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Pharmaceutical Screening of Bat Feces and Their Applications and Risks in Traditional Chinese Medicine

Kou-Toung Chung [1], Ching-Lung Lin [2], Wu-Chang Chuang [3], Ming-Chung Lee [4], Li-Wen Chen [5] and Chung-Hsin Wu [2,*]

[1] Department of Health Nutrition and Chemical Engineering, Army Academy of ROC, Taoyuan City 320, Taiwan; chungkoutoung@gmail.com
[2] School of Life Science, National Taiwan Normal University, Taipei 117, Taiwan; linchinglung1@gmail.com
[3] Sun Ten Pharmaceutical Co., Ltd., New Taipei City 231, Taiwan; cwctd331@sunten.com.tw
[4] Brion Research Institute of Taiwan, New Taipei City 231, Taiwan; mileslee@sunten.com.tw
[5] Department of Science Education and Application, National Taichung University of Education, Taichung City 403, Taiwan; liwenchen@mail.ntcu.edu.tw
* Correspondence: megawu@ntnu.edu.tw; Tel.: +886-2-77496363

Abstract: Bat feces have been reported in traditional Chinese medicine (TCM) books to have the effect of reducing fever and improving eyesight, but the mechanism of vision improvement still needs further research. To this end, we used 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and liquid chromatography/tandem mass spectrometry (LC/MS/MS) to analyze the antioxidant capacity of and the types of vitamins in bat feces. We hoped to screen the pharmacological components of bat feces and to explain the role that these components may play in treating visual deterioration. Our results found that bat feces had a good antioxidant capacity and mainly contained vitamins B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B3 (nicotinic acid), and B5 (pantothenic acid). Although these vitamins may help to maintain the health of the optic nerve and cornea, the vitamin content of bat feces is low, but the heavy metal content is high, as shown using inductively coupled plasma–mass spectrometry (ICP-MS) analysis. Therefore, we suggest that the use of bat feces as TCM to improve vision should be strictly restricted.

Keywords: antioxidant capacity; luminous sand; heavy metals; inductively coupled plasma–mass spectrometer; liquid chromatography/tandem mass spectrometry; vitamins

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1. Introduction

Bat feces were first recorded as a traditional Chinese medicine (TCM) in Shennong's "Classic of Materia Medica" [1] and were called Luminous Sand in the Song Dynasty's "Rihuazi Materia Medica" [2]. Luminous sands are oblong particles with slightly pointed ends, 5 to 7 mm in length, and about 2 mm in diameter. They have a rough surface and are in the form of small brown particles or powder. When observed under a microscope, brown or yellow–brown shiny insect body fragments can be seen. The ancient book "Compendium of Materia Medica" reported that bat feces have the medicinal effects of reducing fever, improving eyesight, activating blood circulation, and eliminating metabolic accumulation [3]. Modern medicine believes that bat feces contain vitamin A, which can be used to treat night blindness and relieve eye bleeding [4]. Vitamin A is a light-sensitive substance on the surface of the retina. It can help the formation of rhodopsin in the photoreceptor cells. Through the decomposition and regeneration of rhodopsin, the eyes can detect light. People who lack vitamin A often develop night blindness.

Compared with other mammals, bats have the greatest variability in dietary strategies, spanning insectivorous, carnivorous, or frugivorous types. The food types of most bats are insects and other small arthropods. In addition, these diverse dietary habits of bats provide high-quality ecological and environmental services to the ecosystem [5]. For example,

insectivorous bats eat a large number of pests and can be used as a natural pesticide [6]. Since bats consume a rich and nutritionally diverse diet, the variability in different dietary strategies also appears to influence the composition of bat feces [7,8]. The Luminous Sand reported by ancient Chinese medicinal materials matches the description of the feces of insectivorous bats. It has been reported that the active ingredient in insectivorous bat feces for treating eye diseases may be insect eyes, which are rich in vitamin A and have the effect of reducing heat and improving eyesight [9]. Our observations revealed that the main component of insectivorous bat feces is undigested insect body fragments, and the number of insect eyes contained in the feces is very small. It is not accurate to say that insect eyes are the active ingredients of bat feces.

In view of the fact that there are still very few studies related to bat feces and relevant reports on the mechanism of bat feces in relieving eye diseases are also quite lacking, we hope to clarify the feasibility of bat feces as a TCM for relieving eye diseases by screening the contents of bat feces. In this study, we used 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and liquid chromatography/tandem mass spectrometry (LC/MS/MS) to analyze the antioxidant capacity of and the types of vitamins in insectivorous bat feces. We hope that screening the contents of bat feces can provide relevant evidence to explain whether bat feces can alleviate eye diseases.

2. Materials and Methods

2.1. Bat Feces Preparation

Fecal samples of *Hipposideros armiger* terasensis and *Miniopterus fuliginosus*, the most common species in Taiwan, were collected from three locations in northern Taiwan. *Hipposideros armiger* terasensis is the largest insectivorous bat among the Chiropterans in Taiwan. They use ultrasonic waves to search for large insects, such as scarabs and beetles. In addition to flying insects, they also prey on insects perched on tree trunks or leaves. The types of insects they feed on vary with the season and whether they reproduce or not. These bats are commonly found in natural caves, artificial tunnels, or abandoned buildings in low- and medium-altitude areas. *Miniopterus fuliginosus* feeds on small insects and is a typical cave bat that uses caves or tunnels as its main habitat. During the summer, both species total as many as hundreds or thousands of individuals. Fecal samples were collected from dung piles beneath bat colonies in caves. We mainly collected fresh fecal pellets, avoiding the contamination of the samples with old feces. We collected fecal samples using sterile forceps in sterile microcentrifuge tubes, kept them on ice, transported them to the laboratory, and processed them within 24 h. In the laboratory, we prepared pooled fecal samples for QC by combining and homogenizing approximately 5 g of thawed aliquots from 10 individual fecal samples. These samples were shaken at 4 °C for 5 min and sonicated in ice water for 5 min to obtain low-, medium-, and high-level QC fecal homogenates. Aliquots (50 µL) of the homogenate were then placed in a series of 1.5 mL Eppendorf tubes and stored at −80 °C until analysis.

