Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal¹⁻³

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ABSTRACT

Background: We have previously shown that gut microbial fermentation of prebiotics promotes satiety and lowers hunger and energy intake in humans. In rodents, these effects are associated with an increase in plasma gut peptide concentrations, which are involved in appetite regulation and glucose homeostasis.

Objective: Our aim was to examine the effects of prebiotic supplementation on satiety and related hormones during a test meal for human volunteers by using a noninvasive micromethod for blood sampling to measure plasma gut peptide concentrations.

Design: This study was a randomized, double-blind, parallel, placebo-controlled trial. A total of 10 healthy adults (5 men and 5 women) were randomly assigned to groups that received either 16 g prebiotic/d or 16 g dextrin maltose/d for 2 wk. Meal tolerance tests were performed in the morning to measure the following: hydrogen breath test, satiety, glucose homeostasis, and related hormone response.

Results: We show that the prebiotic treatment increased breath-hydrogen excretion (a marker of gut microbiota fermentation) by ≈3-fold and lowered hunger rates. Prebiotics increased plasma glucagon-like peptide 1 and peptide YY concentrations, whereas postprandial plasma glucose responses decreased after the standardized meal. The areas under the curve for plasma glucagon-like peptide 1 and breath-hydrogen excretion measured after the meal (0–60 min) were significantly correlated (r = 0.85, P = 0.007). The glucose response was inversely correlated with the breath-hydrogen excretion areas under the curve (0–180 min; r = −0.73, P = 0.02).

Conclusion: Prebiotic supplementation was associated with an increase in plasma gut peptide concentrations (glucagon-like peptide 1 and peptide YY), which may contribute in part to changes in appetite sensation and glucose excursion responses after a meal in healthy subjects. Am J Clin Nutr 2009;90:1236–43.

INTRODUCTION

The recent growing incidence of metabolic diseases, including diabetes and obesity, in Western countries is associated with changes in eating habits. Obesity is the result of a complex interaction between genetic and environmental factors. Among the latter, changes in eating habits leading to increased fat intake and decreased dietary fiber intake are involved in the incidence of these metabolic diseases (1, 2). Several dietary fibers are often cited as being of particular interest with regard to their putative role in the management of these metabolic disorders because they affect food intake, body weight, glucose homeostasis, plasma lipid profile, and associated risk factors for cardiovascular disease (3). A number of recent studies provide novel insights that might help establish a link between dietary nondigestible carbohydrate that changes the composition of gut microbiota and obesity and insulin resistance (4–8). These compounds are called prebiotics because they promote the growth of certain bacteria (eg, bifidobacteria) whose number correlates with an improvement of several features of metabolic syndrome (9). In the search to determine the role of prebiotics in the control of body weight and fat mass development, a recent study showed that supplementation with a prebiotic had a significant benefit for the maintenance of an appropriate body mass index and fat mass in primarily nonobese young adolescents, in addition to its benefit in bone mineralization (10). Taken together, these human studies provide evidence that the modulation of gut microbiota by using prebiotics affects energy homeostasis and body weight gain. However, few data about the putative mechanisms involved in these effects are provided. We and others have previously published experimental data showing that the modulation of gut peptide secretion [glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), and/or ghrelin] in rodents after feeding with fermentable dietary fibers could constitute a link between the outcome of microbial fermentation in the lower part of the gut and metabolic consequences (eg, decreased food intake, body weight and fat mass development, and improved insulin sensitivity) (11–20).

Several results support the relevance of prebiotic fermentation in appetite management in healthy and obese humans (21–24). To date, few data are available that concomitantly describe the influence of prebiotics on appetite sensation, gut peptide secretion, and metabolism in humans. Interestingly, one study reports that...
prebiotic (oligofructose) consumption (20 g/d) significantly elevated plasma GLP-1 after a mixed meal (4). On the other hand, a decrease in ghrelin and an elevation in serum PYY was observed with prebiotic supplementation in obese individuals (23). However, investigations trying to relate the kinetic measurement of these peptides to sensations related to food intake remain difficult. Indeed, gut peptide measurement requires a large amount of plasma, which is classically sampled by using an invasive method with an indwelling intravenous cannula.

