Carotenoid Content and Profile of Some Commercially Available Eggs and In Vitro Bioaccessibility of Lutein and Zeaxanthin from Organic Egg Yolks

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Abstract

The present study investigated the carotenoid content and composition of several commercial eggs produced in different housing systems and the amount of macular xanthophylls that becomes available for absorption by the intestinal cells (bio-accessible) after the ingestion of boiled organic egg yolks. The highest total carotenoid content was observed in the case of organic egg yolks (7.02 mg/100 g). Carotenoid concentration varied not only between egg yolks obtained through different housing methods (free-range, barn and battery-cage), but also between egg yolks from the same housing method obtained by different producers. Except for organic egg yolks that had lutein and zeaxanthin as the major carotenoids, canthaxanthin was observed in all the investigated egg yolks to a different extent. Both lutein and zeaxanthin displayed a high bioaccessibility from boiled organic egg yolks (86% and 91%, respectively).

Keywords: macular xanthophylls; egg yolk; simulated digestion; bioaccessibility.

INTRODUCTION

Aside from being a great source of high-quality proteins, eggs represent an affordable source of oxygenated carotenoids, mainly lutein and zeaxanthin. These two xanthophylls, known collectively as the "macular pigment", accumulate in the macular region of the human retina and are considered to have a great contribution in the prevention and treatment of cataract and age-related macular degeneration (AMD) (Krinsky et al., 2003, Vishwanathan et al., 2009). Moreover, their presence in the serum and brain of older population (octogenarians and centenarians) and infants has been associated with improvements in cognitive function (Johnson, 2012, Johnson et al., 2013, Vishwanathan et al., 2014). Considering that animals are not able to synthesize carotenoids de novo, the content and composition of these bioactive compounds in animal tissues and products are affected by the animal’s diet. By manipulating the complete feed of laying hens, the level of some important nutrients such as selenium, vitamin E, n-3 fatty acids and carotenoids can be enhanced in eggs (Surai et al., 2000, Skřivan and Englmaierová, 2014, Panaite et al., 2019). Yolk color, one of the most important attributes of eggs from the consumer’s point of view, can be modified...
in an effort to satisfy the demand. Non-homogenous or pale yolks are usually associated with different health problems of the laying hen and in order to avoid this, producers deliberately include either natural or synthetic xanthophylls in their diet (Baker and Günther, 2004). The preferred yellow-orange coloration of egg yolks can be achieved organically by including plants with a high xanthophyll concentration such as corn (Zea mays L.) or alfalfa (Medicago sativa L.) in the complete feed or artificially by adding mixtures of canthaxanthin with other carotenoids and xanthophylls without exceeding the maximum concentration of 80 mg/kg in the complete feedingstuff (Breithaupt, 2007).

Despite being consumed in most households around the world, information about carotenoid concentration and profile in commercial egg yolks of different rearing systems (organic, free-range, barn and battery-cage) is still insufficient. Consumers are nowadays able to choose between many options available at different prices having only yolk color as a tool for differentiating between “good” or “excellent” eggs, without being aware of the possible contribution of synthetic carotenoids to the overall color. Many consumers consider that the yolk coloration relies exclusively on the housing system and diet of the laying hens. Furthermore, consumers usually assume that the amount of a bioactive compound present in a food product becomes completely available for the human body immediately upon ingestion. Conscious choices are very seldom made not necessarily due to the lack of research but also because of the inefficient communication between scientific findings and consumers.

In view of the fact that eggs represent a popular food product in Romania (Popescu, 2018), the main aim of the present study was to obtain information about the carotenoid concentration and profile of some eggs available on the Romanian market and to evaluate the in vitro bioaccessibility of the main xanthophylls present in organic egg yolks through an internationally recognized in vitro digestion method (Minekus et al., 2014).

