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Polystyrene microplastic particle size and concentration effects on *in vitro* apparent nutrient digestibility and ruminal degradation kinetics

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ABSTRACT

Context. Microplastics are present in ruminant production systems. **Aims.** This study quantified the effects of polystyrene microplastic particle size and concentration on *in vitro* feed digestibility and ruminal degradation kinetics. **Methods.** The Ankom filter bag method was used to investigate the effects of two sizes of microplastic particles (5 and 15 μm) at five concentrations (0.0, 0.5, 1.0, 3.0, and 5.0 g/L), and five incubation times (96, 72, 48, 24, and 6 h) on the *in vitro* dry matter degradability (IVDMD) of several different silage types. The Tilley and Terry method was used to assess if results were replicable to another common *in vitro* method. For the Tilley and Terry method, the effects of two sizes of microplastic particles (5 and 15 μm) at five concentrations (0.0, 0.5, 1.0, 3.0, and 5.0 g/L), and two incubation times (96 and 48 h) on the IVDMD of one silage type were investigated. **Key results.** Microplastic exposure of any combination of particle size and concentration did not affect the IVDMD for any of the silage types or *in vitro* methods investigated, irrespective to the incubation time ($P > 0.05$). **Conclusions.** In isolation of host effects, virgin polystyrene microplastic particles do not impact silage digestibility when 5 or 15 μm in size and at concentrations ≤ 5.0 g/L of ruminal liquor. **Implications.** Research is necessary to confirm whether *in vitro* findings can be transferred to *in vivo* models and to investigate other polymers and weathered plastic.

Keywords: Ankom (DaisyTM IncubatorII) filter bag method, environmental microplastics, *in vitro* dry organic matter degradability (IVDMD), plastic dosage and size, rumen, ruminants, silages, Tilley and Terry in vitro digestion method.

Introduction

Microplastics are present throughout ruminant production systems. Preliminary research has shown that this poses a threat to livestock health, productivity, and consumer perceptions of meat and milk products (Olmo and Holman 2025). To countenance this conclusion, the impact of microplastic ingestion on ruminant performance traits, such as feed use efficiency, must be confirmed.

In vitro methods using rumen fluid fermentation under controlled conditions are non–invasive techniques that allow microplastic effects on ruminal digestibility to be investigated. Tassone *et al.* (2024), for example, used the Ankom filter bag method (Daisy incubation) to find that microplastic particles (polyethylene terephthalate) reduced the digestibility of crude protein in a hay diet. Low levels of microplastic were found to impact on the intestinal phase of digestion and medium to high levels of microplastic impacted on the ruminal phase of digestion (Tassone *et al.* 2024). These same authors did not directly investigate size of microplastic particles, necessitating research into the effect of alternative sizes and concentrations (Tassone *et al.* 2024). An *in vivo* study by Chang *et al.* (2024) demonstrated comparable effects of microplastics on feed digestion in the rumen and noted microplastic particle size-dependent effects. Three sizes of polystyrene microplastic particles (25, 50 and 100 μm) were administered at 150 mg/day to lambs for 60 days. Lambs exposed to microplastics had jejunum epithelium damage and inflammation which increased in severity with increasing particle size. Also observed were significantly reduced average daily gain (lowest in sheep exposed to 100 μm microplastics), feed conversion efficiency (highest in sheep exposed to 100 μm microplastics), nutrient digestibility and rumen pH (Chang *et al.* 2024). Albeit,

dry matter intake and rumen and colon epithelium were unchanged (Chang *et al.* 2024). Experimental exposure studies on other species, such as Nile Tilapia fingerlings, human intestinal epithelial cells and chickens, also suggest reduced nutrient digestibility (Stock *et al.* 2019; Yin *et al.* 2023; Mahmood *et al.* 2024).

It has been observed that experimental exposure studies have used exaggerated doses of microplastics (Bucci *et al.* 2020). This means that studies assessing the effects of environmentally realistic doses and sizes of microplastics in ruminant production systems are lacking (Lenz *et al.* 2016). This is despite factors such as plastic type, size, concentration and duration of exposure affecting the impact of microplastics on these systems (Bucci *et al.* 2020; Siddiqui *et al.* 2023).

