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The Possibility of Using Bee Drone Brood to Design Novel Dietary Supplements for Apitherapy

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Abstract: Drone brood is a little-known bee product, often treated as beekeeping waste or natural varroosis bait. Obtaining drone brood from beehives does not weaken the bee family, which is why this product is used as natural medicine in Eastern European countries. The main objective of this work was to design an innovative dietary supplement containing freeze-dried drone brood (DB) enriched with calcium ions (3:1). As the calcium component, inorganic calcium carbonate (CC) and ground chicken eggshells (ES) were used. Bioaccessibility of hormones, selected nutrients (proteins and amino acids), non-nutritive polyphenols from pure drone brood (DB), and designed supplements (DB + CC, DB + ES) were analyzed using an in vitro gastrointestinal system. It was shown that drone brood components are better bioaccessible from the DB + ES compared to DB + CC and DB capsules. An increase was achieved by up to 93.33%, 21.29%, 105.14%, and 52.34% for testosterone, estradiol, calcium, and polyphenols, respectively. Drone brood proteins were completely digested to free amino acids which was confirmed by SDS-PAGE electrophoresis and high-performance thin layer chromatography (HPTLC). Due to the demonstrated synergistic action of drone brood and the calcium of eggshells, the newly proposed two-ingredient supplement seems to be an efficient treatment to equalize hormonal and calcium deficiency in osteoporosis; however, its application requires further studies.

Keywords: drone brood; testosterone; protein; polyphenols; bioaccessibility

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1. Introduction

Bee drone brood is a rarely used bee product, which is characterized by a rich chemical composition, which makes it biologically active. It contains approximately 40% protein, 30% reducing sugars, but also hormones, mainly sex hormones: testosterone (0.29–0.31 nmol/100 g), estradiol (431.2–847.90 nmol/100 g), progesterone (42.6–51.3 nmol/100 g), and prolactin (344.6–475.4 nmol/100 g) [1]. Additionally, it is also a good source of vitamins and bioelements, mainly phosphorus, potassium, calcium, iron, and zinc. Freeze-dried drone brood (named Apilarnil or Apistimul) is available for sale mainly in Eastern European countries as a dietary supplement or one of its ingredients. Thanks to preservation by freeze-drying, it can be stored for up to two years [2]. The rich chemical composition and confirmed biological activity of drone brood could indicate its potential use to equalize the hormonal system in people of both sexes [3]. Due to this, drone brood is applied in the treatment and prevention of various diseases, including osteoporosis and male infertility, hypothyroidism, liver diseases, psychiatry, adaptogenic therapies, as well as in malnutrition in the treatment of children [4–9]. However, there is a lack of scientific data confirming the effectiveness of drone brood in vivo, and the in vitro bioaccessibility of its components is unknown.

Osteoporosis is a global problem that affects people of both sexes, more often women than men, and the incidence of its occurrence clearly increases in people over 60 years of age [10,11]. Sex hormone deficiency has adverse effects on bone growth and modeling,

ultimately reducing peak bone mass and setting the stage for osteoporosis in later life. This disorder is characterized by the thinning of bone tissue, which results in low bone mass and a disturbed bone structure, increasing the likelihood of bone fractures [12,13]. A properly balanced diet plays a very important role in the prevention of osteoporosis and can reduce age-related bone loss and fracture risk. The crucial issue is proper calcium (1000–1200 mg/day) and vitamin D (800–1200 IU/day) supplementation [13,14]. However, a greater benefit is achieved when calcium is introduced into the diet in small proportions, avoiding oscillator spikes that could lead to cardiovascular complications [15]. Recently, plant-derived phytoestrogens with estrogenic effects (e.g., genistein, daidzein, icariin, dioscin, and *Ginkgo biloba* extracts) were also proposed as agents for prophylactics of steroid-induced osteoporosis, which could provide a safer alternative to primary pharmacological strategies [16–18].

Recently, shells of chicken eggs have been proposed as a natural source of calcium in the prevention and treatment of osteoporosis [19–21]. This natural product, which is bakery waste, is an interesting alternative to the currently used inorganic forms of calcium. Moreover, eggshells are an inexpensive calcium source and are accessible at home. Chicken eggshells contain about 95% calcium carbonate and 3.5% of glycoproteins and proteoglycans; therefore, they could be a valuable natural source of calcium with a higher solubility compared to the oyster shells that are currently used [22]. Additionally, the inner membrane of the eggshell contains glucosamine, chondroitin sulphate, hyaluronic acid, type I collagen, and a large number of microelements such as magnesium, strontium, zinc, barium, and fluorine, making them an excellent biomaterial for the production of new dietary supplements that positively affect bone metabolism [22–25]. Furthermore, a better calcium absorption from eggshells was found than from calcium carbonate, due to its porous structure which facilitates digestion [26,27].

The aim of this study was to combine beneficial compounds of drone brood (hormones, proteins, antioxidants, and bioelements) and hen eggshells to design a new natural dietary supplement with enhanced bioaccessibility tested in vitro. The use of drone brood for the production of dietary supplements could broaden the range of products offered by beekeeping and increase beekeepers' income.

2. Materials and Methods

2.1. Chemicals

Chemicals: 1-propanol, ninhydrin, ethyl acetate, methanol, formic acid NP reagent, PEG 400, 1-butanol, 2-propanol, boric acid, aniline-diphenylamine-phosphoric acid reagent. Standards: valine, histidine, leucine, glycine, aspartic acid, proline, lysine, glutamic acid, ferulic acid, ellagic acid, vanillin, kaempferol, apigenin 7-glucoside, Laemmli Buffer, Coomassie Brilliant Blue G-250, HCl, NaCl, $NaHCO_3$, pepsin from porcine gastric mucosa and NIST1515 certified reference material were obtained from Sigma Aldrich (St. Louis, MO, USA), Lipancrea from Polfa (Warszawa, Poland), the Elisa Test Kit for testosterone (abx574314) and estradiol (abx574169) from Abbexa (Cambridge, UK), the Calcium Assay Kit ab102505 from Abcam (Cambrige, UK). Bradford reagent: Bio-Rad Protein Assay Dye Reagent Concentration (Hercules, CA, USA), ROTI®Mark BI-PINK (Carl Roth GmbH, Karlsruhe, Germany).

2.2. Drone Brood Collection

Drone brood (7-day-old) samples were collected from one apiary in the southeastern part of Poland (Podkarpackie Voivodeship) in June 2022. The drone brood (total 200 g) of *Apis mellifera carnica* was manually selected from the one-half drone frame, immediately sealed in sterile containers, and transferred to the laboratory. The samples were homogenized using a tissue homogenizer with 7 mm plastic Omni Tips TM (TH 02, Omni International, Kennesaw, GA, USA). The material was frozen at −70 °C and then freeze-dried (using Alpha 1–2, LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode,

Germany). Dehydration was carried out for 72 h by cooling the sample to −55 °C at a standard pressure of 0.1 bar. Lyophilizate was used to prepare the capsules.