**MATERIALS AND METHODS**

The water used throughout the study was treated in a Milli-Q water purification system. Chemicals and reagents used were of analytical or HPLC grade. For the preparation of the simulated digestive fluids, pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545), porcine bile extract (B8631), KCl, KH₂PO₄, NaHCO₃, NaCl, MgCl₂(H₂O)₆, (NH₄)₂CO₃ and CaCl₂ were acquired from Sigma-Aldrich (Steinheim, Germany). Lutein and zeaxanthin (purity ≥95% and ≥98%, respectively) were purchased from Extrasynthese (Lyon, France). Eggs were purchased based on their availability from a local supermarket (Cluj-Napoca, Romania) and experiments were performed before the expiry date. Eggs from different farming methods such as organic (code 0), free-range (code 1), barn (code 2) and cage (code 3) were included in the present study. Table 1 illustrates the investigated commercial eggs, along with their measured weight and yolk to white ratio.

**Table 1.** The Selected commercial eggs used in the current study along with their respective weight and yolk to white ratio.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Name</th>
<th>Weight (g ± SD) †</th>
<th>Yolk to White Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Producer 1, code 1</td>
<td>P1C1</td>
<td>64.53 ± 0.20</td>
<td>0.42</td>
</tr>
<tr>
<td>Producer 1, code 2</td>
<td>P1C2</td>
<td>72.56 ± 0.56</td>
<td>0.41</td>
</tr>
<tr>
<td>Producer 1, code 3</td>
<td>P1C3</td>
<td>64.17 ± 0.12</td>
<td>0.54</td>
</tr>
<tr>
<td>Producer 2, code 1</td>
<td>P2C1</td>
<td>62.33 ± 0.21</td>
<td>0.37</td>
</tr>
<tr>
<td>Producer 2, code 2</td>
<td>P2C2</td>
<td>71.71 ± 0.15</td>
<td>0.41</td>
</tr>
<tr>
<td>Producer 3, code 3</td>
<td>P3C3</td>
<td>58.77 ± 0.13</td>
<td>0.39</td>
</tr>
<tr>
<td>Producer 4, code 0</td>
<td>P4C0</td>
<td>61.65 ± 0.30</td>
<td>0.62</td>
</tr>
</tbody>
</table>

† mean ± SD (n = 3)

**Carotenoid extraction from egg yolks**

Approximately 2 g of each egg yolk was weighed (in triplicate) in amber glass round bottom flasks. A ternary solvent mixture of methanol:ethyl acetate:petroleum ether (1:1:1, v/v/v) was added and samples were stirred for 30 minutes. After filtration, the residue was re-extracted and the combined extracts were partitioned in a separating funnel with water and diethyl ether. The carotenoid-containing ether phase was collected in a graduated cylinder, the volume was noted and the absorbance was read at λmax = 450 nm. By using rotary evaporation (Heidolph MR Hei-End, Schwabach, Germany), the solvent was removed and the samples were subjected to saponification using methanolic KOH (30%, w/v). After 4 hours, the samples were washed with aqueous NaCl, dried with anhydrous sodium sulphate, evaporated to dryness with a rotary evaporator and stored at -20°C prior to HPLC analysis.
Spectrophotometric measurement of total carotenoid content

The total carotenoid content was determined using a spectrophotometric method (Britton, 1995), employing a UV-Vis Spectrophotometer (Jasco V-530). Total carotenoid content, expressed as mg carotenoids/100 g egg yolk, was estimated by applying the following equation:

\[ X \text{ (mg carotenoids)} = \frac{\text{Abs} \times V \times 1000}{A_{1\text{cm}}^{1\%} \times 1 \times 100} \]

where Abs = absorbance measured at 450 nm, V = sample volume (mL), \( A_{1\text{cm}}^{1\%} \) = absorption coefficient, which is defined as the theoretical absorbance of a solution of 1% (w/v) concentration (i.e. 1 g in 100 mL) in a cuvette with a path length (l) of 1 cm. An average value for \( A_{1\text{cm}}^{1\%} \) of 2500 was used (Britton, 1995). In order to avoid any pigment loss, the analysis was carried out under subdued light. The UV-Vis absorption spectrum was recorded in the range of 300-600 nm and for total carotenoid quantification the absorbance was read at 450 nm.