The realistic environmental exposure of livestock to microplastics is unknown, with only a few studies offering quantifications of microplastics in animal faeces and feed, based on small sample sizes and reported in inconsistent units. In ruminant faeces, a wide range of 74–50,583 microplastic particles have been detected per kg (Olmo and Holman 2025) and in a study on cattle feed, 36 ± 63 microplastic particles were detected per kg wet weight (Wu *et al.* 2021). Hence, an accurate depiction of the ingestion of microplastics is unclear.

We aimed to initiate research on the effect of microplastic particles on ruminant digestion by investigating the *in vitro* digestibility of silage exposed to ruminal liquor containing different concentrations and sizes of polystyrene microplastic. To more closely reflect natural exposure *in lieu* of accurate data, lower concentrations were investigated compared to previous *in vitro* studies involving other species. To reflect microplastics sizes more likely to penetrate the gastrointestinal barrier, microplastics < 15 μm were selected. Two methods of *in vitro* digestion were used with the results expected to inform industry of a pathway by which environmental microplastics could impact livestock production systems.

Materials and methods

Samples and consumables

This study used two sizes of microplastic particles based on polystyrene microspheres (5 and 15 μm) with a particle density of 1.05 g/cm^3 (Product No. 79633 and 74964, Sigma–Aldrich, AUS). Four types of silages with known *in vivo* digestibility were used, representing a diverse range of ensiled forages in terms of species and quality used in Australian production systems (Bailes *et al.* 2020; Table 1). Silage nomenclature was standardised according to Bailes *et al.* (2020) to ensure consistency and direct comparability with previous findings. The silages were prepared by drying at 80°C for 24 h before being ground through a 1 mm screen (Bailes *et al.* 2020). Rumen contents, both fluid and particulate matter, were manually collected from 5 canulated steers (AEC approval ORA 22-25-4) and prepared by blending half of the rumen fluid in a blender to extract microorganisms from the feed particles before all of the fluid, both blended and unblended, was filtered through coarse and then fine muslin cloth. For both *in vitro* techniques, a buffer solution was then prepared by combining rumen fluid and a buffered artificial saliva solution at a 1:4 v/v ratio. The buffered artificial saliva was prepared by combining 1.6 L of 39.0 ± 0.5°C distilled water with 59.50 g of Na_2HPO_4, 62.70 g $NaHCO_3$, 3.00 g NaCl, 3.65 g KCl, 0.38 g $MgCl_2$, 0.80 g urea, 0.80 g $(NH_4)_2SO_4$, and

Table 1. The botanical composition (dry matter basis), nutritive composition, and *in vivo* digestibility of the silages investigated.

Silage no.	Description	DM (g/kg)	WSC (g/kg DM)	N (g/kg DM)	CP (g/kg DM)	*In vivo* digestibility (g/kg DM)
5	72% Annual ryegrass (*Lolium rigidum* Gaud.) 26% Oats, early cut, 2% Great brome (*Bromus diandrus* Roth.)	376	208	23.9	144.0	780
11	100% Forage sorghum · sorghum (S. *bicolor* L. Moench ×*Sorghum sudanense* Stapf.)	345	157	9.2	57.5	640
13	49% Wheat (*Triticum aestivum* L.) 47% Annual ryegrass 3% Wild radish (*Raphanus raphanistrum* L.) 1% Wild oats (*Avena fatua* L.)	530	184	11.6	72.5	550
22	49% Subterranean clover 23% Silver grass 18% Lucerne 7% Barley grass 3% Annual ryegrass	361	85.4	33.4	231.0	706

Adapted from Bailes *et al.* (2020).
Abbreviations included DM, dry matter; WSC, water soluble carbohydrates; N, nitrogen; CP, crude protein.

0.26 g of $CaCl_2$ (McDougall 1948; Kaiser and Kerr 2003). The rumen contents, fluid, and liquor were each held at 39.0 ± 0.5°C throughout this process. Where possible, glassware and metal containers and instruments were used for sample storage and preparation and during analysis.