2.3. Chicken Eggshell Collection and Processing

For this study, organic (n = 30) and farm cage (n = 30) hen eggs were collected. The shells were separated from the eggs, and the membrane was additionally separated from the shells. First, the microbiological purity of fresh eggshells was verified. The shells were washed with distilled water (10 mL) to obtain an assay solution, which was then applied to a 2% agar medium (0.1 mL of each sample). The samples were incubated in an incubator at 37 °C for 24 h. After this time, the colonies grown on the plates were evaluated. The colony smear was observed under a microscope (Delta Optical Genetic Pro, Mińsk Mazowiecki, Poland) to determine the types of bacteria. The shells were heated for 30 min at 100 °C, cooled, and then ground in an electric mill (MMK-06M, Milanówek, Poland). The eggshell powder was again controlled in terms of microbiological quality. Briefly, 1 g of the appropriate thermally treated powder was suspended in 10 mL of distilled water. The suspension was analyzed as described above. The obtained eggshell powder was stored tightly closed in a desiccator under strictly controlled environmental conditions until analysis.

2.4. Preparation of a Dietary Supplement Based on Drone Brood and Eggshell Powder

To prepare the supplement, gelatin shells (0.90 mg each) purchased in one of the local stores were used. The composition of the prepared versions of the supplements (DB + ES and DB + CC) is shown in Table 1. For comparison, capsules containing single components in the doses used were also prepared (ES, CC, and DB).

Table 1. The composition of prepared dietary supplements.

Supplement	Code	Drone Brood	Eggshell of Organic Eggs	Calcium Carbonate
Organic Ca	ES	-	250 mg (=95 mg Ca)	-
Inorganic Ca	CC	-	-	250 mg (=100 mg Ca)
Hormones	DB	300 mg =0.0024 nmol testosterone =1.99 nmol estradiol	-	-
Hormones + Organic Ca (3.2:1)	DB+ES	300 mg	250 mg	-
Hormones + Inorganic Ca (3:1)	DB+CC	300 mg	-	250 mg

Each of the given variants was filled in one gelatin capsule (90 mg each) using a manual encapsulator (Capsule Connection, LCC, Prescott, AZ, USA). Three capsules were prepared in each variant.

2.5. Analysis of the Mineral Composition of Eggshells Using the ICP-OES

The content of selected minerals in the hen eggshells was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Thermo iCAP 6500 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The samples were subjected to a mineralization process with nitric acid (temp. 200 °C) (Millestone Ethos One, Łomianki, Warsaw, Poland). The detection limit for each element was set at a level greater than 1 µg/L. The curve fit coefficients (R^2) for all elements tested were above 0.99. All analyses were performed in three independent replicates for each sample. The repeatability of the target was expressed as a relative standard deviation (RSD), and the recoveries of the target were 92–106%, respectively. The method has been validated using a certified reference material (NIST1515). The response of the equipment was periodically checked against known standards. To identify the appropriate measurement lines and avoid possible interference, the method of adding an internal standard was used. Yttrium and ytterbium ions at concentrations of 2 mg/L and 5 mg/L were used as internal standards.

2.6. Calcium Assay Test

For the evaluation of soluble calcium content, a colorimetric Calcium Assay Kit (ab102505) was used. To 50 µL of the analyzed sample, 90 µL of a chromogenic reagent was added. Then, 60 µL of a calcium assay buffer was added. The samples were mixed and incubated at room temperature for 10 min and protected from light. Absorbance was measured on a microplate reader at a wavelength of 575 nm. The results were calculated according to the equation of the calibration curve prepared for calcium standards attached to the kit, within a concentration range of 0.0625–2 µg/sample ($y = 0.8432x$, $R^2 = 0.9985$).

2.7. In Vitro Bioaccessibility of the Supplement Components

The study of the bioaccessibility of the designed supplement components was carried out according to the instructions of Vitali et al. [28] with minor modifications. Each capsule variant was analyzed in duplicate. As the digested mixture, 1 capsule each of the prepared variants was used. A total of 10.5 mL of distilled water was added and heated at 37 °C for 10 min in a laboratory incubator to dissolve the gelatin shell. After this time, a control sample (not digested) was taken. The samples were dispersed with 0.5 mL of pepsin in gastric juice (0.5 mg/mL of pepsin in 0.1 M HCl; adjusted to pH 2 with 5 M HCl) and incubated in a lab incubator for 1 h at 37 °C; every 15 min the samples were shaken for 2 min. After neutralization (1 M $NaHCO_3$), the gastric (stomach) fraction was taken. The samples were supplemented with simulated intestinal juice (bile: pancreatin, 12:2 g/L), NaCl: KCl (120:5 mmol/L) and incubated for the next 2 h under the same conditions as described above. The digested samples were then centrifuged at 4100× *g* for 20 min, and selected parameters were determined in the obtained supernatants. Until further analysis, the samples were stored at −20 °C. In the case of the ES and CC capsules, only calcium and polyphenol content was measured, whereas in the case of the DB, DB + ES and DB + CC samples, the evaluated compounds included calcium, testosterone, estradiol, protein and polyphenolic compounds' content, as well as amino acids' profile, and FRAP-reducing power.

The bioaccessibility index BI [%] for calcium, hormones, polyphenols, and antioxidants was calculated according to the following equation:

$$BI\ [\%] = (Bdigested/Bundigested) \times 100$$

where BI [%]—bioaccessibility of bioactive compounds; Bdigested—the concentration of bioactive compounds in digested (gastric/intestinal) phase; Bundigested—the concentration of the bioactive compound in the nondigested phase.

The soluble protein digestibility was calculated using the same formula. The percentage calculated in gastric and intestinal fractions was assumed to correspond to undigested proteins.

2.8. Soluble Protein Assay

The protein content in the samples was determined by the Bradford method according to Sidor et al. [29]. The absorbance was read at 595 nm using a microplate reader (EPOCH 2, BioTek, Winooski, VT, USA). The results were calculated based on a calibration curve 1.8–250 µg/per sample ($y = 0.0424x$, $R^2 = 0.9658$). Bovine albumin was used as a standard.

2.9. Protein Profiling by SDS-PAGE

For the determination of proteins in supplement fractions after in vitro digestion, 30 µL of each sample was heated for 5 min at 100 °C with 15 µL of a standard Laemmli buffer immediately before application. After cooling, denatured samples (20 µL of each) were applied to a 12.5% denaturing gel (with 5% stacking gel). Electrophoresis was carried out initially at 50 V (15 min) and then at 150 V for 2 h using Bio-Rad PowerPac 3000 (Bio-Rad Laboratories, Hercules, CA, USA). The prestained ROTI® Mark BI-PINK (Carl Roth GmbH, Karlsruhe, Germany) was used as a molecular weight marker. After electrophoresis, the gel was stained with colloidal Coomassie Brilliant Blue G-250 overnight and then washed for 24 h with a decolorizing solution consisting of ethanol, acetic acid, and water in order

to remove the dye. The gel was scanned and processed with Epson Perfection V850 Pro (Simatupang, Jakarta Selatan, Indonesia).