2.2. Preparation of Standards

For LC/MS/MS, ultrapure water, methanol, and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (nicotinamide), vitamin B3 (nicotinic acid), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B8 (biotin), vitamin B9 (folic acid), vitamin B12 (cyanocobalamin), vitamin C (ascorbic acid), vitamin A (retinol), vitamin D3 (cholecalciferol), vitamin E (α-tocopherol), vitamin K1 (phylloquinone), and LC–MS ultrapure formic acid were purchased from Sigma-Aldrich (St. Louis, MI, USA). All standards were prepared under UV-shielded light. The calibration standard solutions of the vitamins were prepared in 10% methanol in the concentration range of 5–1000 ng/mL (ppb) and stored at −80 °C until use.

2.3. Determination of Antioxidant Capacity of Fecal Samples

In this study, we evaluated the antioxidant activity of the fecal samples using 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH, D9132, Sigma-Aldrich Co., St. Louis, MO, USA). We first diluted fecal samples with distilled water to prepare solutions of different concentrations, then added 100 µL of 1.5 mM/mL DPPH (Sigma-Aldrich Co.) to each well of a 96-well plate, and then added fecal samples of different concentrations. We incubated a mixture of stool samples with DPPH for 30 min at room temperature. Subsequently, we measured the absorbance at 517 nm using a microplate spectrophotometer (µQuant, Biotek Instruments, Inc., Winooski, VT, USA). To obtain more objective experimental data, we performed three separate DPPH determinations for each concentration of fecal samples. To benchmark the antioxidant function of fecal samples, we measured the absorbance of blank methanol and L-ascorbic acid (A5960, Sigma-Aldrich) as controls. The antioxidant activity of fecal samples was calculated using the formula for calculating DPPH radical scavenging activity, as follows: antioxidant activity of fecal samples (%) = 100 × [(absorbance of fecal samples + DPPH) − (absorbance of fecal samples)]/[(absorbance of DPPH) − (absorbance of methanol)].

2.4. Vitamin Analysis

2.4.1. Water-Soluble Vitamins

(A) Liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipment:
   (1) Quaternary pump: Shimadzu LC-20AD;
   (2) Autosampler: Shimadzu SIL-20AC;
   (3) Photodiode array detector: Shimadzu SPD-M20;
   (4) Mass detector: Shimadzu LCMS-8040.

(B) Sample preparation:
   (1) Place 1 g of sample powder in a 50 mL centrifuge tube, add 9 mL of 10 mM ammonium acetate aqueous solution, vortex for 1 min, and then ultrasonicate for 15 min;
   (2) Add another 10 mL of chloroform (chloroform) to the centrifuge tube and vortex for 1 min;
   (3) Centrifuge at 3500 rpm for 10 min, take out the supernatant, filter it with a 0.22 µm filter membrane, and use the filtrate as the test solution.

(C) LC/MS/MS analysis method:
Chromatography column: Raptor Biphenyl (2.7 µm, 100 × 2.1 mm);
Column temperature: 35 °C.
Mobile phase: as shown in Table 1;
A: 5 mM ammonium acetate, 0.1% formic acid in water;
B: 5 mM ammonium acetate, 0.1% formic acid in methanol.

Table 1. Mobile-phase gradient of LC/MS/MS analysis for water-soluble vitamins.

Time (min)	A, %	B, %
Initial	100	0
1.00	100	0
6.80	0	100
8.80	0	100
9.00	100	0
12.00	100	0

Flow rate: 0.4 mL/min;
Injection volume: 15 µL.
Mass spectrometry conditions:

Ion source: electrospray ionization (ESI+);

Ion source interface voltage (probe voltage): 4.5 kV;
Nebulizing gas flow: nitrogen, 3.0 mL/min;
Drying gas flow: 15.00 L/min;
Collision gas: argon, 230 kPa;
Desolventization tube temperature (DL temp.): 250 °C;
Heating module temperature (heat block temp.): 400 °C;
Quantitative ion pair: as shown in Table 2.

Table 2. Quantitative ion pair of mass spectrometry for water-soluble vitamins.

Vitamin	Quantitative Ion Pair	Qualitative Ion Pair
	Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)	Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)
B1 (thiamine)	265.00→122.10	265.00→144.10
B2 (riboflavin)	377.20→243.10	377.20→198.10
B3 (nicotinamide)	123.00→80.10	123.00→96.10
B3 (nicotinic acid)	123.90→80.10	123.90→78.10
B5 (pantothenic acid)	220.20→90.10	220.20→202.10
B6 (pyridoxine)	170.00→152.10	170.00→134.10
B8 (biotin)	245.20→227.20	245.20→123.10
B9 (folic acid)	441.90→295.10	441.90→176.20
B12 (cyanocobalamin)	678.60→147.10	678.60→359.20

2.4.2. Vitamin C

(A) Liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipment:
  (1) Quaternary pump: Shimadzu LC-20AD;
  (2) Autosampler: Shimadzu SIL-20AC;
  (3) Photodiode array detector: Shimadzu SPD-M20;
  (4) Mass detector: Shimadzu LCMS-8040.

(B) Sample preparation:
  (1) Place 1 g of sample powder in a 50 mL centrifuge tube, add 9 mL of 10 mM ammonium acetate aqueous solution, vortex for 1 min, and then ultrasonicate for 15 min;
  (2) Add another 10 mL of chloroform (chloroform) to the centrifuge tube and vortex for 1 min;
  (3) Centrifuge at 3500 rpm for 10 min, take out the supernatant, filter it with a 0.22 μm filter membrane, and use the filtrate as the test solution.