Therefore, in the present study, we have adapted the methodology of gut peptide measurement in human subjects. We did this through a noninvasive blood sampling micromethod approach to measuring the relation between prebiotic fermentation, appetite sensations, and plasma gut peptide profiles involved in the regulation of appetite and insulin secretion in healthy subjects.

SUBJECTS AND METHODS

Test subjects

In total, 10 healthy volunteers (5 men and 5 women) aged 21–38 y (mean ± SD age: 26.3 ± 6 y) with normal body mass indexes (in kg/m²; mean ± SD: 21.6 ± 0.99) participated in the study. Subjects were recruited by local advertisement (initial recruitment began 4 February 2008) and were free from acute and chronic diseases or use of medications that might influence study outcomes (eg, antibiotics). A diet evaluation consisting of both food-frequency questionnaires and a 3-d diet record was obtained to identify and exclude individuals with a typical fiber intake >30 g fiber/d. A total of 30 subjects participated in the validation of the food-frequency questionnaire; 10 subjects were selected for the study on the basis of nutritional exclusion criteria observed after the dietary habits evaluation (ie, probiotic, prebiotic, or symbiotic consumption) and randomly assigned to prebiotic (3 women and 2 men) or dextrin maltose (2 women and 3 men) treatment. Throughout the 2-wk experimental phase of the study, the subjects lived at home and prepared their own meals while consuming prebiotics or a dextrin maltose supplement, as described in the protocol section below. Subjects were instructed to eat until they were comfortably full and to try not to gain or lose weight consciously. The study protocol was approved by the Ethical Committee of Université Catholique de Louvain, and written informed consent was obtained from each subject.

Protocol

Subjects were randomly assigned in a double-blind, parallel, placebo-controlled design. Outpatient investigation consisted of a 2-wk experimental phase. Subjects were given a prebiotic fiber, prebiotics (Orafti Synergy1; Beneo-Orafti, Tienen, Belgium), or dextrin maltose (dextrin maltose, Caloreens; Nestle Clinical Nutrition, Brussels, Belgium) as a powdered supplement. Both the dextrin maltose and the prebiotic treatment had a similar taste and appearance and were provided to the subjects in identical opaque packages. Both the subjects and the investigators were blinded to the treatment.

The prebiotic (Orafti Synergy1) (6.27 kJ/g) was fully soluble fructan and consisted of a mixture of glucosyl-(fructosyl)α-fructose and (fructosyl)α,β-fructose extracted from chicory roots (Chicorium intybus). This fructan was not digested, but it was highly fermented in the colon. The dextrin maltose (Caloreens) (16.7 kJ/g) is also a fully soluble carbohydrate, but it is totally digested and therefore not fermentable. Daily supplements were divided into 2 portions of 8 g each to be eaten during breakfast and dinner. To assess compliance, subjects were given preweighed bags (8 g) for daily supplement consumption and were instructed to return empty bags for monitoring. They were instructed to consume the entire amount of the 2 bags daily.

Study design and procedure

The study was divided into 4 different periods: a training session (3 d before the start of the study), the first test day (day 0), a treatment period (2-wk treatment period), and the second test day (day 14), as shown in Figure 1A.

Before the start of the study (3 d before), all subjects attended a training session at which they were invited to participate in a day of training with a free-choice buffet breakfast as described in Figure 1A and by Cani et al (21). The subjects were also instructed in the use of the rating scales (for appetite and potential adverse effects), prebiotic or dextrin maltose ingestion (inclusion in food and/or adequate beverage), and how to self-report their food intake.

The subjects were instructed to abstain from alcohol and strenuous physical activity for 2 d before the first test day (day 0) and during the entire intervention phase (ie, during the 2-wk treatment period) until day 14 to ensure a similar macronutrient balance on the test days.

At day 0 (ie, before the beginning of the treatment) and at day 14 (ie, at the completion of the 2-wk period of prebiotic and dextrin maltose treatment), the subjects were invited to self-report food consumed during breakfast and during the entire test day (including food and beverages consumed between meals). A food-frequency questionnaire (over a 1-wk period) and 24-h recall were recorded on days 0 and 14 and validated with the dietician.