2.10. Hormonal Activity Determination

Estradiol and testosterone in the undigested (10% suspension) and digested samples were tested with the use of immunoenzymatic ELISA test kits (abx574314, abx574169 for testosterone and estradiol, respectively), strictly according to the manufacturer's manual (Abbexa, Cambridge, UK). The absorbance was immediately measured at 450 nm and recalculated according to the prepared calibration curve in pg/mL for testosterone, $y = -0.408\ln(x) + 2.968$, ($R^2 = 0.945$) and for estradiol, $y = -0.644\ln(x) + 4.977$, ($R^2 = 0.914$). The results were expressed in nmol/capsule [1].

2.11. Antioxidants Assay

2.11.1. FRAP Test

The FRAP (Ferric Reducing Antioxidant Power) test was performed according to Sidor et al. [29]. The results were expressed as equivalent Trolox umol (TE) per one capsule (µmol/capsule) from the Trolox calibration curve, prepared for Trolox in the range 25–300 nmol TE/mL ($y = 0.152x$, $R^2 = 0.9989$).

2.11.2. Total Phenolic Content (TPC) Assay

The total content of phenolic compounds was determined using the Folin–Ciocalteu reagent according to Sidor et al. [29] and adapted to use microplate analyses. The results were calculated based on a calibration curve prepared for gallic acid in the range 0–125 µg GAE/mL ($y = 0.336x$, $R^2 = 0.9914$).

2.12. HPTLC Analysis

A comparative analysis of free amino acids and polyphenolics for undigested and digested fractions of supplements were performed on HPTLC Silica Gel 60 F254 plates (20 × 10 cm) purchased from Merck (Darmstadt, Germany). The selected fraction extracts were applied to the plate (10 µL for polyphenols, amino acids were analyzed applying samples in volumes corresponding to the same protein concentration) as 8 mm bands from the lower edge of a plate at the rate of 150 nL/s using a semiautomated HPTLC application device (Linomat 5, CAMAG, Muttenz, Switzerland).

A chromatographic separation was performed in a chromatographic tank saturated for 20 min with the appropriate mobile phase (Table 2) and developed at a distance of 70 mm. The results obtained were documented using an HPTLC imaging device (TLC Visualizer, CAMAG) under white light (for amino acids and sugars) with a total of 366 nm (for polyphenols). In addition, each plate was derivatized using an automated derivatizer of TLC plates (CAMAG Derivatizer). The obtained chromatographic images were analyzed using the HPTLC software (Vision CATS, CAMAG).

Table 2. Details of separations performed on HPTLC Silica gel 60 F_{254}.

Identified Group	Mobile Phase (v:v:v)	Derivatization Reagent	Used Standards
Amino acids	1-propanol, H_2O (7:3)	ninhydrin	valine, histidine, leucine, glycine, aspartic acid, proline, lysine, glutamic acid
Polyphenols	ethyl acetate, methanol, water, formic acid (50:4:4:2.5)	NP reagent, PEG 400	ferulic acid, ellagic acid, vanillin, kaempferol, apigenin 7-glucoside

2.13. Statistical Calculations

All calculations were made in triplicate. For the data obtained, mean values and standard deviations were calculated. Significant differences were calculated by one-way ANOVA followed by Tukey's test ($p = 0.05$), differences compared to the undigested samples were checked with Student's t-Test ($p = 0.05$), and standard deviations for the obtained results were calculated. All calculations were made using the Statistica 13.3 software (StatSoft, Tulsa, OK, USA).

3. Results and Discussion

Two natural raw materials were used to produce the natural dietary supplement: freeze-dried drone brood and ground chicken eggshells, which were mixed in a fixed proportion and sealed in gelatin shells purchased in one of the local stores. At the beginning, the quality of the raw materials was checked, and the drone brood was selected based on previous research [29]. As a source of natural calcium, the use of two raw materials was considered: eggshells from organic and farmed eggs. The selection of the material was preceded by a comparative analysis of the chemical content and evaluation of the microbiological purity of the shells.

3.1. Eggshell Quality Analysis

The quality of eggshells as a potential natural source of bioavailable calcium was first evaluated regarding its mineral content and microbial contamination. Table 3 summarizes the results of the mineral profile of powder from hen eggshells obtained from various hen rearing systems. The main bioelement present in eggshell powder was found to be calcium (31.9 g/100 g on average) in the form of calcium carbonate, which constitutes 96% of the mineral composition. This element was accompanied by significant amounts of magnesium (average 483.1 mg/100 g), sulfur (average 446.99 mg/100 g), sodium (96.12 mg/100 g), and potassium (average 52.45 mg/100 g). Additionally, eggshells have been shown to contain trace amounts of the essential elements, chromium and copper (values in the range of 0.04–0.3 mg/100 g), and only traces of cadmium and arsenic were found, as well as lead and nickel. Furthermore, the effect of the hen rearing system was found negligible ($p < 0.05$). The results presented are comparable to those reported by Brun et al. [20] who showed that eggshells are essentially composed of calcium (382 mg/g), sodium (5.1 mg/g), phosphorus (4.4 mg/g), and potassium (1.4 mg/g). Furthermore, Bartter et al. [24] showed a similar level of calcium in eggshells, in the range of 360 to 400 mg/g of eggshell.

Although microbiological contamination was detected for 80% of the fresh shells tested, numerous (uncountable) colonies were found for 15% of the samples only (Figure S1). Surprisingly, the caged eggs were more contaminated than the organic ones. Rod-type bacteria and streptococci were identified mainly in the microscopic image (Figure S2). The microbiological analysis of the heat-treated powders did not show any grown colonies in both caged and farm egg powders.

3.2. Supplement Design

Supplements based on drone brood and chicken eggshell powder have been prepared on the basis of recommendations that clearly declare the daily need for each component separately. For drone brood, a safe daily intake of 70 to 900 mg of drone brood per day was suggested [2]. The average daily calcium intake from both foods and supplements is 1156 mg for men and 1009 mg for women [30]. Assuming the dosage of the designed supplement is two capsules a day, the recommended daily dose will be filled by an adult, on average, in 66% for drone brood (max dose) and 50% for calcium (1000 mg on average).

Table 3. The mineral content of the powdered shells of the eggs of various hen rearing systems.