(C) LC/MS/MS analysis method:
LC analysis conditions:
Chromatography column: Raptor Biphenyl (2.7 μm, 100 × 2.1 mm);
Column temperature: 35 °C:
Mobile phase: as shown in Table 3;
A: 5 mM ammonium acetate, 0.1% formic acid in water;
B: 5 mM ammonium acetate, 0.1% formic acid in methanol.

Table 3. Mobile phase gradient of LC/MS/MS analysis for vitamin C.

Time (min)	A, %	B, %
Initial	100	0
2.40	100	0
4.40	89	11
4.60	70	30
6.50	68	32
6.70	0	100
7.00	100	0

Flow rate: 0.2 mL/min;
Injection volume: 5 μL.
Mass spectrometry conditions:

Ion source: electrospray ionization (ESI+);
Ion source interface voltage (probe voltage): 4.5 kV;
Nebulizing gas flow: nitrogen, 3.0 mL/min;
Drying gas flow: 15.00 L/min;
Collision gas: argon, 230 kPa;
Desolventization tube temperature (DL temp.): 250 °C;
Heating module temperature (heat block temp.): 400 °C;
Quantitative ion pair: as shown in Table 4.

Table 4. Quantitative ion pair of mass spectrometry for vitamin C.

Vitamin	Quantitative Ion Pair	Qualitative Ion Pair
	Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)	**Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)**
C	177.10→95.10	177.10→141.20

2.4.3. Fat-Soluble Vitamins

(A) Liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipment:
   (1) Quaternary pump: Shimadzu LC-20AD;
   (2) Autosampler: Shimadzu SIL-20AC;
   (3) Photodiode array detector: Shimadzu SPD-M20;
   (4) Mass detector: Shimadzu LCMS-8040.

(B) Sample preparation:
   (1) Take 0.25 g of sample powder and place it in a 15 mL centrifuge tube, add 1.5 mL of pure water and 1.5 mL of methanol (methanol), shake with a vortex mixer for 1 min, and then shake with ultrasonic for 20 min;
   (2) Add another 10 mL of n-Hexane to the centrifuge tube and vortex for 5 min;
   (3) Centrifuge at 3500 rpm for 10 min, and place 1 mL of supernatant into a glass centrifuge tube;
   (4) Blow dry with nitrogen in a 40 °C water bath and add 1 mL of methanol (methanol) to dissolve and mix evenly;
   (5) Filter with a 0.22 μm filter membrane and use the filtrate as the test solution.

(C) LC/MS/MS analysis method:
Chromatography column: Raptor Biphenyl (2.7 μm, 100 × 2.1 mm);
Column temperature: 35 °C;
Mobile phase: as shown in Table 5;
A: 5 mM ammonium acetate, 0.1% formic acid in water;
B: 5 mM ammonium acetate, 0.1% formic acid in methanol.

Table 5. Mobile phase gradient of LC/MS/MS analysis for fat-soluble vitamins.

Time (min)	A, %	B, %
Initial	100	0
2.40	100	0
4.40	89	11
4.60	70	30
6.50	68	32
6.70	0	100
7.00	100	0

Flow rate: 0.4 mL/min;
Injection volume: 5 μL.
Mass spectrometry conditions:

Ion source: electrospray ionization (ESI+);
Ion source interface voltage (probe voltage): 4.5 kV;
Nebulizing gas flow: nitrogen, 3.0 mL/min;
Drying gas flow: 15.00 L/min;
Collision gas: argon, 230 kPa;
Desolventization tube temperature (DL temp.): 250 °C;
Heating module temperature (heat block temp.): 400 °C;
Quantitative ion pair: as shown in Table 6.

Table 6. Quantitative ion pair of mass spectrometry for fat-soluble vitamins.

Vitamin	Quantitative Ion Pair	Qualitative Ion Pair
	Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)	**Precursor Ion (*m*/*z*)→Product Ion (*m*/*z*)**
A	269.30→93.10	269.30→119.10
D3	385.20→367.40	385.20→259.30
E	431.10→165.20	431.10→137.10
K1	451.30→187.20	451.30→185.10

2.5. Heavy Metals Analysis

(A) Inductively coupled plasma–mass spectrometry (ICP-MS) conditions:
Agilent 7500a.

(B) Sample preparation:

(1) Take 0.4 g of the sample powder and place it in a microwave digestion bottle, add 8 mL of nitric acid, let it stand for about 10 min, and then digest it in a microwave digester. The operating conditions of microwave digestion are as shown in the table below;

Stage #	Max Power (W)	Ramp (min)	Temperature (°C)	Hold (min)
1	1200	15	175	05:00
2	1200	5	200	15:00

(2) After the digestion is completed, cool to room temperature and transfer to a 100 mL quantitative flask. Wash the microwave digestion flask with pure water. Put the washing liquid into the quantitative flask, dilute it with pure water to a constant volume, mix evenly, and filter through a 0.45 μm filter membrane. The filtrate was the finished product sample solution, and this solution was used as the test solution.

(C) ICP-MS analysis method.

(1) Method settings:
Acquisition mode: spectrum;
Peak pattern: full quant;
Every mass integration time: 0.33 s;
Repetition: three times.

(2) Peristaltic pump program:
Uptake speed: 0.35 rps;
Uptake time: 30 s;
Stabilization time: 30 s.

(3) Analysis conditions.
Plasma Parameters:

Plasma radio frequency power: 500~1600 W, normal setting 1200 W;
Sampling depth: 3.0–23.0 mm, normal setting 10 mm;
Carrier gas flow rate: 0.00–2.00 L/min, normal setting is 1 L/min;
Auxiliary gas flow rate: 0.00–2.00 L/min, normal setting is 0.22 L/min;
Nebulizer pump: 0.00–0.50 rps, normal setting is 0.1 rps;
Premix chamber temperature (S/C temp): 2 °C.