Ankom filter bag *in vitro* digestion method

This experiment used a factorial design and the Ankom filter bag method (Ankom Technology 2021) to investigate the effect of two sizes of microplastic particles (5 and 15 μm), five concentrations of microplastic particles (0.0, 0.5, 1.0, 3.0, and 5.0 g/L), and five incubation times (96, 72, 48, 24, and 6 h) on the *in vitro* digestibility of various silages. Before use, filter bags (pore size: 25 μm, F57 filter bags, Ankom Technology, USA) were pre–rinsed with acetone for 3–5 min and air dried. Silage samples of 0.500 ± 0.005 g were weighed into individual filter bags, before all filter bags were heat sealed and inspected to ensure their integrity. Sample blanks were prepared without the inclusion of any silage. Four silage samples representing a range of parent forages were used in the study: Silage 5, Silage 11, Silage 13 and Silage 22 (Table 1).

Fifteen to 24 filter bags were placed into each jar. Each jar contained one blank filter bag, one filter bag each with Silage 11, Silage 13 and Silage 22, and 11 to 20 filter bags with Silage 5. Filter bags were placed into jars to cover the range of incubation times (96, 72, 48, 24, and 6 h) to ensure that all the filter bags could be removed at the same final timepoint (0 h). Microplastic particles were added to 20 individual digestion jars, filled with 2 L of rumen liquor/buffer solution, to achieve the corresponding concentrations. This meant digestion jar was the experimental unit and the filter bags were pseudoreplicates.

Four digestion jars were controls (0 g/L microplastic particles), two jars received 0.5 g/L microplastics of 5 μm, three jars received 1 g/L microplastics of 5 μm, two jars received 3 g/L microplastics of 5 μm, one jar received 5 g/L microplastics of 5 μm, three jars received 0.5 g/L microplastics of 15 μm, two jars received 1 g/L microplastics of 15 μm, two jars received 3 g/L microplastics of 5 μm, and one jar received 5 g/L microplastics of 5 μm. After addition of the bags at the first incubation time (96 h) and at each of the subsequent time periods (72, 48, 24, and 6 h), all jars were gassed with CO_2, for 1–2 min to achieve and maintain an anaerobic environment, and continuously rotated within incubators set to 39.0 ± 0.5°C (Daisy™ Incubator^{II}, Ankom Technology, USA). The rumen/buffer solution was refreshed after the initial 48 h incubation to ensure microbial integrity. At the end of the incubation period the rumen liquor was drained from all jars and the filter bags washed with cold tap water until the rinsing water was clear and there was no obvious residue on the exterior of the filter bags. Washed filter bags were dried at 80°C for 24 h and cooled in a desiccator before weighing. The percentage loss of dry matter (dry matter degradability, IVDMD) was calculated.

Tilly and Terry *in vitro* digestion method

This experiment used a factorial design and a modified Tilley and Terry (1963) method to investigate the effect of two sizes of microplastic particles (5 and 15 μm), five concentrations of microplastic particles (0.0, 0.5, 1.0, 3.0, and 5.0 g/L), and two incubation times (96 and 48 h) on the *in vitro* digestibility of Silage 5 only. Tubes containing 0.500 ± 0.005 g of silage were allocated to treatment groups and were combined with 200 mL of rumen liquor/buffer solution, purged with CO_2, and sealed using stoppers fitted with a Bunsen valve, with fresh rumen/buffer solution made for the 48 h incubation. The flasks were manually agitated (swirled) every 12 h and held at 39°C for their assigned incubation time.

For the 96 h incubation time, four flasks containing Silage 5 were exposed to each combination of microplastic particle size and concentration, and two sample blank flasks were exposed to no microplastics. For the 48 h incubation time, an additional six to eight flasks were exposed to each treatment combination except for concentrations of 3.0 or 5.0 g/L. Flasks containing Silage 11, Silage 13 and Silage 22 were included as controls and were not exposed to microplastics. At 96 and 48 h, eight and 12 flasks were designated as controls, respectively. When the total incubation period was completed, the contents of each tube was filtered under vacuum, into a pre–weighed corresponding numbered porosity two sintered glass crucible containing a large spoonful of celite to promote filtering. The residue within the crucibles was dried at 80°C for 48 h and cooled in a desiccator before weighing and IVDMD calculation.

The two *in vitro* methods were included to assess whether the effects were replicable across methods. While the Ankom filter bag method can be preferred owning to its superior throughput compared to the Tilley and Terry method, this was not apparent in this study because an entire Daisy incubator digestion jar could represent only one experimental unit due to the plastic particles being smaller than the Ankom filter bags pore size (25 μm) and the potential for the microplastics to leach out.