HPLC-DAD analysis of carotenoids from egg yolks and micellar phase

Carotenoid separation was performed on a HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a SPD-M20A diode array detector and an YMC C30 reversed phase column (250 × 4.6 mm i.d., 5 µm particle size) using solvent A: methanol/tert-butyl methyl ether/water (83:15:2, v/v/v) and solvent B: tert-butyl methyl ether/methanol/water (90:7:3, v/v/v) as follows: 0 min 1% solvent B, 50 min 55% B; 51 min 60% B; 52 min 60% B; min 54 1% B, and afterwards the column was equilibrated for 15 minutes. The flow rate was 1 mL/min and the injection volume 20 μL. Carotenoids were identified by comparing their retention time, elution order and absorption spectra characteristics with those of the standards (lutein and zeaxanthin) and with literature data. External calibration was performed with lutein and zeaxanthin (1-200 μg/mL) for the quantitative analysis.

Static in vitro digestion method

For the simulated digestion the internationally recognized in vitro digestion method guidelines (Minekus et al., 2014) were followed. This standardized in vitro digestion protocol was developed by a consortium of scientists in the frame of a COST action as a common “starting point” to the end that the results obtained after the simulated gastrointestinal digestion studies could be compared and correctly interpreted.

The protocol used in the current investigation is shown in greater detail in Figure 1. The organic eggs were boiled for 10 minutes at 100°C and placed under running tap water for 5 minutes. The yolks were separated from whites and manually shredded. Approximately 1 g egg yolk was weight (in triplicate) in a falcon tube and combined with water to reach the volume of 6.25 mL. In order to simulate the gastric phase, several solutions were added: simulated gastric fluid (SGF), calcium chloride, porcine pepsin solution, water up to 12.5 mL (final digestion volume for the gastric phase) and the pH was reduced to 3 using HCl 1 M. As recommended by Minekus et al. (2014), the pepsin solution was prepared in simulated gastric fluid so as to obtain the concentration of 2000 U mL\(^{-1}\). The mixture was incubated for 2 hours at 37°C in a shaking water bath (Memmert GmbH + Co. KG, Schwabach, Germany).

To simulate the small intestinal phase, simulated intestinal fluid (SIF) was added along with calcium chloride, porcine pancreatin solution, bile solution, water to reach the final volume of 25 mL and the pH was adjusted to 7 with NaOH 1 M. The porcine pancreatin solution and the bile solution were made up in simulated intestinal fluid. As egg yolk constitutes a high-lipid matrix, the concentration of pancreatin was based on lipase activity which was calculated so as to reach 2000 U mL\(^{-1}\). Bile solution was added to achieve the recommended concentration of 10 mM. In the same manner to the gastric phase, the final digestion mixture was incubated at 37°C for 2 hours.

The digested samples were centrifuged at 4°C for 2 hours (4800x g; Eppendorf 5810 R, Hamburg, Germany) and the micellar fraction was collected and filtered (0.2 μm filter). Carotenoid extraction was performed as indicated in a previous study (Tudor et al., 2020a). All experiments were conducted under subdued light.

Bioaccessibility (%) was calculated as the ratio between the concentration of macular xanthophyll found in the micellar phase and its concentration in the boiled egg yolk:

\[ \text{Bioaccessibility (\%)} = \left( \frac{[\text{macular xanthophyll}]_{\text{micellar phase}}}{[\text{macular xanthophyll}]_{\text{boiled egg yolk}}} \right) \times 100 \]
Figure 1. Schematic representation of the simulated digestion method used for the investigation of macular xanthophylls bioaccessibility, SGF - simulated gastric fluid; SIF - simulated intestinal fluid.

Statistical analysis
All analyses were performed in triplicate and the results are presented as mean ± SD. Significant differences between samples were analysed with one-way ANOVA post hoc tests, and pairwise multiple comparisons were conducted using Tukey's test. Significant differences were reported based on P < 0.05. Statistical analyses were performed using the SPSS program (SPSS Inc., Chicago, IL, USA). Chromatograms were acquired using OriginPro, Version 9.5 (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSIONS
Total carotenoid content and major compounds of the selected commercial egg yolks
In the current study, the highest carotenoid content was found in the case of organic egg yolks (P4C0), 7.02 mg/100 g. The highest yolk to white ratio (0.62) was also observed for this egg class (Table 1). Egg yolks obtained from free-range (code 1), barn (code 2) and battery-cage (code 3) farming methods had the total carotenoid content ranging from 0.84 mg/100 g (P2C1) to 1.56 mg/100 g (P1C3), except for the egg yolks obtained by a producer in battery-cage housing system (P3C3) which had a distinguished total carotenoid content of 5.3 mg/100 g (Figure 2).