	Cage Rearing [mg/100 g d.m. ± SD]	Organic Farming [mg/100 g d.m. ± SD]	Average Content [mg/100 g d.m. ± SD]	Percentage [%]
Al	0.12 ± 0.29 [a]	0.48 ± 0.30 [b]	0.30 ± 0.11	0.0009
As	0.04 ± 0.07 [a]	0.03 ± 0.04 [a]	0.04 ± 0.009	0.0001
Ca	32,362.79 ± 503.94 [a]	31,417.52 ± 678.54 [a]	31,890.15 ± 473.06	96.15
Cd	0.002 ± 0.004 [a]	0.002 ± 0.001 [a]	0.002 ± 0.00	0.0001
Cr	0.12 ± 0.08 [b]	0.004 ± 0.008 [a]	0.08 ± 0.00	0.0002
Cu	0.13 ± 0.03 [b]	0.05 ± 0.030 [a]	0.09 ± 0.02	0.0002
Fe	4.30 ± 1.69 [a]	4.86 ± 1.33 [a]	4.58 ± 1.33	0.14
K	48.24 ± 0.75 [a]	56.67 ± 1.40 [a]	52.45 ± 2.75	0.16
Mg	508.73 ± 4.95 [b]	457.49 ± 8.15 [a]	483.11 ± 3.60	1.45
Mn	0.20 ± 0.09 [a]	0.21 ± 0.103 [a]	0.20 ± 0.06	0.0006
Mo	n.d.	n.d.	n.d.	n.d.
Na	87.26 ± 0.54 [a]	104.99 ± 1.52 [b]	96.12 ± 0.54	0.02
Ni	n.d.	n.d.	n.d.	n.d.
P	194.49 ± 2.83 [b]	139.68 ± 4.35 [a]	167.08 ± 2.83	0.57
Pb	n.d.	n.d.	n.d.	n.d.
S	445.83 ± 3.14 [a]	448.15 ± 12.47 [a]	446.99 ± 3.14	1.34
Sr	20.93 ± 0.17 [a]	32.15 ± 1.31 [b]	23.28 ± 1.31	0.07
Zn	0.48 ± 0.53 [a]	0.44 ± 0.02 [a]	0.44 ± 0.01	0.001
TOTAL	33,673.82	32,662.23	33,164.91	100%

d.m.—dry mass; n.d.—not detected; [a,b]—the means marked with different letters in the rows differ statistically significantly.

3.3. In Vitro Digestion Study

The development of an in vitro model to predict bioaccessibility should include digestive transformations of food into material ready for assimilation and absorption/assimilation steps that predict absorption into the intestinal epithelial cells [31]. Bioavailability can be defined as the amount or fraction of components that are released from food ingested in the gastrointestinal tract and become available for absorption in the intestine [31]. In the laboratory, bioaccessibility assessment involves the digestion of food using selected procedures, which simulate gastric and small intestinal digestion [32]. The bioaccessibility of components of the designed supplement (DB + ES), including calcium, hormones, proteins, polyphenols, and reducing power, was evaluated, and the results are summarized in Table 4. For completeness, digestion was performed under identical conditions for the inorganic calcium supplement variant (DB + CC), single components (DB, ES and CC), and capsule shells alone (Table 4). The release of individual ingredient groups in the simulated gastrointestinal tract has been considered separately and is described below (Figure 1). The comparison of the calculated values of the bioaccessibility index (BI%) for both variants of the supplement (DB + ES and DB + CC) compared to pure brood (DB) are shown in Table 4.

Table 4. Comparison of the bioaccessibility of protein, calcium, testosterone, estradiol, polyphenolic compounds, and antioxidants in the tested fractions.

Sample	Tested Fraction before/ after Digestion	Proteins [mg/Capsule ± SD]	Calcium [mg/Capsule ± SD]	Testosterone [nmol/Capsule ± SD]	Estradiol [nmol/Capsule ± SD]	TPC [mgGAE/Capsule ± SD]	FRAP [µmolTE/Capsule ± SD]
ES (250 mg/capsule)	Undigested	6.09 ± 2.13	78.7 ± 15.52	n.t.	n.t.	n.d.	n.d.
	Gastric phase	4.22 ± 0.67 *	72.45 ± 0.37	n.t.	n.t.	n.d.	n.d.
	Intestinal phase	0.88 ± 1.53 *	82.60 ± 1.24	n.t.	n.t.	n.d.	n.d.
CC (250 mg/capsule)	Undigested	n.t	100.00 ± 17.102	n.t.	n.t.	n.d.	n.d.
	Gastric phase	n.t.	9.20 ± 0.12 *	n.t.	n.t.	n.d.	n.d.
	Intestinal phase	n.t.	10.28 ± 0.04 *	n.t.	n.t.	n.d.	n.d.
DB (300 mg/capsule)	Undigested	114.22 ± 3.84	0.10 ± 0.02	0.011 ± 0.00	1.28 ± 0.07	2.28 ± 0.30	8.37 ± 0.56
	Gastric phase	18.18 ± 2.63 *	0.29 ± 0.03 *	0.0019 ± 0.00 *	n.d.	1.74 ± 0.35 *	3.52 ± 0.36 *
	Intestinal phase	13.62 ± 2.96 *	0.72 ± 0.10 *	0.0063 ± 0.00 *	0.35 ± 0.02 *	2.12 ± 0.60	4.41 ± 0.56 *
DB + ES (300 mg + 250 mg/capsule)	Undigested	45.19 ± 5.49	43.34 ± 4.12	0.0060 ± 0.00	1.08 ± 0.03	2.56 ± 0.11	3.54 ± 0.06
	Gastric phase	7.62 ± 1.15 *	38.96 ± 1.01 *	0.0025 ± 0.01 *	n.d.	1.56 ± 0.19 *	2.81 ± 0.25 *
	Intestinal phase	2.99 ± 0.36 *	45.57 ± 0.11	0.0098 ± 0.03 *	0.23 ± 0.07 *	1.34 ± 0.30 *	2.88 ± 0.09 *
DB + CC (300 mg + 250 mg/capsule)	Undigested	40.95 ± 2.00	55.05 ± 3.41	0.012 ± 0.0 0	1.11 ± 0.04	1.97 ± 0.08	3.47 ± 0.13
	Gastric phase	22.10 ± 4.25 *	5.55 ± 0.50 *	0.0048 ± 0.03 *	n.d.	1.42 ± 1.40 *	4.21 ± 1.54 *
	Intestinal phase	14.23 ± 10.57 *	6.66 ± 0.13 *	0.0136 ± 0.03	0.30 ± 0.08 *	1.22 ± 1.75 *	3.33 ± 0.60
Control (capsule alone)	Undigested	<0.035	n.t.	n.t.	n.t.	0.05.	n.d.
	Gastric phase	n.d.	n.t.	n.t.	n.t.	n.d.	n.d.
	Intestinal phase	n.d.	n.t.	n.t.	n.t.	n.d.	n.d.

Results marked * differ statistically significantly compared to the undigested fraction within the supplement. n.d.—not detected, n.t.—not tested.