Ion Lenses:

Extract 1: −200–10 V, normal setting −120 V;
Extract 2: −200–0 V, normal setting −39 V;
Einzel 1,3: −200–100 V, normal setting −80 V;
Einzel 2: −200–100 V, normal setting 8 V;
Omega bias: −200–100 V, normal setting −41 V;
Omega (+): −200–100 V, normal setting 9 V;
Omega (−): −200–100 V, normal setting 9 V;
QF focus: −200–100 V, normal setting 9 V;
Plate bias: −50–50 V, normal setting −10 V.

2.6. Statistical Analysis

All data are shown as the mean ± standard error of the mean (SEM). Differences among different groups were assessed using a one-way analysis of variance (ANOVA). The Student–Newman–Keuls multiple comparisons post hoc test was performed if a significant F-value was obtained. Significance was defined as $p < 0.05$.

3. Results

3.1. Antioxidant Capacity of Bat Feces Treatment

We determined the antioxidant capacity of bat feces using the DPPH assay, as shown in Figure 1. DPPH is a stable free radical that can be used to measure the free radical scavenging activity of antioxidants. The DPPH method can be used in aqueous and non-polar organic solvents and can be used to examine hydrophilic and lipophilic antioxidants. Our results show that the free radical scavenging activity exceeded 50% when the bat feces treatment concentration was in the range of 1–100 mg/mL. The results indicate that bat feces had very good antioxidant capacity at appropriate concentrations and could effectively eliminate free radical damage.

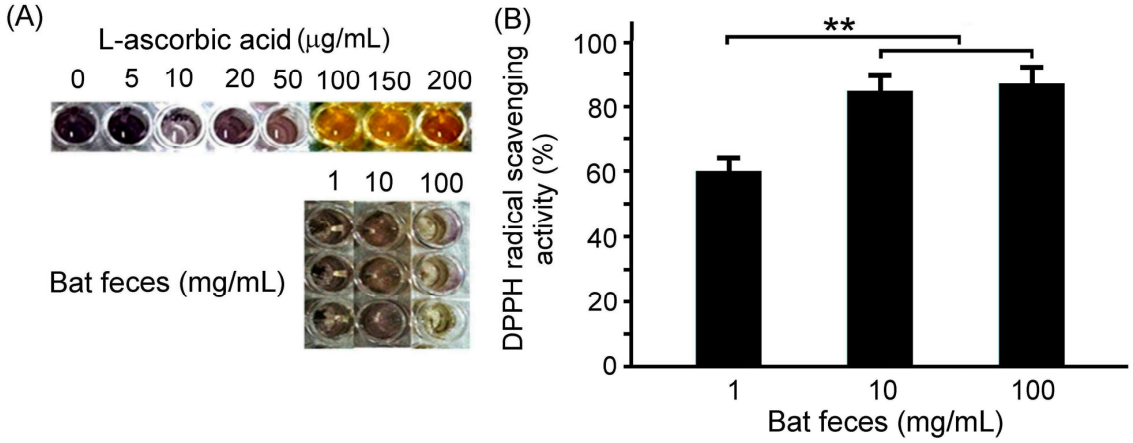

Figure 1. Antioxidant capacity of bat feces. (**A**) DPPH free radical assay under bat feces treatments with concentrations of 1–100 mg/mL. (**B**) Quantified radical scavenging activity under bat feces treatments with concentrations of 1–100 mg/mL. Data are shown as mean ± SEM, ** $p < 0.01$, and the number of replications was at least three for each bat feces treatment.

3.2. Quantification of Vitamins in Bat Feces Using LC/MS/MS Analysis

We determined whether the fat-soluble vitamins A, D3, E, and K1 were present in bat feces using LC/MS/MS analysis, as shown in Figure 2. We found that bat feces contained very low amounts of fat-soluble vitamins A, D3, E, and K1, if any, as the contents of these vitamins in bat feces were all below the detection threshold and they could not be detected using LC/MS/MS analysis.