Figure 2. Total carotenoid content of the selected egg yolks (mg/100 g). P1C1, producer 1, code 1; P1C2, producer 1, code 2; P1C3, producer 1, code 3; P2C1, producer 1, code 2; P2C2, producer 2, code 2; P3C3, producer 3, code 3; P4C0, producer 4, code 0.
In a similar manner, the carotenoid concentration found in the yolks of different eggs purchased from a local supermarket in Germany varied greatly from 2.1 mg/100 g to 3.4 mg/100 g in the case of organic egg yolks, from 1.2 mg/100 g to 2.5 mg/100 g for free-range egg yolks, from 1.2 mg/100 g to 2.9 mg/100 g for yolks of barn egg production and from 0.7 mg/100 g to 2.9 mg/100 g for egg yolks produced through cage system (Schlatterer and Breithaupt, 2006). In another study on 120 eggs obtained through different feeding systems in Bangladesh, the total carotenoid content determined by a HPLC method ranged from 1.7 mg/100 g to 2.5 mg/100 g (Islam et al., 2017). Using a spectrophotometric method, Bovšková et al. (2014) found the highest total carotenoid content in the yolk of eggs from household production (7.25 mg/100 g) and the lowest from those of organic production (2.13 mg/100 g). In contrast, in a study carried out by Bunea et al. (2017) the total carotenoid content in egg yolks from organically fed hens ranged from 1.68 mg/100 g to 8.73 mg/100 g egg yolk.

Apart from the housing system, age and genetic factors (Mugnai et al., 2014, Ko et al., 2020), the laying hens’ diet has a major role in the carotenoid content and composition of the egg yolk. The wide variation in the total carotenoid content occurs most probably due to the different plants present in the complete feed that may possess a higher or a lower carotenoid concentration (Meléndez-Martínez et al., 2021). For instance, diets based on wheat, soy and barley yield a lower xanthophyll concentration in the egg yolk than those based on corn (Nys, 2000).

In this study, with the exception of the organic egg yolks which had lutein and zeaxanthin as the major carotenoids (Figure 3), in all the carotenoid profiles of the investigated egg yolks a synthetic carotenoid was observed, namely canthaxanthin. In the case of the egg yolks with the aforementioned elevated total carotenoid content (P3C3), an unusually high signal for canthaxanthin was observed (Figure 4).

The yellow-orange coloration of egg yolk appears due to the presence of lutein and zeaxanthin, the two major carotenoids of this animal-derived food matrix. β-Carotene can be found as a minor carotenoid in egg yolks due to its pro-vitamin A activity that enables its deposition in egg yolk only after the hens’ vitamin A requirement is achieved (Hammershøj et al., 2010, van Ruth et al., 2011). In view of the consumer’s association of yolk color with the overall quality of eggs, most of the eggs produced in conventional poultry farming contain in addition to lutein and zeaxanthin synthetic xanthophylls. Canthaxanthin, β-apo-8′-carotenoic acid ethyl ester or citranaxanthin are commonly used to supplement the feed of laying hens (Schlatterer and Breithaupt, 2006). Synthetic carotenoids are prohibited by organic regulations in the diet of laying hens therefore the profile of carotenoids in egg yolk can be used as a tool for the discrimination between organic and conventional eggs (van Ruth et al., 2011). In the case of organic production, the concentration of lutein and zeaxanthin in the egg yolk is naturally elevated by including more plants rich in these dihydroxy-xanthophylls in the diet of laying hens.

**Figure 3.** HPLC-DAD chromatogram of the major carotenoids in organic egg yolk (P4C0, producer 4, code 0). Peaks: 1, lutein; 2, zeaxanthin.
Figure 4. HPLC-DAD chromatogram of the major carotenoids in cage egg yolk (P3C3, producer 3, code 3). Peaks: 1, lutein; 2, zeaxanthin; 3, canthaxanthin. Canthaxanthin was identified based on the absorption spectra and the elution order previously reported in literature.