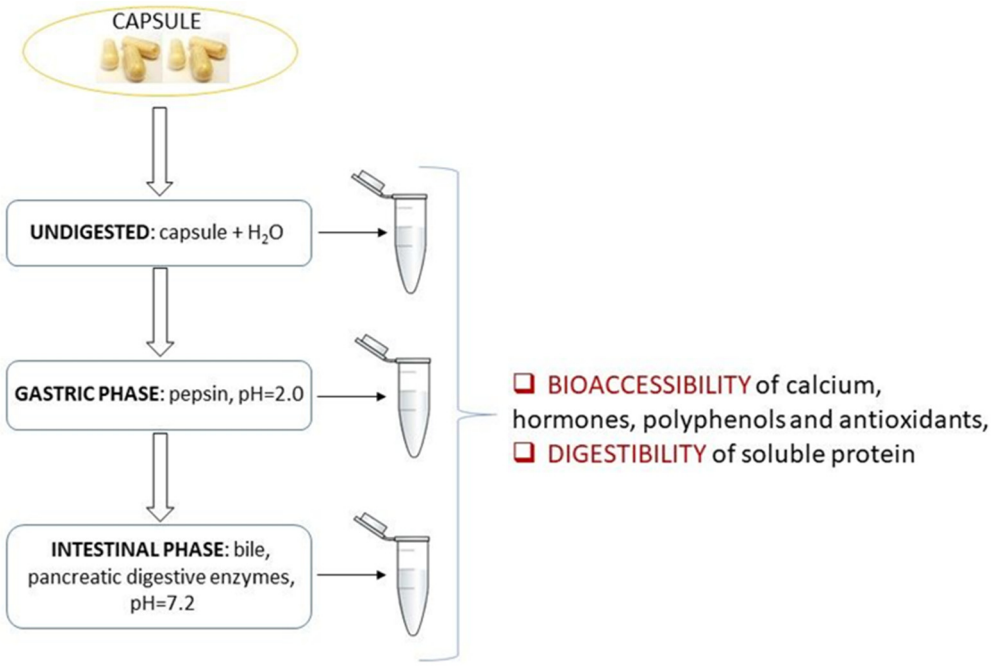


Figure 1. Diagram of the in vitro digestion performed.

3.4. Calcium

The percentage of calcium absorbed from supplements, as with that from foods, depends not only on the source of calcium but also on the total amount of elemental calcium consumed at one time; as the amount increases, the percentage absorbed decreases. Absorption from supplements is highest at doses of 500 mg or less. For example, the body absorbs about 36% of a 300 mg calcium dose and 28% of a 1000 mg dose [30]. During the simulated in vitro digestion of the capsules produced, the ability of calcium available to assimilation in an organism was checked (Table 4). Although a higher amount of calcium was contained in calcium carbonate (CC) than the eggshell capsule (ES) (by 21.3%), the calcium showed a much greater release capacity (92.05% in the stomach and 104.82% in

the intestines) compared to calcium carbonate (9.21% in the stomach and 10.28% in the intestines) (Figure 2b). What is valuable, a high bioaccessibility index of calcium from drone brood (DB) was demonstrated (290% in the stomach and 720% in the intestine). Calcium from DB + ES was released to a much greater extent (89.89% and 105.14% in the stomach and intestines, respectively) compared to DB + CC, where the calcium absorption capacity was only 10.08% in the stomach and 12.09% in the intestines. Kusumi et al. [33] studied the digestibility of calcium supplied in various forms and found that eggshell calcium was significantly more available than synthetic calcium carbonate. This result suggests that eggshell powder dissolves more easily in gastric juice due to its porous structure, making it easier to absorb compared to synthetic calcium compounds. Due to the favorable bioaccessibility of calcium, eggshells have been proposed as an additive to various types of foods, in order to increase the supply of this element, especially in the diet of people exposed to its deficiencies [24,34].

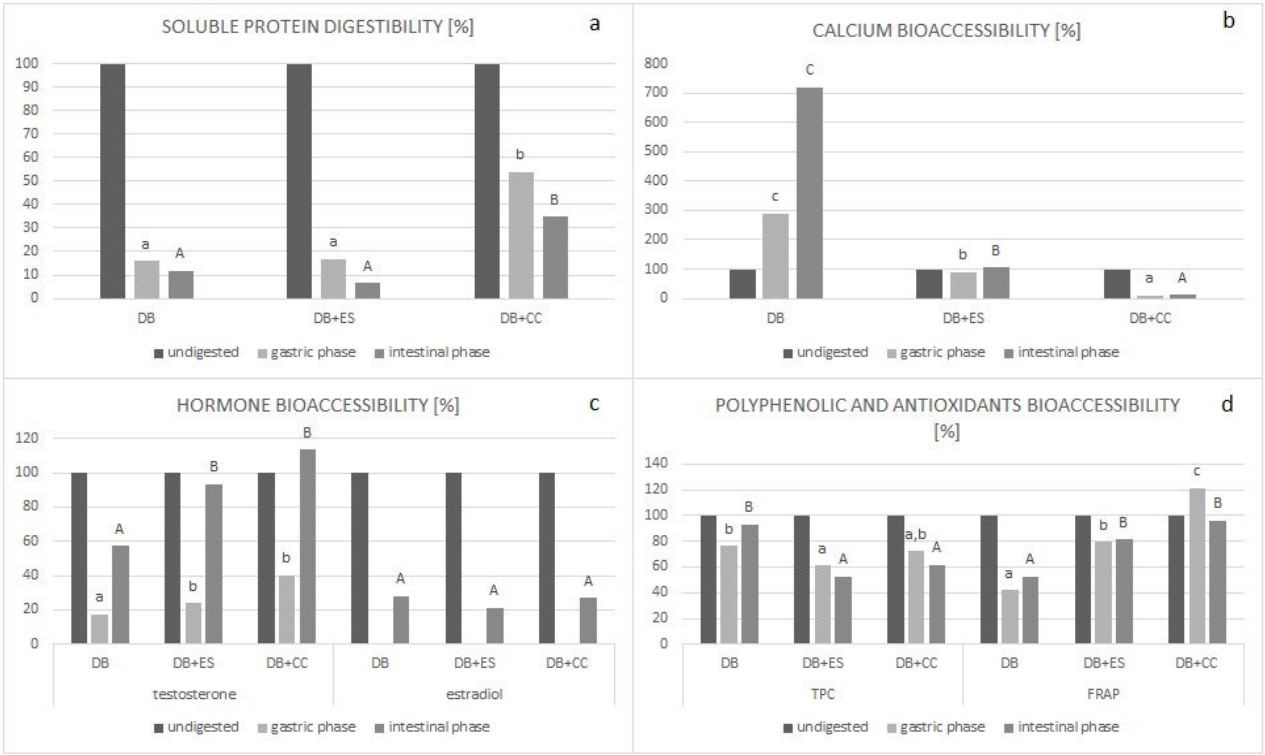


Figure 2. Digestibility index [%] of (**a**) proteins, and bioaccessibility index [%] of (**b**) calcium, (**c**) hormones, (**d**) polyphenols, and the reducing power of the tested capsule fractions. ^{a,b,c}—the results marked with different letters differ statistically significantly between the gastric fractions of the supplements ($p < 0.05$); ^{A,B,C}—the results marked with different letters differ statistically significantly between the intestinal fractions of the supplements ($p < 0.05$).

The effect of eggshell calcium on changes has long been tested in in vitro and in vivo studies. Sakai et al. [26] found that eggshell calcium is more efficient compared to calcium carbonate in increasing bone mass in women, indicating its potential use as a calcium supplement in human nutrition. Omelka et al. [27] evaluated the effects of calcium-rich chicken eggshell powder and the inorganic form of calcium carbonate on osteoporotic bone structure using an animal model of ovariectomized rats. They observed that chicken eggshell powder more effectively ameliorates bone loss in ovariectomized rats than inorganic calcium. Eggshell treatment significantly lowered bone resorption, increased plasma calcium levels, the relative volume of trabecular bones, and secondary osteon population density, which together improved bone strength. As an explanation of observed differences, the authors presented the differences in the bioavailability of calcium carbonate, which is

only around 20–30% [35]. On the other hand, the calcium bioavailability in eggshells can be influenced and increased by the presence of other minerals, important for bone health.