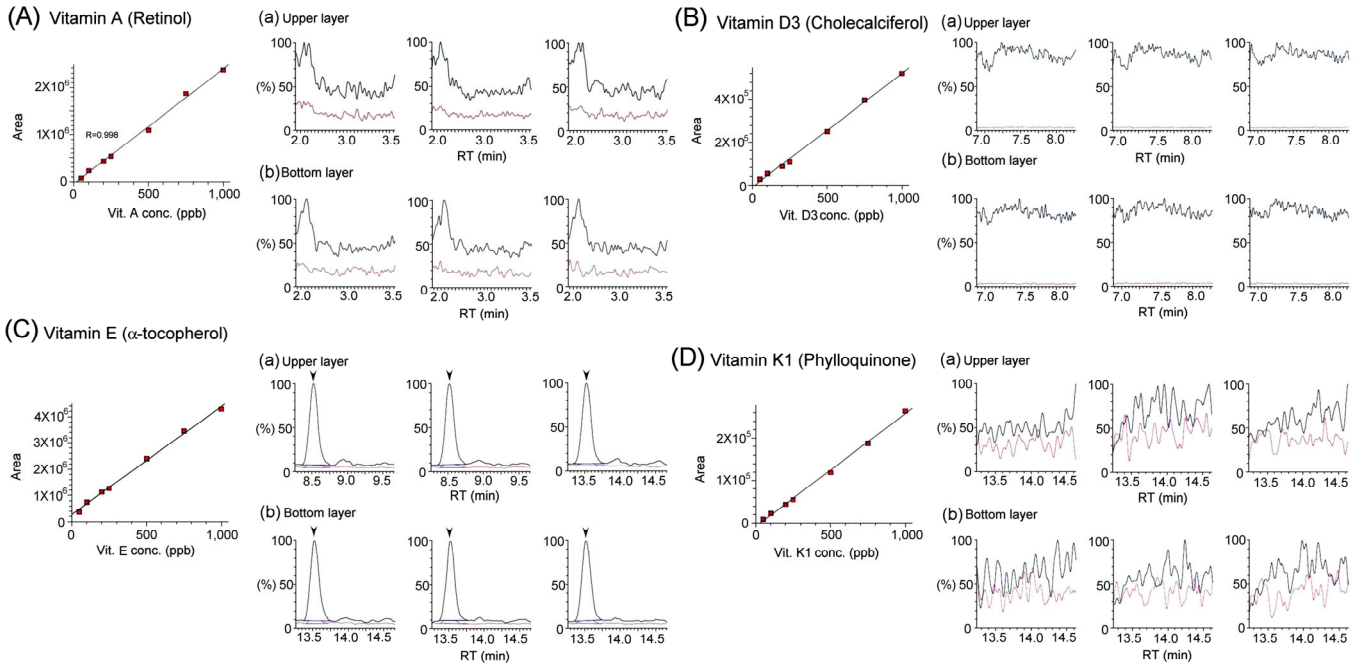

Figure 2. Representative chromatograms of fat-soluble vitamins A, D3, E, and K1 (**A**–**D**) in the (**a**) upper and (**b**) bottom layers of bat feces using LC/MS/MS analysis. The red lines represent the sample detection concentration. The arrows represent the peaks of the standard. Bat feces contained very low amounts of fat-soluble vitamins A, D3, E, and K1, if any, as the contents of these vitamins in bat feces were all below the detection threshold and could not be detected using LC/MS/MS analysis. The number of replications was at least three for each vitamin.

We determined whether the water-soluble vitamin C was present in bat feces using the LC/MS/MS analysis in Figure 3. The results showed that the vitamin C content in bat feces was below the detection threshold and could not be detected. The contents of the vitamin C in bat feces were all below the detection threshold and could not be detected using LC/MS/MS analysis.

We determined the potential presence of water-soluble B vitamins in bat feces using LC/MS/MS analysis, as shown in Figure 4A–I. Our research results found that bat feces contained detectable amounts of vitamins B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B3 (nicotinic acid), B5 (pantothenic acid), and B6 (pyridoxine); however, B8 (biotin), B9 (folic acid), and B12 (cyanocobalamin) were below the detection threshold of LC/MS/MS analysis and could not be detected. Regardless of being in the upper or lower layers of bat feces, the contents of vitamins B3 (nicotinic acid), B3 (nicotinamide), and B5 (pantothenic acid) were much higher than the content of the other B vitamins. Moreover, except for vitamin B3 (nicotinamide), the contents of vitamins B1, B2, B3 (nicotinic acid), B5, and B6 in the lower layer of bat feces were slightly higher than those in the upper layer of bat feces.

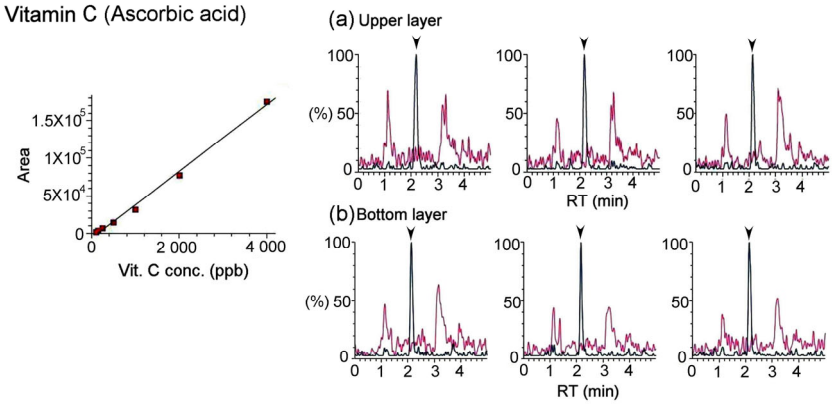

Figure 3. Representative chromatograms of water-soluble vitamin C in the (**a**) upper and (**b**) bottom layers of bat feces by LC/MS/MS analysis. The red lines represent the sample detection concentration. The arrows represent the peaks of the standard. The vitamin C content in bat feces was below the detection threshold and could not be detected. The contents of the vitamin C in bat feces were all below the detection threshold and could not be detected using LC/MS/MS analysis. The number of replications was at least three for each vitamin.

Table 7 summarizes the results from Figures 2–4. As demonstrated by our LC/MS/MS analysis, bat feces contain detectable amounts of vitamins B1, B2, B3, B3 (nicotinic acid), B5, and B6; however, vitamins A, C, B8, B9, B12, D3, E, and K1 were below the detection threshold of LC/MS/MS and could not be detected. Although bat feces contains detectable amounts of vitamins B1, B2, B3, B3 (nicotinic acid), B5, and B6, the contents of these vitamins in bat feces were still quite low. Except for vitamin B3, the contents of vitamins B1, B2, B3 (nicotinic acid), B5, and B6 in the lower layer of bat feces were slightly higher than those in the upper layer of bat feces. In the past, the traditional concept was that bat feces contained a small amount of vitamin A. Obviously, the results from Table 7 do not support this concept.

Table 7. Vitamins in bat feces determined via LC/MS/MS analysis.

Vitamins		Bat Feces	
		Upper Layer	Bottom Layer
Water-Soluble Vitamins	B1(thiamine)	3.44 ± 0.05	2.22 ± 0.02
	B2 (riboflavin)	6.75 ± 0.34	2.37 ± 0.21
	B3 (nicotinamide)	52.53 ± 1.50	70.41 ± 1.46
	B3 (nicotinic acid)	19.67 ± 0.36	16.13 ± 0.49
	B5 (pantothenic acid)	62.63 ± 2.34	41.38 ± 0.33
	B6 (pyridoxine)	0.05 ± 0.02	0.04 ± 0.02
	B8 (biotin)	N/A	N/A
	B9 (folic acid)	N/A	N/A
	B12 (cyanocobalamin)	N/A	N/A
	C (ascorbic acid)	N/A	N/A
Fat-Soluble Vitamins	A (retinol)	N/A	N/A
	D3 (cholecalciferol)	N/A	N/A
	E (α-tocopherol)	N/A	N/A
	K1 (phylloquinone)	N/A	N/A

Unit: ng per g bat feces. N/A: Not Available.