On the test days (days 0 and 14) and after an overnight fast, the subjects were invited to a free-choice buffet breakfast. The subjects were instructed to finish the breakfast in 15 min (Figure 1B). Food and drinks were weighed before and after the meal, and the energy intake was calculated. Appetite ratings were assessed on 100-mm visual analog scales (VASs) with the text expressing the most positive and the most negative ratings anchored at each end: satiety, “I cannot eat another bite”; hunger, “I have never been more hungry” (25–27). VASs were used to assess satiety and hunger after the test breakfast. Sensations were recorded at the beginning of the meal at time 0 (fasting) and throughout the period after breakfast until lunch at 30, 60, 120, and 180 min, postprandially. Blood samples, appetite ratings (hunger, satiety), and fermentation (hydrogen breath test) were measured at intervals during the study as described in Figure 1B. Potential adverse effects were monitored daily during the entire treatment period by using subjective scales rated as “absent,” “light,” “moderate,” or “severe” for the following symptoms: nausea, diarrhea, flatulence, borborygmus, abdominal cramps, stomach cramps, gastrointestinal reflux, abdominal rumbling, and thirst.

Reported energy, macronutrients, and fiber intakes were calculated by using the program Nutrilog SAS (Marans, France), and prebiotic intake (insulin-type fructans) was calculated by
taking into account the content reported in food stuff by van Loo et al (28) and our updated databank compilation (PD Cani and NM Delzenne, unpublished data, 2008 and 2009).

Physiologic variables

Finger-prick blood samples (240 μl) were drawn repeatedly in heparinized capillaries to measure the concentrations of plasma glucose, insulin, and gut hormones [GLP-1, PYY, GIP, and pancreatic polypeptide (PP)]. Briefly, blood samples were taken at 0 (fasting), 10, 30, 60, and 120 min postprandially (Figure 1B). Capillary blood (60 μl) was taken in <30 sec and directly flushed within tubes containing protease inhibitors, such as dipeptidyl peptidase IV (DPPIV) inhibitor (Millipore’s DPPIV inhibitor; St Charles, MO) and phenylmethanesulfonyl fluoride. Plasma was immediately removed and stored at −80°C for further analysis. Blood glucose concentrations were determined repeatedly at 0 (fasting), 30, 60, 120, and 180 min postprandially (Figure 1B) as an indicator of colonic fermentation by using an ambulatory MicroH2 breath test (MicroH2; Micromedical, Basingstoke, United Kingdom). Gut hormone concentrations were determined in duplicate in 25 μL of plasma by using a human gut hormone multiplex kit (Millipore) and by using Luminex Technology (Bio-Plex; Bio-Rad, Nazareth, Belgium). This multiplex assay kit was to be used for the simultaneous quantification of the following human gut hormones: GLP-1 (active), GIP (total), PYY (total), PP, and insulin. The sensitivity levels of the assay (in pg/mL) correspond to the following: GIP, 0.2; GLP-1, 5.2; PP, 2.4; insulin, 44.5; and PYY, 8.4. The interassay variation (%CV) was <19%, and the intra-assay variation (%CV) was <11%.

Statistical analysis

Results are expressed as means ± SEMs. A 2-factor repeated-measures ANOVA (model I) was used to compare the differences
between groups for the hydrogen breath test; appetite ratings (VAS); and plasma concentrations of GIP, GLP-1, PYY, PP, glucose, and insulin; differences at individual time points were determined by using a Bonferroni post hoc analysis when the interaction between treatment and time was statistically significant ($P < 0.05$). A Student’s $t$ test was used to compare the area under the curve (AUC) changes in exhaled hydrogen and blood glucose concentrations. The correlations were analyzed by using Pearson’s correlation in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). The level of significance was set at $P < 0.05$.

RESULTS

The study compliance was excellent (only one subject returned 2 full packets, which indicated missed doses). Minor gastrointestinal disorders were reported on the first 3 d of prebiotic treatment, 66% of the subjects reported flatulence (40% light, 50% moderate, and 10% severe), and 26.6% of the subjects reported light abdominal rumbling. The amount of prebiotics consumed before starting the study was not significantly different between men and women ($6.7 \pm 1.1$ and $6.2 \pm 0.9$ g/d for women and men, respectively; mean value: $6.4 \pm 0.7$ g/d). The macronutrient intake (protein, carbohydrate, fat, and dietary fibers excluding supplements) during the test days (day 0 and 14) was equivalent in the dextrin maltose and prebiotic groups (Table 1), whereas the total dietary energy intake was lowered by $\approx 6\%$ under prebiotic treatment, but this was not significant (Table 1).