3.5. Steroid Hormones

In studies of the content of sex hormones involved in bone formation, it was found that during capsule digestion, the bioaccessibility of both hormones increased (Table 4). The testosterone in the DB + ES capsule has been shown to have an absorption capacity in the intestine than that contained in the DB capsule. Furthermore, a lower absorption capacity of the hormone analyzed from the DB + CC capsule was found by 50% compared to DB + ES. The greatest ability of testosterone to be released from the matrix was demonstrated in the intestinal phase, where it was emulsified in the presence of bile acids while its bioaccessibility increased (Figure 2c). Testosterone has a very low solubility in water. Preparations with testosterone in the composition show an absolute bioavailability of 4% in the state after a meal. When eating a medium to high fat meal, testosterone in the digestive tract is combined with fat by bile acids. Once emulsified, testosterone is incorporated into chylomicrons and absorbed by the lymph [36]. This may also be related to zinc, which is naturally present in eggshells (0.44 mg/100 g of dry weight) and which facilitates the release and absorption of testosterone [37].

The study of estradiol content showed its presence in the undigested and intestinal fraction. The presence of calcium is not favorable for the release of this hormone; however, the effect was 27.02% higher in the case of DB + CC capsules compared to the DB + ES ones (Table 4). Due to the hydrophobic nature of both hormones, their release in the gastrointestinal tract is similar; they become bioavailable only after emulsification of the digested content, which occurs in contact with bile [37].

Sex steroid hormones play a key role in the development and maintenance of the skeleton in both men and women. Estradiol and testosterone levels, especially the free or bioavailable fractions, decline with increasing age in both men and women and likely contribute to bone loss and fractures [38]. Menopause and the accompanying loss of ovarian estrogens are associated with a decrease in bone mineral density (BMD). During male andropause, testosterone levels decline, knocking down estrogen, thereby reducing any protective benefits and reducing bone rejuvenation and bone mineral density (BMD). By replacing lost hormones, hormone balance can be restored, and bone health can be improved. However, long-term hormone replacement therapy (HRT) is controversial because of increases in the risk of breast carcinoma, endometrial cancer, thromboembolic events, and cardiovascular diseases [39].

Little is known about the impact of drone brood on the body hormonal balance. The latest research [40] confirmed the stimulatory effects of the application of bee drone larvae (BDL) on the beneficial androgenic effects and growth performance of male goats and male kids, but treating the female animals, used for reproductive purposes, provided inconsistent results [41]. Supplementation with DBH stimulated the early stages of folliculogenesis in gilts, but provoked atresia in the last stage of follicular development. However, the results cited confirmed the biological properties of the bee drone brood as an ideal additive for growth promotion in animal husbandry instead of banned hormonal anabolics.

3.6. Protein

In vitro protein digestibility was tested on soluble protein content, protein degradation profiles (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)) and HPTLC profiles of free amino acids. The analysis of protein digestibility in the prepared capsules showed that the ES capsule itself contained a small amount of soluble protein, probably derived from the cuticle (Table 4). Drone brood is the main source of soluble protein in the capsules analyzed. Among all the prepared preparations, the highest protein content was found in the DB capsule with the highest share of drone brood (300 mg of drone brood/capsule), compared to DB + ES or DB + CC (300 mg of drone brood/capsule of 550 mg). In the gastric phase, it was found that the protein is best digested in the DB

and DB + ES capsules (84.09 and 83.13%, respectively). A significantly lower percentage of protein digestion was also observed in the DB + CC (46.03%) capsule. In the intestinal fractions after in vitro digestion was found, a high level of protein digestion in the DB + ES capsule at a level of 60.76% was present. Significantly lower results were shown for the DB + CC capsule, which was 25.15% lower compared to the DB + ES capsules (Figure 2a).

SDS-PAGE protein profiling was performed to understand changes in protein content during in vitro digestion of the supplement capsules. Most of the proteins came from the drone brood (Figure 3). The undigested DB sample showed a rich protein profile of 10, 12, 25, 26, 40, 42, 61, 70, 74, 80, 115, 130, and 175 kDa. In all the samples, further digestion in the stomach resulted in the elimination of a large amount of protein and the generation of fuzzy bands (10, 42, and 72 kDa). This indicates that the organized protein network began to disintegrate in the gastric phase, and most of the protein macromolecules disintegrated rapidly. In the intestinal fractions of the preparations, mainly bands with a mass of 10, 26, 42, 74, and 130 were identified, and their intensity was higher compared to the non-digested fractions. This may indicate the participation of digestive enzymes (lipase, amylase, and protease) in the protein profile of the intestinal fraction, whose molecular weights are 48, 56, and 22 kDa, respectively [42].