Statistical analysis

Data were analysed in *R* statistical software (RStudio Team 2024). The response of IVDMD to the experimental factors microplastic exposure (yes, no), incubation time, microplastic size, microplastic concentration and silage type was estimated by linear models. 'Control' was included as a factor level for size and concentration to classify jars which contained microplastic concentrations of 0 g/L and size of 0 μm. The linear models were designed to estimate mean response at every combination of time, size, concentration and silage type. As Silage 5 had a greater number of pseudoreplicates in the filter bag study, linear models were based on Silage 5 only. Only when silage types were compared, were data from other

silage types included. Other silage types were not exposed to microplastics in the Tilley and Terry study.

Silage 5 data from the Ankom filter bag *in vitro* digestibility method were analysed using the model:

$$\text{Particle size} \times \text{Particle concentration} \times \text{Incubation time}$$

Silage 5, Silage 11, Silage 13 and Silage 22 data from the Ankom filter bag *in vitro* digestibility method were analysed using the model:

$$\text{Particle size} \times \text{Particle concentration} \times \text{Silage type}$$

Data from the Tilley and Terry *in vitro* digestibility method were analysed using the treatment model:

$$\text{Particle size} \times \text{Particle concentration}$$

Null hypothesis significance tests for the fixed effects were conducted by analysis of variance derived from the model. Predicted mean responses under each treatment combination and standard errors of the predicted means were also calculated. Differences were considered to be significant when $P < 0.05$.

Results

Ankom filter bag *in vitro* digestion method

For Silage 5 samples, microplastic exposure did not affect IVDMD at any of the incubation times investigated ($P = 0.456$; Fig. 1). There was a numerical but non–significant difference in IVDMD between Silage 5 samples that were and were not exposed to microplastics exposed and incubated for 24 h ($P = 0.226$, Fig. 1). IVDMD for Silage 5 samples was found to increase with incubation time ($P < 0.001$) until 72 h, whereafter IVDMD stabilised. Microplastic particle size and concentration did not affect IVDMD for Silage 5 samples at any of the incubation times investigated ($P = 0.390$; Fig. 2).

Microplastic exposure did not affect IVDMD for Silage 5, Silage 11, Silage 13, or Silage 22 samples after 96 h of incubation ($P = 0.485$; Fig. 3). Similarly, microplastic particle size and concentration did not have significant independent or interaction effects on IVDMD across the four silage types investigated ($P = 0.622$).

Tilley and Telly method

The aforementioned absence of an effect of microplastics on IVDMD of Silage 5 over incubation time was replicated by the Tilley and Terry *in vitro* digestion method ($P = 0.300$; Fig. 4). The IVDMD for Silage 5 was numerically higher after 96 h of incubation than 48 h of incubation but this difference was not significant ($P = 0.169$). Similarly, there was no interaction effect of microplastic particle size and concentration on IVDMD for Silage 5 ($P = 0.657$).

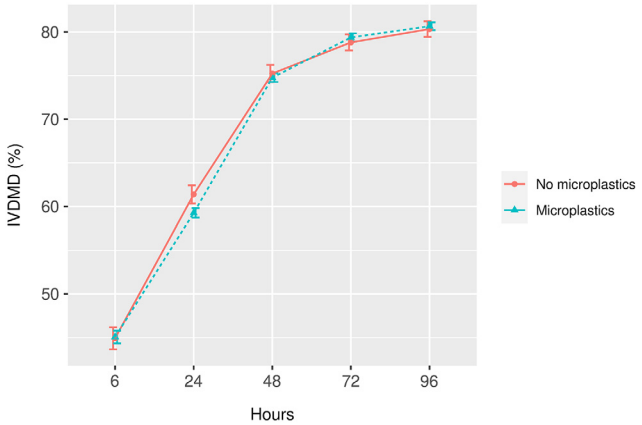

Fig. 1. Effect of microplastics on predicted mean *in vitro* dry organic matter degradability (IVDMD) of Silage 5 over time using the Ankom filter bag *in vitro* method. Error bars represent standard error.

The digestibility observed by the Tilley and Terry method reflected that of the Ankom filter bag method with IVDMD, being highest for Silage 5 (predicted mean ± standard error: 77.2 ± 0.9%) and lowest for Silage 13 (60.4 ± 2.8%; $P < 0.001$).