Figure 5. Percentages of macular xanthophylls lutein and zeaxanthin, canthaxanthin and other minor carotenoids from selected egg yolks (P1C1, producer 1, code 1; P1C2, producer 1, code 2; P1C3, producer 1, code 3; P2C1, producer 2, code 1; P2C2, producer 2, code 2; P3C3, producer 3, code 3; P4C0, producer 4, code 0).
In compliance with the legal requirements, lutein and zeaxanthin were the major xanthophylls and accounted for 80% of the carotenoids in the organic egg yolks (Figure 5). In a similar manner, Pintea et al. (2012) found the percentage of lutein and zeaxanthin 83% in the egg yolks of organically raised ISA Brown and Araucana hens. Also, the contribution of lutein and zeaxanthin in organic egg yolks from 24 Dutch farms was 85% (van Ruth et al., 2011). This high percentage of lutein and zeaxanthin in the organic egg yolks is most probably the result of the farming practices, laying hens having access to the outdoors and to feed material containing a higher xanthophyll concentration.

As illustrated in Figure 5, canthaxanthin concentration ranged between 16.3% - 19.8% in free-range egg yolks (P1C1 - P2C1), 9.5% - 14.3% in barn egg yolks (P1C2 - P2C2) and 8.7% - 28% in battery-cage egg yolks (P1C3 - P3C3). Using an isocratic HPLC method, Brulc et al. (2013) found the concentration of canthaxanthin 10.6% in free-range egg yolks, 10.4% in barn egg yolks and 8.7% in battery-cage egg yolks. In several eggs purchased from supermarkets in the Netherlands and New Zealand, canthaxanthin varied considerably from 4.4% to 58.4% in free-range eggs and from 4.6% to 40.7% in barn eggs (van Ruth et al., 2011).

Canthaxanthin is a ketocarotenoid regarded as an efficacious synthetic pigment due to its high coloration ability and deposition efficiency (Akiba et al., 2000, Grashorn and Steinberg, 2002). Moreover, the high stability and the reduced cost make this particular synthetic red xanthophyll more appealing to egg producers. The European Union (EU) legislation (Council Directive 70/524/EEC) has established a maximum amount of 8 mg/kg canthaxanthin in the complete feed of laying hens owing to the undesirable side effect at high dosages, i.e. the formation of small crystals in the human retina (Baker and Günther, 2004). However, when used within the proposed maximum limit canthaxanthin is considered safe (EFSA, 2014).

**Bioaccessibility of macular xanthophylls from organic egg yolks**

Although the xanthophyll content in egg products is not as high as in plant sources (Perry et al., 2009), lutein proved to be more bioavailable from eggs than from lutein supplements or from dark green leafy vegetables such as spinach (Chung et al., 2004). Being a fat-in-water emulsion with the lipid content of 32.6% (Belitz et al., 2009), egg yolk serves as an excellent carrier for carotenoids, facilitating their gastrointestinal digestion and subsequent absorption (Schweiggert and Carle, 2015).

Based on the high total carotenoid content and the absence of synthetic carotenoids, organic egg yolks were further used for the investigation of lutein and zeaxanthin bioaccessibility. Figure 6 illustrates the carotenoid profile of the boiled egg yolk before and after the simulated gastrointestinal digestion. In our study, lutein concentration in boiled egg yolks was higher than that of zeaxanthin (3.41 mg/100 g as against 1.4 mg/100 g). Other available publications reported a variable lutein to zeaxanthin ratio in boiled egg yolks from 0.7 (Asensio Grau et al., 2018) to 1.3 (Rodrigues et al., 2017) and 1.9 (Nimalaratne et al., 2012). However, the above-mentioned studies did not specify the husbandry system of the investigated eggs. The ratio of lutein to zeaxanthin in the eggs yolks of nine organically fed hen breeds ranged from 0.85 to 3.46 (Bunea et al., 2017). The content of xanthophylls in the complete feedingstuffs differs, being generally in favour of lutein (Breithaupt, 2007, Grashorn, 2016) and it is for this reason that in most of the investigated egg yolks zeaxanthin concentration is lower than that of lutein. Moreover, as discussed in a recently published review paper (Tudor and Pintea, 2020b), the occurrence of zeaxanthin in most foods is lower compared to lutein and egg yolk is considered an important commonly consumed source of this xanthophyll in human nutrition.