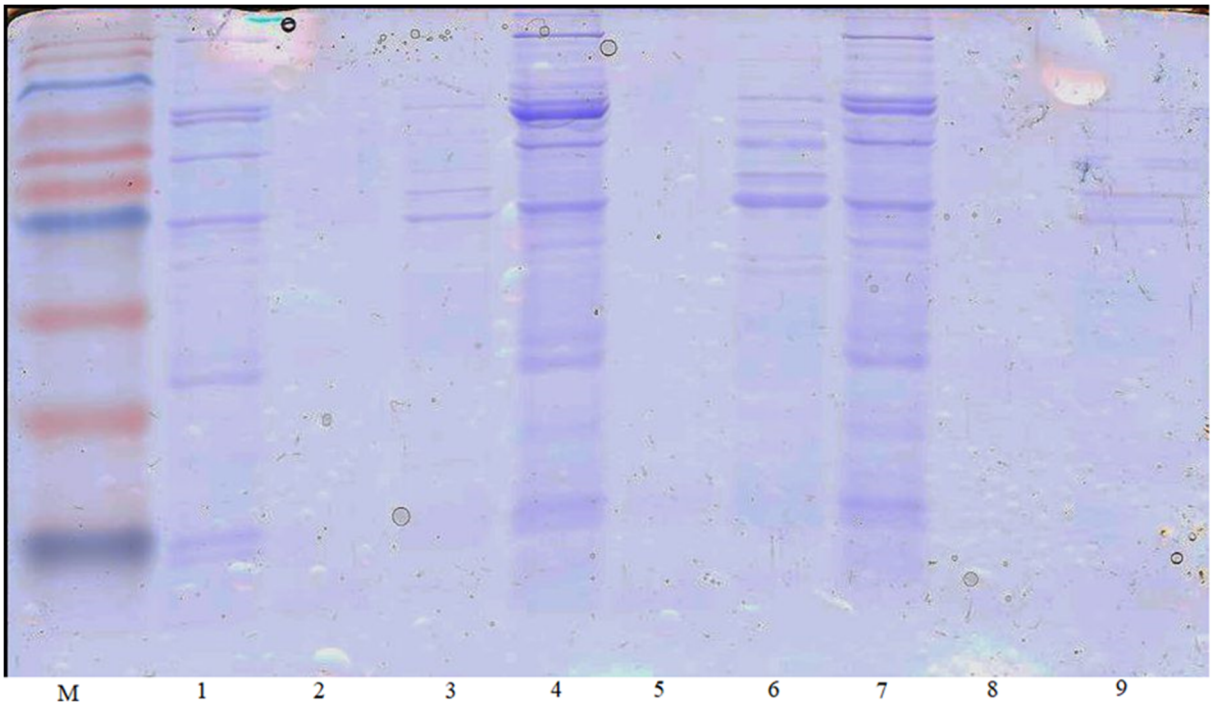


Figure 3. Protein profiles of fractions obtained by SDS-PAGE. M—molecular weight marker; 1—DB (undigested); 2—DB (gastric phase); 3—DB (intestinal phase); 4—DB + ES (undigested); 5—DB + ES (gastric phase); 6—DB + ES (intestinal phase); 7—DB + CC (undigested); 8—(gastric phase); 9—DB + CC (intestinal phase).

Similar observations were made by other researchers [43]. The authors tested the protein content by electrophoresis in soy milk. They showed that after digestion in the oral cavity, the protein content did not change, which could be influenced by the lack of enzymes responsible for protein digestion in the oral cavity. After 5 and 60 min of digestion in the stomach, the number of bands and their intensity decreased significantly compared to the undigested sample, with a significant decrease in protein content after 5 min. However, after 30 and 120 min of digestion in the intestines, they observed an intensification of some bands with a mass of 25 and 35 kDa, compared to the gastric fraction.

3.7. Free Amino Acids

The qualitative free amino acid profile during in vitro digestion was determined by HPTLC, and the results are shown in Figure 4. The undigested samples showed a very similar amino acid profile, where glycine (Rf = 0.28), valine (Rf = 0.45), and leucine (Rf = 0.55) were identified. A slightly weaker band intensity, i.e., lower amino acid content, was observed for the DB + CC gastric fraction. Fractional analysis after the end of digestion (intestinal phase) showed an intensification of previously visible bands, mainly glycine, valine, and leucine. The higher content of amino acids in the intestinal fraction is the result of protein breakdown during digestion. Protein content at each stage decreases, while free amino acids increase. In an earlier study, Ref. [29] characterized the amino acid profile of drone brood using high performance thin layer chromatography (HPTLC) for the first time, and we found more amino acids, including histidine, lysine, proline, glycine, valine, leucine, and tyrosine. However, the separation was conducted under different conditions; therefore, the obtained results cannot be unequivocally compared. Margaoan et al. [44] also found a richer amino acid profile for Apilarnil (freeze-dried drone brood). The authors identified the highest amounts of proline, alanine, lysine, and glycine, and the total content of free amino acids was 1830.07 mg/100 g of Apilarnil.

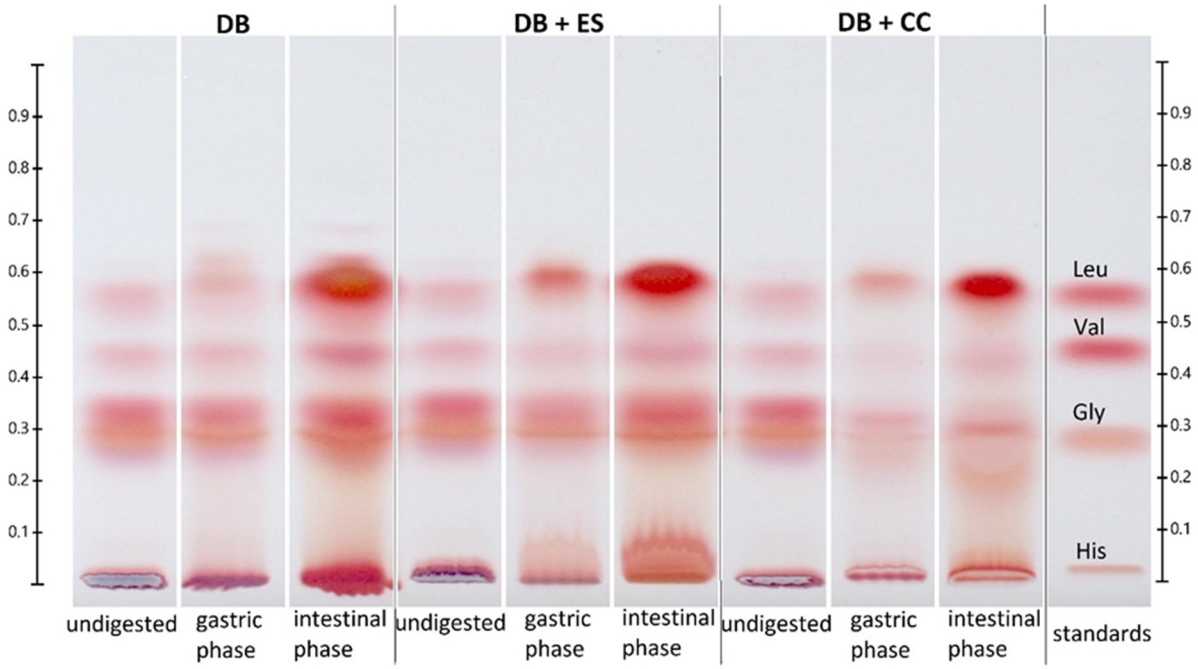


Figure 4. Increase in free amino acids in drone brood (DB) and designed supplements (DB + ES and DB + CC) during in vitro digestion.

3.8. Antioxidants

The analysis of the bioaccessibility of antioxidant compounds was carried out using the FRAP method (Table 4). The capsule containing the only drone brood was found to have the greatest reducing potential, which is due to its highest concentration in the capsule. No reducing potential was found in the ES and CC capsules; therefore, the addition of calcium in both forms reduces the share of drone brood in the capsule by 45.46%, which in turn reduces the antioxidant properties of the undigested fraction, compared to the DB capsule. Taking into account the reduction of the share of drone brood in the DB + ES and DB + CC capsules (up to 54.54% in both cases), a reduction in the reducing potential was found in the DB + ES capsule by 22.36%, and in the DB + CC capsule by 23.90%. The decrease in antioxidant properties in supplements appears to indicate that calcium may block the release of antioxidant compounds or there are interactions between ingredients. After digestion in the stomach and intestines, the FRAP values of all samples

decreased, which may be due to the digestion of the compounds. Similar results were obtained by Seraglio et al. [45], who examined the availability of antioxidant compounds in varietal honeys and showed a decrease in the antioxidant potential in the intestinal fraction compared to the undigested fraction (by up to 33%).

