U.S. Environmental Protection Agency (EPA).[28] For children, the highest EDI on day 24 are also in the liver and uterus/ovary, with values of 0.211 and 0.148 μg kg^{-1} day^{-1}, representing 4.22 and 2.96% of the TDI for Ag,[28] respectively. For comparison, the contribution of drinking water to overall silver exposure varies significantly by region, ranging from 1% in Canada to 20% in France.[44] It is important to note that the EDI values presented here reflect silver ion accumulation only and do not account for intact silver nanoparticles, which could potentially increase the total silver exposure.

AgNPs are a promising strategy to replace antimicrobial growth promoters; however, the bioaccumulation of AgNPs in animal tissues, particularly in edible organs such as the liver, kidneys, and muscles, raises questions about the potential transfer of Ag into the human food chain. This is particularly important because AgNPs may remain in the body for extended periods, potentially leading to toxic effects if consumed at high levels. Therefore, comprehensive studies on the bioaccumulation and excretion of AgNPs are critical to establishing safe usage guidelines. The present study showed that Ag accumulates in different tissues over time, but their concentration tends to decrease once they are withdrawn from the animals' diet.

Elemental analysis further revealed that AgNPs influence the distribution and concentration of macro elements and essential trace elements, particularly Co, Cu, Fe, Ni, and Zn in key tissues. The observed shifts in ionomic profiles indicate that AgNPs may alter mineral metabolism, especially that of trace elements, potentially affecting physiological functions and growth. These findings highlight the need for further research to determine the long-term effects of nanoparticle exposure on mineral homeostasis and establish safe guidelines for the use of AgNP in animals.

Existing studies often vary in terms of nanoparticle size, coating materials, dosage, and experimental conditions, leading to inconsistent results. This lack of standardized protocols makes it difficult to compare findings across studies and draw definitive conclusions about the long-term safety of AgNPs for both human health and the environment. Furthermore, there is a need for comprehensive models that can simulate real-world exposure scenarios, including chronic low-dose exposure and

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the effects of AgNPs on different tissues over time. Developing standardized methodologies is essential to accurately evaluate the bioavailability, accumulation patterns, and potential toxic effects of AgNPs in animals and humans as well as to understand their environmental fate and impact after excretion.

While this study provides information about the bioaccumulation of Ag from NPs and its impact on ionomic profiles, certain toxicological analyses, such as histological evaluation, were beyond its scope. Histological data could offer detailed insights into tissue-specific damage or cellular changes induced by AgNPs. However, the primary focus of this work was to investigate systemic bioaccumulation and mineral homeostasis alterations as proxies for potential toxicological effects. Similar approaches have been employed in prior studies to identify broader systemic impacts of nanoparticle exposure.[8,17,52] Future research integrating histological analyses will provide a more comprehensive understanding of tissue-level responses and help validate the systemic disruptions observed here.

This study also did not include pharmacokinetic or pharmacodynamic (PK/PD) analysis or aggregation studies of AgNPs in blood. These analyses are critical for elucidating the absorption, distribution, and biophysical characteristics of NPs within biological systems. However, the exploratory nature of this study aimed to identify bioaccumulation patterns and ionomic changes across multiple tissues. Previous studies highlight the importance of aggregation in determining nanoparticle biodistribution and interaction with biological barriers.[53] Future studies could integrate PK/PD data and aggregation analyses to complement the findings presented here and deepen our understanding of nanoparticle behavior in vivo. Additionally, while cellular level imaging techniques, such as TEM, would provide direct localization of nanoparticles, the use of ICP-MS offered robust quantitative insights into bioaccumulation across tissues, aligning with the study's systemic focus.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.4c00624.

Calculation of excretion estimative, changes in elemental concentrations across different tissues, and summary of silver nanoparticle bioaccumulation studies in vivo: characteristics, dosage, accumulation, and methodology (PDF)

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■ ABBREVIATIONS USED

AAS:atomic absorption spectroscopy
AES-ICP:atomic emission spectroscopy inductively coupled plasma
AgNPs:silver nanoparticles
AgNPs@Carb:silver nanoparticles complexed with carbohydrates
CONAMA:National Environment Council (Brazil)
EDI:estimated daily intake
EFSA:European Food Safety Authority

EPA:Environmental Protection Agency
ICP-MS:inductively coupled plasma mass spectrometry
ICP-OES:inductively coupled plasma optical emission spectrometry
ICSD:Inorganic Crystal Structure Database
NAA:neutron activation analysis
NOAEL:no observed adverse effect level
NPs:nanoparticles
PCA:principal component analysis
PK/PD:pharmacokinetic/pharmacodynamic
PVP:polyvinylpyrrolidone
SP-ICP-MS:single-particle inductively coupled plasma mass spectrometry
SP-ICP-MS:single-particle inductively coupled plasma mass spectrometry
TDI:tolerable daily intake
TDI:tolerable daily intake
TEM:transmission electron microscopy
VICH:International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products
XRD:X-ray diffraction

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