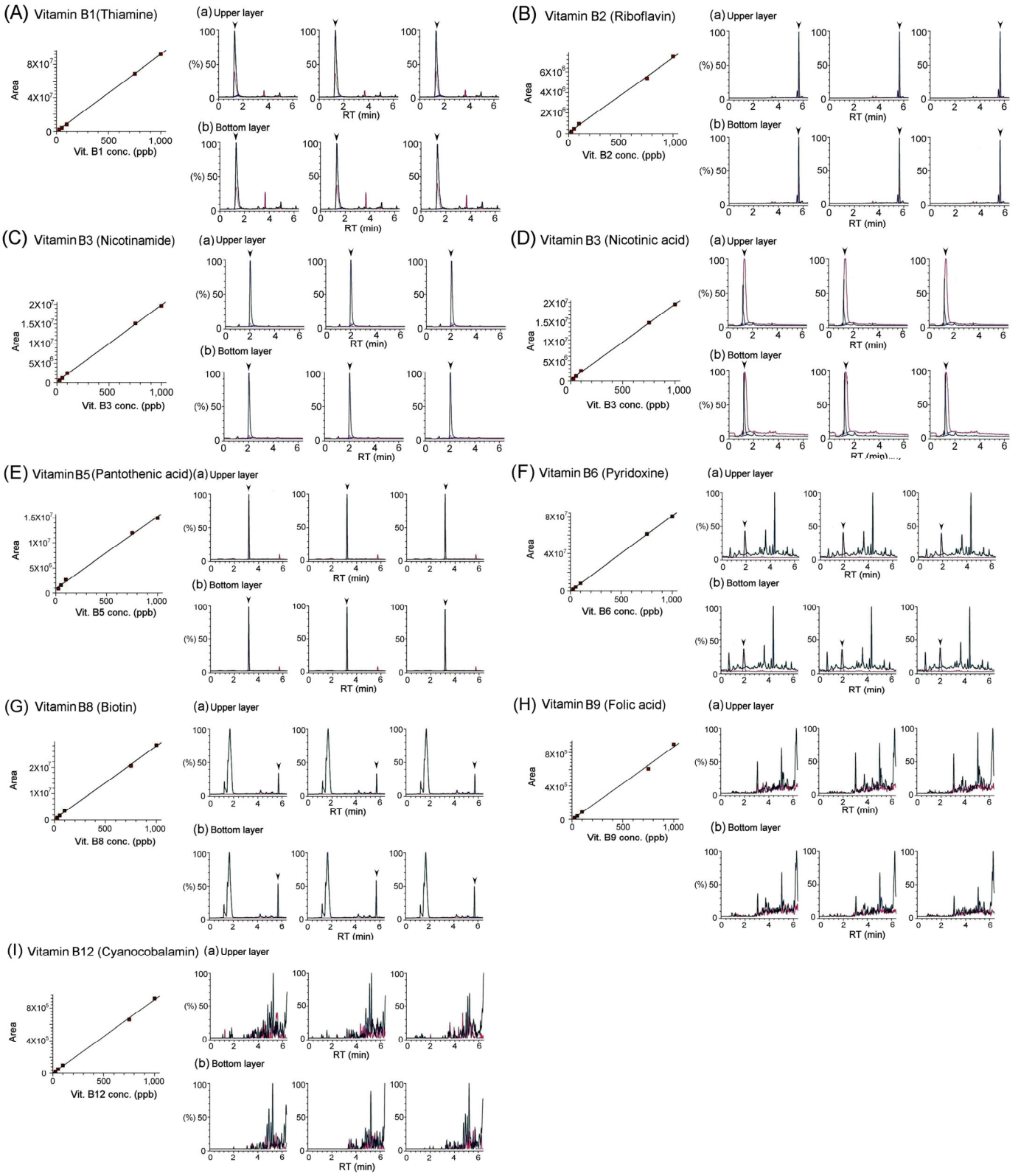

Figure 4. Representative chromatograms of water-soluble vitamins C, B1, B2, B3, B5, B6, B8, B9, and B11 (**A**–**I**) in the upper and bottom layers of bat feces by LC/MS/MS analysis. The red lines represent the sample detection concentration. The arrows represent the peaks of the standard. Bat feces contain detectable amounts of vitamins B1, B2, B3, B3 (nicotinic acid), B5, and B6; however, B8, B9, and B12 were below the detection threshold of LC/MS/MS analysis and could not be detected. Regardless of being

in the upper or lower layers of bat feces, the contents of vitamins B3, B3 (nicotinamide), and B5 were much higher than the contents of the other B vitamins. Moreover, except for vitamin B3, the contents of vitamins B1, B2, B3 (nicotinic acid), B5, and B6 in the lower layer of bat feces were slightly higher than those in the upper layer of bat feces. The number of replications was at least three for each vitamin.

3.3. Quantification of Heavy Metals in Bat Feces Using ICP/MS Analysis

Bat feces are an ancient traditional method of treating visual degradation, so it is important to test their heavy metal contents to ensure that they are safe for consumption. We used ICP/MS to analyze the contents of seven heavy metals, including chromium (Cr), manganese (Mn), copper (Cu), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), in bat feces samples. As reported in Table 8, we detected Cr, Mn, Cu, As, Cd, Hg, and Pb in most bat feces samples. Among these detected heavy metals, we detected extremely high levels of Mn and Cu, with concentrations of 55.53 and 46.25 ppm, respectively. Although TCM does not regulate standards for Mn and Cu contents, the testing standards stipulate that the unsubdivided heavy metal limit benchmark specification must be less than 20 ppm. The bat feces samples far exceeded the heavy metal standards of TCM. In addition, as can be seen from the results in Table 8, the As content of the bat feces samples was much higher than the heavy metal standards of TCM. Arsenic is a widely distributed and toxic metalloid element in nature. Therefore, the use of bat feces in TCM to treat visual degradation has considerable health risks.