It is commonly accepted that antioxidant activity is formed mainly by polyphenols which have anti-inflammatory and anticancer properties [46]. Long-term consumption of foods rich in polyphenols, mainly of plant origin, has been shown to improve conditions of osteoporosis [47]. Due to this, the polyphenol bioaccessibility of the capsules produced was evaluated and is shown in Figure 2d. The content of phenolic compounds in the undigested fraction ranged from 1.97 to 2.28 mg/capsule (for DB + CC and DB, respectively). These compounds were not detected in the prepared eggshell (ES) and calcium carbonate (CC) capsules, which confirms that the drone brood is the only source of phenolic compounds in the designed supplements. The capsule prepared on the basis of brood and eggshells was found to show a 23% higher content of phenolic compounds compared to the DB + CC capsule, which may indicate that calcium carbonate–a synthetic compound–reduces the release of polyphenols from drone brood, whereas compounds derived from eggshells support the release of polyphenols from the matrix. Phenolic compounds were the most bioaccessible in the drone brood (76.31 and 92.98% in the gastric and intestinal fractions, respectively). In addition, the phenolic compounds with DB + ES also showed high bioaccessibility: 60.93 and 52.34% in the gastric and intestinal phases, respectively. However, the recorded values were lower than in the case of DB capsules by 11.33% and 9.37% in the gastric and intestinal fraction.

The bioaccessibility of phenolic compounds was conducted in an extended simulation of in vitro digestion for various samples. As the total content of phenolic compounds, the researchers assumed the sum of extractable and hydrolysable fractions, with the hydrolysable fractions being several times higher than the extractable fractions, discussed in this paper as the undigested phase [28,45]. Seraglio et al. [45] analyzed the bioavailability of phenolic compounds from varietal honeys using a digestion simulation method analogous to our method. The authors showed that the sum of individual phenolic compounds in the undigested fraction was 46.66% lower compared to the intestinal fraction. The possibility of a food matrix, as well as slight differences in digestion conditions, may have influenced the different behavior between studies.

Changes in the polyphenol profile during in vitro digestion of the supplement were confirmed by HPTLC (Figure 5). In the undigested samples, two faint bands can be identified at Rf = 0.10 and 0.12 (blue and brown, respectively). The blue-colored band can be attributed to ellagic acid, previously detected by this method [29]. In addition, in the zone of low Rf values, a blue color is visible, which may indicate the presence of phenolic acids, e.g., chlorogenic. In samples taken after the intestinal phase of digestion, an additional intense blue band appeared at Rf = 0.45–0.49. This may indicate the release of additional phenolic compounds from the matrix due to digestive processes. Polyphenolic compounds are bound to protein structures in tissues; therefore, as a result of loosening their structures, they can be released in the course of digestion.

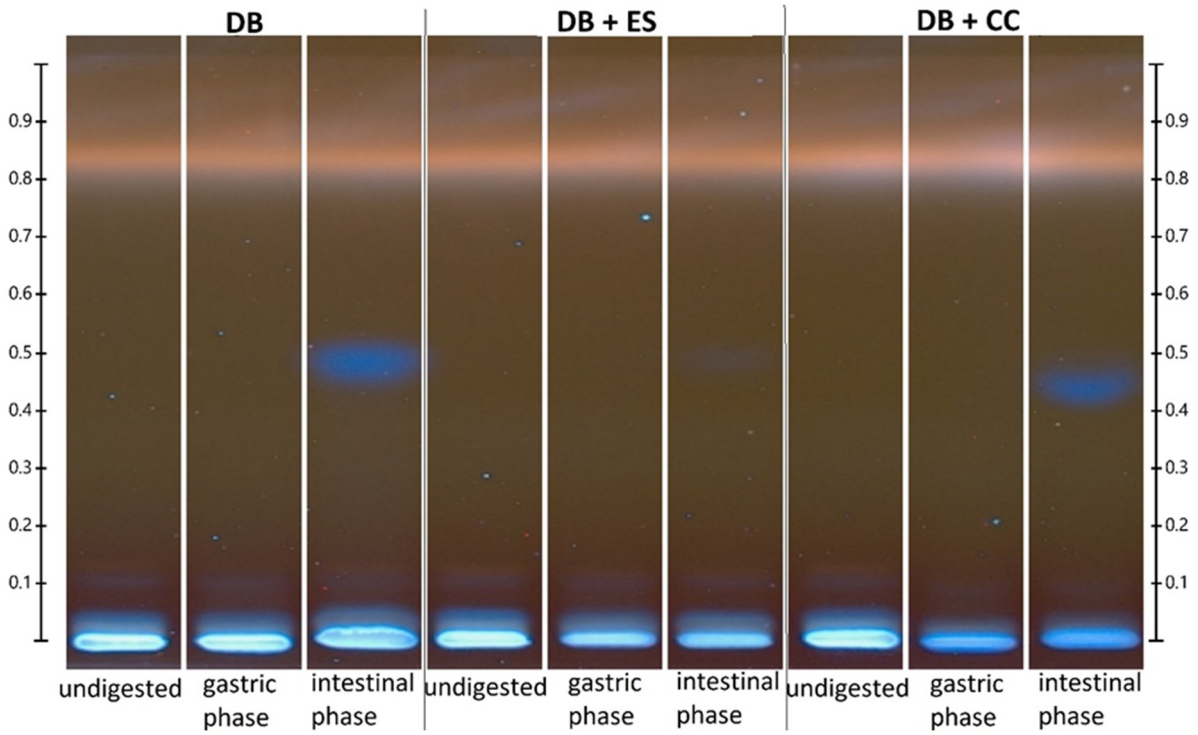


Figure 5. The change of the polyphenolic profile of prepared supplements DB + ES and DB + CC during in vitro digestion.

4. Conclusions

The novel dietary supplement composed of drone brood enriched with organic calcium was designed. For the first time, the bioaccessibility in vitro of the main components (steroid hormones, calcium, proteins, and polyphenols) from pure drone brood compared to designed supplements was demonstrated. A higher bioaccessibility of calcium from eggshells than form calcium carbonate was found, which is in line with literature data. It was shown that the proposed combination (drone brood with eggshells) increases the bioaccessibility of important compounds of drone brood, including hormones, polyphenols, and proteins which were rapidly digested into amino acids. This indicates a beneficial synergistic interaction between both components. However, bioaccessibility studies constitute the initial stage of the in vitro evaluation of the pharmacological potential of the proposed two-component supplement. The next step must include the assessment of bioavailability. Finally, before the recommendation of the designed preparation in the prevention/symptom relief of osteoporosis, the confirmation of its effectiveness and dose-related toxicity during in vivo studies are required. However, in the case of a positive result, the use of drone brood for the production of dietary supplements could expand the range of products offered by beekeeping and increase the income of beekeepers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13084687/s1. Figure S1. Microbiological purity of organic and caged eggshells. Figure S2. Exemplary microscopic images of microorganisms grown from inoculation of egg shells, where: (**a**) Gram-positive bacteria with a spherical shape, aggregated in clusters, microorganisms resemble bacteria of the *Streptococcus* family; (**b**) Gram-positive, rod-shaped bacteria most likely of the genus *Bacillus*, streptococci also visible; (**c**) Gram-positive bacteria with a spherical shape, clustered together, probably bacteria of the *Streptococcus* family. The presence of yeast cells has also been demonstrated.

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