In the present study, the bioaccessibility of lutein and zeaxanthin from boiled organic egg yolks were 86% and 91%, respectively (Table 2). Even though the bioaccessibility of macular xanthophylls from egg yolks is regarded as superior in most scientific publications, actual research on this topic is scarce. In a similar manner to the present study, Rodrigues et al. (2017) investigated lutein and zeaxanthin bioaccessibility from boiled egg yolks through the standardized static in vitro digestion method (Minekus et al., 2014) and reported a high bioaccessibility for both lutein and zeaxanthin (83% and 90%, respectively). Furthermore, the authors noticed an increase in the bioaccessibility of lutein (from 52% to 69%) after the addition of boiled eggs to a mixed vegetable salad. In another study that employed the same in vitro digestion method but adjusted in order to mimic the gastrointestinal conditions of patients with exocrine pancreatic insufficiency (EPI), the bioaccessibility of lutein from boiled eggs ranged from 27% to 104% and zeaxanthin bioaccessibility from 26% to 98% (Asensio Grau et al., 2018).

It is generally assumed that the bioaccessibility of xanthophylls is higher compared to carotenones (Rodríguez-Amaya, 2016). The elevated bioaccessibility of lutein and zeaxanthin from egg yolk could also be explained by their lipid-dissolved form and by the high content of saturated and monounsaturated fatty acids (SFA and MUFA) present in this matrix (Bunea et al., 2017), which have been previously observed to promote the bioaccessibility of xanthophylls (Tudor et al., 2020a).
Figure 6. HPLC-DAD chromatograms of the organic egg yolk before (a) and after (b) in vitro digestion. Peaks: 1, lutein; 2, zeaxanthin.

Table 2. Micellar concentration and bioaccessibility of lutein and zeaxanthin from organic egg yolks

<table>
<thead>
<tr>
<th>Micellar Concentration (mg/100 g of yolk ± SD)†</th>
<th>Bioaccessibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>2.93 ± 0.14</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>1.27 ± 0.19</td>
</tr>
</tbody>
</table>

† mean ± SD (n = 3)

On another note, Nimalaratne et al. (2015) found the bioaccessibility of lutein 44% and that of zeaxanthin 45% from boiled eggs when using a dynamic in vitro gastrointestinal model (TIM-1). Needless to say, all results obtained by in vitro digestion methods require validation through human nutritional studies. However, numerous publications confirm the high bioavailability of lutein and zeaxanthin from egg yolk. For instance, the intake of one egg per day for 5 weeks enhanced serum lutein (+26%) and zeaxanthin (+38%) concentrations in older adults with the mean age of 79 (Goodrow et al., 2006). Likewise, the daily consumption of 3 whole eggs for 12 weeks has been shown to increase the plasma concentration of both lutein (+21%) and zeaxanthin (+48%) in participants with metabolic syndrome (Blesso et al., 2013).

CONCLUSIONS

This study provides insight into the quality of some eggs available on the Romanian market regarding the carotenoid content and profile and into the bioaccessibility of lutein and zeaxanthin from boiled egg yolks produced through organic production. The high total carotenoid content (7.02 mg/100 g), the carotenoid profile composed mostly of lutein and zeaxanthin (80%) and the absence of synthetic compounds in the organic egg yolks reflected their proper production method. The total carotenoid content varied between the egg yolks obtained from free-range (code 1), barn (code 2) and battery-cage (code 3) farming methods and, in contrast to the egg yolks obtained through organic production, the synthetic carotenoid canthaxanthin was observed in all of the yolk samples, ranging from 8.7% to 28%. An increasing number of publications emphasize the relationship between several eye-related disorders and macular pigments lutein and zeaxanthin. In the current study, the bioaccessibility of lutein and zeaxanthin from boiled organic egg yolk, an animal-derived source of macular xanthophylls, were 86% and 91%, respectively.

Author Contributions: C.T. and A.P. conceived and designed the experiments; M.A.P., C.T. and E.C.G. performed the investigations; C.T. and M.A.P. collected the data and interpreted the results; F.C. contributed to statistical analysis and original manuscript revision; C.T. and M.A.P. wrote the original draft of the manuscript; A.P. supervised the experimental work; A.P. and C.T. revised the original manuscript.
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Conflicts of Interest
The authors declare that they do not have any conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

REFERENCES