Table 8. Heavy metals in bat feces determined using ICP/MS.

Types of Heavy Metals	Heavy Metals in Bat Feces (ppm)	Limitation Standards of Heavy Metals in TCM (ppm)
Chromium (Cr)	2.87 ± 0.38	--
Manganese (Mn)	55.53 ± 4.48	--
Copper (Cu)	46.25 ± 3.51	--
Arsenic (As)	5.57 ± 0.68	2.0
Cadmium (Cd)	0.39 ± 0.07	1.0
Mercury (Hg)	0.33 ± 0.07	0.2
Lead (Pb)	2.29 ± 0.37	5.0

No sub-item: heavy metal limit benchmark specification ≦ 20 ppm.

4. Discussion

Retinal photoreceptor cells in the eye can generate a large amount of free radicals when they convert light into nerve potential. The overproduction of free radicals in the eye can exacerbate damage to the human retina. As a result, oxidative stress can cause a significant increase in related retinal diseases, such as retinitis pigmentosa, age-related macular degeneration, and glaucoma [10]. Our results using the DPPH radical scavenging assay showed that bat feces had good antioxidant capacity. Therefore, we believe that bat feces may alleviate retinal damage through antioxidant stress. In future research, more methods should be used to comprehensively evaluate the antioxidant activity of bat feces.

Compared with other mammals, bats have the most variable dietary strategies, as they can be insectivores, carnivores, or various frugivores. This variability in dietary strategies may affect the composition of bat feces. A previous study suggested that bat feces mainly contain urea, uric acid, cholesterol, a small amount of vitamin A, and heavy metals, such as zinc, manganese, and copper [11]. Our results did not show the presence of vitamin A in bat feces (Figure 2; Table 7). Vitamins are essential for life-sustaining functions, including enzymes that promote fat and carbohydrate metabolism, and have direct and indirect antioxidant properties. Except for vitamin D, animal bodies cannot produce sufficient amounts of vitamins; therefore, most vitamins must be obtained from the diet [12]. Furthermore, most vitamins obtained from the diet are mainly absorbed from

the proximal small intestine and dietary vitamins should, therefore, not reach the distal intestine and feces; thus, it is reasonable that there is no or very little vitamin A in bat feces.

Bat feces have been used as TCM in the past to alleviate eye-related diseases. When humans ingest bat feces and enter the body system, most of their contents should be excreted as human feces. The part of the contents of bat feces that can be absorbed by the human gastrointestinal system should mainly be vitamins. Our results showed that bat feces contain a small amount of vitamin B complex (Figure 4, Table 7). Why do bat feces contain significant amounts of B vitamins? The animal body requires minuscule amounts of B vitamins, which cannot be obtained from the diet. It is reported that B vitamins can be self-synthesized by the intestinal microbiota [13]. Furthermore, many B vitamins have been shown to be self-synthesized in the human body [14]. B vitamins in the distal intestine also play a crucial role in maintaining intestinal microbiota homeostasis and host health through multiple mechanisms. Vitamin B1 is currently widely used to treat neuropathic pain [15], and past studies have shown that food supplements containing vitamin B1 can improve dry eye symptoms in glaucoma patients [16,17]. Vitamin B2 can act as an antioxidant in the body, and its deficiency can cause visual disorders, such as conjunctivitis and cataracts [18]. Oral vitamin B3 has a protective effect in treating or preventing glaucoma and other aging-related neurodegenerative diseases [19]. B vitamins are known to prevent age-related macular degeneration (AMD), and a high-dose vitamin B5 intake can help to reduce AMD and alleviate vision loss caused by AMD [20]. Although these B vitamins may help to maintain the health of the optic nerve and cornea, their content in bat feces is not high. Whether there are other components of bat feces that can protect the human retina through antioxidant capacity requires further exploration.

The use of bat feces as TCM is still a controversial issue. Bat feces may be effective vectors and natural reservoirs for many infectious viruses, bacteria, and fungi. Bats are known to be natural hosts of many zoonotic viruses and may be responsible for numerous outbreaks, including the ongoing COVID-19 pandemic [21]. In addition, bat feces are optimal substrates for the propagation and spread of fungi, including pathogenic histoplasmosis and fatal cryptococcosis [22]. Our results also showed that the heavy metal content in bat feces, determined by ICP-MS analysis, exceeded the limitation standards, which may also cause harm to human health. There are many disadvantages to the use of bat feces as TCM materials. For example, the chemical composition and content of bat feces are not fixed, and they can be affected by many factors, such as diet, health status, age, food contamination, and disease. When the composition and content of bat feces are uncertain, their clinical efficacy is difficult to determine. Additionally, bat feces do not meet sanitary standards. Epidemiological surveys in the past have shown that the occurrence of diseases such as hepatitis, enteritis, dysentery, cholera, and parasites is closely related to feces-based TCM materials.

5. Conclusions

Our results indicate that bat feces have good antioxidant capacity that may be partly due to them containing the vitamin B complex, but the content of these vitamins is still quite low. Whether there are other reasons for bat feces to produce good antioxidant capacity needs further exploration. Although bat feces have been used as TCM in the past to alleviate eye-related diseases, the possible mechanism by which bat feces alleviate retinal damage in humans still requires further study. On the other hand, our research results found that most bat feces contain excessive levels of harmful heavy metals. Therefore, we suggest that the use of bat feces as TCM to improve vision should involve the strict checking of their contents and harmful substances.