Discussion

Collectively, these results demonstrate that virgin polystyrene microplastic exposure has no effect on silage IVDMD at the comparatively low concentrations and small particle sizes tested. This confirms the research of Tassone *et al.* (2024), who found no effect of polyethylene terephthalate microplastics on *in vitro* dry matter degradability using slightly higher concentrations of microplastics (5–15 g/L). This assertion is partially explained by Chatman *et al.* (2024), who found no effect of polyethylene microplastics on microbial phylogenetic diversity and β–diversity in chicken caecal contents during a 24 h *in vitro* incubation (Chatman *et al.* 2024). Hence, it seems that rumen microbial populations may have had low bioreactivity to the microplastic treatments.

However, the lack of effect on digestibility appears to be specific to *in vitro* environments that exclude the pathophysiological effects of microplastics on animal cells and the subsequent host effects on digestion (Foster *et al.* 2017). Several *in vivo* studies have demonstrated effects of microplastics on digestion. For example, Chang *et al.* (2024) fed 25–100 μm microplastics to sheep and identified increasing destruction to intestinal epithelium in a microplastic size–dependent manner which coincided with significantly reduced dry matter digestibility, among other effects. Similarly, several poultry studies have shown destruction to intestinal epithelium and significant changes to the gut microbiome in a microplastic concentration–dependent manner (Li *et al.* 2023; Liu *et al.* 2023; Yin *et al.* 2023; Zou *et al.* 2023).

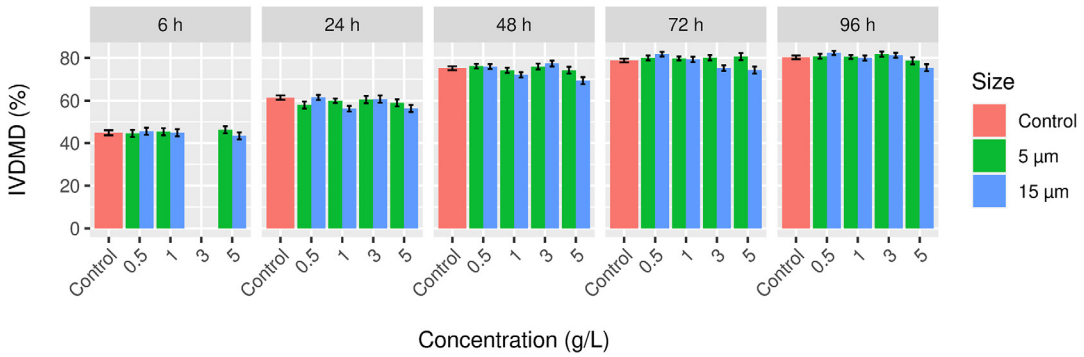

Fig. 2. Effect of microplastic size and concentration on predicted mean *in vitro* dry organic matter degradability (IVDMD) of Silage 5 over time using the Ankom filter bag *in vitro* method. Error bars represent standard error.

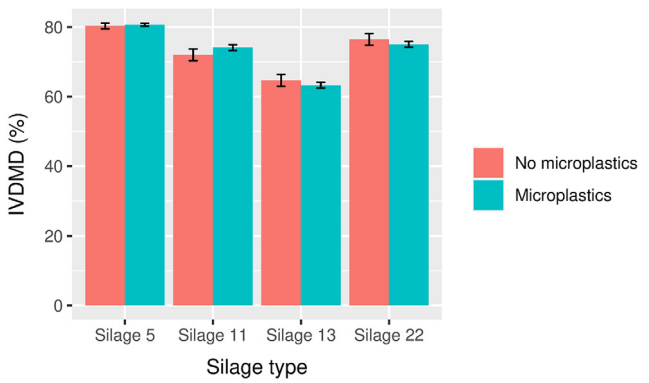

Fig. 3. Effect of microplastic exposure on predicted mean *in vitro* dry organic matter degradability (IVDMD) across silage types after 96 h of incubation using the Ankom filter bag *in vitro* method. Error bars represent standard error.

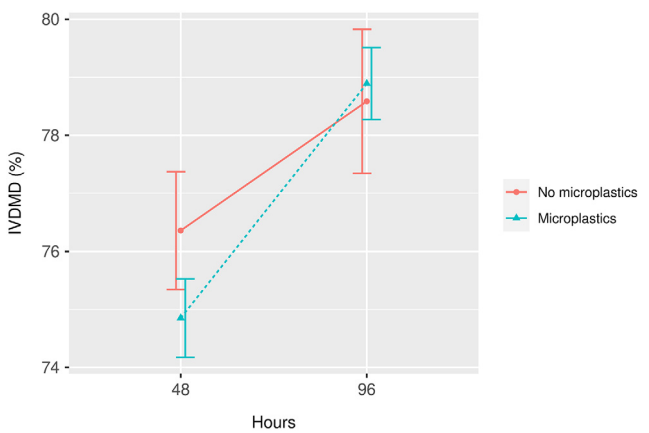

Fig. 4. Effect of microplastics on predicted mean *in vitro* dry organic matter degradability (IVDMD) of Silage 5 over time using the Tilley and Terry *in vitro* method. Error bars represent standard error.

IVDMD was found to be highest for Silage 5 and lowest for Silage 13 ($P < 0.001$), demonstrative of their differences in botanic composition and nutritive profiles (Table 1). However, an explanation for the uniform absence of an effect of microplastics is unclear given that the crude protein percentage and fibre content varied across these silage types (Table 1). Tassone *et al.* (2024) reported that microplastic exposure significantly reduced *in vitro* crude protein degradability and digestibility and NDF degradability when microplastic concentrations were 5–15 g/L and average microplastic particle size was 522 µm. The absence of an effect in the current study may be indicative of the smaller range of microplastic concentrations (0.5–5 g/L) and particle size (5–15 µm) investigated. The comparability of the digestibility results between the *in vitro* digestion methods and previous research on these same silage types (Bailes *et al.* 2020) provides confidence in the results of the current study. Specifically, the absence of any microplastic effects on Silage 5 digestibility was not the result of technical failure but a true indication of no effect.

A limitation of this study was the use of a single virgin plastic polymer, which is not typical of the numerous types of weathered microplastics that livestock are likely to be exposed to. It is possible that the greater direct threat of microplastics to rumen microbes may be through their role as vectors of harmful chemicals and microbes that can adsorb onto their hydrophobic surface, rather than the microplastics themselves. The role of microplastics as vectors was proven by Liao and Yang (2022) who showed that up to 40% of cadmium adsorbed on microplastics were released during *in vitro* ruminant digestion. The current study assumed there was no or minimal adsorbed constituents on the microplastics because they were purchased new (virgin), but this assumption, as well as the role of microplastics as vectors, warrant further research.

Additionally, the microplastic particle sizes and concentrations selected were lesser than that previously investigated, but in both instances, there is little information pertaining to the profile of microplastics likely to be ingested by ruminants. Furthermore, ruminal degradation has been suggested to fragment ingested plastic and thereby introduce microplastics in a range of particle sizes and concentrations to the digestive tract (Quartinello *et al.* 2021). Smaller microplastic particles

may be absorbed and removed from interactions with digesta (Beriot *et al.* 2021). This means that it is unlikely that microplastic particles within the rumen will be of uniform size and constant concentrations, factors that should be considered when applying the current findings or seeking to complete complementary research.

While effort was made to minimise microplastic contamination, it is possible that microplastics leached from the Ankom filter bags which contain polyester. Additionally, the feed types used and the rumen fluid may also have already contained bioaccumulated microplastics. Hence, the treatments should be viewed as additional microplastics, rather than absolute quantities of microplastics.

Conclusion

The study demonstrated that the addition of virgin polystyrene microplastic particles had no effect on the digestibility of silage and ruminal degradation kinetics, when particles were 5 or 15 μm and at concentrations of <5 g/L of ruminal liquor. This outcome was confirmed by two *in vitro* methods for testing feed digestibility, namely the Ankom filter bag method and a modified Tilley and Terry method. These methods did show that IVDMD varied between silage types, depending on their botanical composition and nutritional profile. This was independent to microplastic exposure. When considering the limitations of the current study, it suggests that in isolation of host effects, 5 and 15 μm polystyrene microplastics at ≤ 5.0 g/L of ruminal liquor do not impact on silage digestibility.

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Data availability. Data will be available upon reasonable request.

Conflicts of interest. The authors declare that they have no known conflicts of interest.

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