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References

1. Jin, R.; Zhang, B.; Xue, C.M.; Liu, S.M.; Zhao, Q.; Li, K. [Classification of 365 Chinese medicines in Shennong's Materia Medica Classic based on a semi-supervised incremental clustering method]. *Zhong Xi Yi Jie He Xue Bao* **2011**, *9*, 665–674. (In Chinese) [CrossRef] [PubMed]
2. Li, S.Z. *Compendium of Materia Medica: Bencao Gangmu*; Luo, X.W., Ed.; Beijing Foreign Languages Press: Beijing, China, 2003; ISBN 7119032607.
3. Wu, Y. *Rihuazi Materia Medica*; Anhui Science and Technology Press: Hefei, China, 2005; ISBN 13:9787533732455.
4. Dierenfeld, E.S.; Seyjagat, J. Plasma fat-soluble vitamin and mineral concentrations in relation to diet in captive pteropodid bats. *J. Zoo Wildl. Med.* **2000**, *31*, 315–321. [PubMed]
5. Boyles, J.G.; Cryan, P.M.; McCracken, G.F.; Kunz, T.H. Conservation. Economic importance of bats in agriculture. *Science* **2011**, *332*, 41–42. [CrossRef] [PubMed]
6. Russo, D.; Bosso, L.; Ancillotto, L. Novel perspectives on bat insectivory highlight the value of this ecosystem service in farmland: Research frontiers and management implications. *Agric. Ecosyst. Environ.* **2018**, *266*, 31–38. [CrossRef]
7. Emerson, J.K.; Roark, A.M. Composition of guano produced by frugivorous, sanguivorous, and insectivorous bats. *Acta Chiropterol.* **2007**, *9*, 261–267. [CrossRef]
8. Banskar, S.; Mourya, D.T.; Shouche, Y.S. Bacterial diversity indicates dietary overlap among bats of different feeding habits. *Microb. Res.* **2016**, *182*, 99–108. [CrossRef] [PubMed]
9. Du, H.; Kuang, T.T.; Qiu, S.; Xu, T.; Gang Huan, C.L.; Fan, G.; Zhang, Y. Fecal medicines used in traditional medical system of China: A systematic review of their names, original species, traditional uses, and modern investigations. *Chin. Med.* **2019**, *14*, 31. [CrossRef] [PubMed]
10. Zhang, S.M.; Fan, B.; Li, Y.L.; Zuo, Z.Y.; Li, G.Y. Oxidative Stress-Involved Mitophagy of Retinal Pigment Epithelium and Retinal Degenerative Diseases. *Cell Mol. Neurobiol.* **2023**, *43*, 3265–3276. [CrossRef] [PubMed]
11. Gao, A.; Gong, J.; Zheng, H.; Jia, X.; Wang, M.; Wu, Y.; Zhang, X.; Chen, G.; Ni, S. Research on the traditional Chinese medicine bat feces. *Jilin J. Tradit. Chin. Med.* **2012**, *32*, 1047–1049.
12. Steinert, R.E.; Lee, Y.K.; Sybesma, W. Vitamins for the Gut Microbiome. *Trends Mol. Med.* **2020**, *26*, 137–140. [CrossRef]
13. Xia, Y.; Ji, C.; Li, M.; Zhang, W.; Cheng, X.; Qiu, Y.; Ge, W. Simultaneous quantification of seven B vitamins in human feces by stable isotope label-based high-performance liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2024**, *237*, 115784. [CrossRef] [PubMed]
14. Magnúsdóttir, S.; Ravcheev, D.; de Crécy-Lagard, V.; Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Front. Genet.* **2015**, *6*, 148. [CrossRef] [PubMed]
15. Alemanno, F.; Ghisi, D.; Westermann, B.; Bettoni, A.; Fanelli, A.; La Colla, L.; Danelli, G.; Cesana, B.M. The use of vitamin B1 as a perineural adjuvant to middle interscalene block for postoperative analgesia after shoulder surgery. *Acta Biomed.* **2016**, *87*, 22–27. [PubMed]
16. Nebbioso, M.; Rusciano, D.; Pucci, B.; Zicari, A.M.; Grenga, R.; Pescocolido, N. Treat-ment of glaucomatous patients by means of food supplement to reduce the ocular dis-comfort: A double blind randomized trial. *Eur. Rev. Med. Pharmacol. Sci.* **2013**, *17*, 1117–1122. [PubMed]
17. Ren, X.; Chou, Y.; Wang, Y.; Jing, D.; Chen, Y.; Li, X. The Utility of Oral Vitamin B1 and Mecobalamin to Improve Corneal Nerves in Dry Eye Disease: An In Vivo Confocal Microscopy Study. *Nutrients* **2022**, *14*, 3750. [CrossRef]
18. Pereira, A.; Adekunle, R.D.; Zaman, M.; Wan, M.J. Association Between Vitamin Deficiencies and Ophthalmological Conditions. *Clin. Ophthalmol.* **2023**, *17*, 2045–2062. [CrossRef] [PubMed]
19. Williams, P.A.; Harder, J.M.; Foxworth, N.E.; Cochran, K.E.; Philip, V.M.; Porciatti, V.; Smithies, O.; John, S.W. Vitamin B3 modulates mitochondrial vulnerability and prevents glaucoma in aged mice. *Science* **2017**, *355*, 756–760. [CrossRef] [PubMed]
20. Merle, B.M.J.; Barthes, S.; Féart, C.; Cougnard-Grégoire, A.; Korobelnik, J.F.; Rougier, M.B.; Delyfer, M.N.; Delcourt, C. B Vitamins and Incidence of Advanced Age-Related Macular Degeneration: The Alienor Study. *Nutrients* **2022**, *14*, 2821. [CrossRef]

21. Das, T.; Sikdar, S.; Chowdhury, M.H.U.; Nyma, K.J.; Adnan, M. SARS-CoV-2 prevalence in domestic and wildlife animals: A genomic and docking based structural comprehensive review. *Heliyon* **2023**, *9*, e19345. [CrossRef] [PubMed]
22. Gerbáčová, K.; Maliničová, L.; Kisková, J.; Maslišová, V.; Uhrin, M.; Pristaš, P. The Faecal Microbiome of Building-Dwelling Insectivorous Bats (*Myotis myotis* and *Rhinolophus hipposideros*) also Contains Antibiotic-Resistant Bacterial Representatives. *Curr. Microbiol.* **2020**, *77*, 2333–2344. [CrossRef]