Breath-hydrogen excretion after breakfast

Changes in gut microbial fermentation after the prebiotic treatment were estimated through breath-hydrogen excretion. Breath-hydrogen excretion was equivalent between groups at baseline before the beginning of the treatment (prebiotics: $9.4 \pm 3.4$ compared with dextrin maltose: $8.4 \pm 3.4$, $P > 0.05$). Conversely, prebiotic treatment induced more fermentation at time 0 (fasting) on day 14 (Figure 2). Compared with dextrin maltose, there was a significant time $\times$ treatment effect ($P = 0.0156$) with significantly higher fermentation in the prebiotic group at times 30 and 120 min after the breakfast ($P < 0.05$) (Figure 2). In addition, the hydrogen AUC indicated a 3-fold higher colonic fermentation in volunteers supplemented with prebiotics compared with dextrin maltose (Figure 2).

Hunger and satiety score after breakfast

Data are presented as changes from baseline (in cm) before and after the corresponding treatment of each subject in Figure 3B. Prebiotic treatment significantly lowered hunger VAS scores at time 180 min, whereas this variable remained unchanged after dextrin maltose treatment (Figure 3B). The satiety VAS score tended to be higher in the prebiotic group, but this effect was not significant (time $\times$ treatment $P = 0.1747$; Figure 3A).

Plasma hormones

The absolute plasma concentrations and the changes from baseline (fasting values) for postprandial GIP, GLP-1, PYY, and PP are depicted in Figure 4. The plasma hormone concentrations did not significantly differ between the 2 treatments at time 0 (fasting; Figure 4), even if the prebiotic treatment tended to increase the fasting absolute plasma PYY concentration ($P = 0.1$) compared with the dextrin maltose group. At time 10 min, the incretin hormone GLP-1, which was expressed as changes from baseline, was significantly higher after the prebiotic treatment than after the dextrin maltose treatment (Figure 4B). Plasma PYY concentrations were significantly increased at 10 min in prebiotic-treated subjects compared with dextrin-maltose-treated subjects (Figure 4C). Plasma PP and GIP concentrations were not significantly affected by the treatment (Figure 4, A and D).

Importantly, we showed a positive and significant correlation between plasma GLP-1 concentration and breath-hydrogen excretion (AUC 0–60; Pearson’s correlation $r = 0.85$, $P = 0.007$).

TABLE 1

Dietary energy and nutrient intakes at the end of the 2-wk experimental phase of the study after prebiotic or dextrin maltose supplementation$^1$

<table>
<thead>
<tr>
<th></th>
<th>Dextrin maltose</th>
<th>Prebiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy intake (kcal/d)</td>
<td>2501 ± 418</td>
<td>2339 ± 218</td>
</tr>
<tr>
<td>Protein (% energy)</td>
<td>14 ± 1.5</td>
<td>14.2 ± 1</td>
</tr>
<tr>
<td>Carbohydrate (% energy)</td>
<td>53.8 ± 3.3</td>
<td>52.4 ± 2.1</td>
</tr>
<tr>
<td>Fat (% energy)</td>
<td>26.2 ± 3.9</td>
<td>29.8 ± 2.2</td>
</tr>
<tr>
<td>Dietary fibers, excluding fructans-type fibers (g/d)</td>
<td>17.6 ± 1.6</td>
<td>18.3 ± 2.4</td>
</tr>
<tr>
<td>Fructans-type fibers (g/d)</td>
<td>5 ± 0.9</td>
<td>20.9 ± 0.9$^2$</td>
</tr>
<tr>
<td>Total dietary fibers (g/d)</td>
<td>22.6 ± 1.9</td>
<td>39.2 ± 3.2$^2$</td>
</tr>
<tr>
<td>Dietary energy intake (%)$^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total energy intake</td>
<td>100 ± 13.7</td>
<td>93.5 ± 7.1</td>
</tr>
<tr>
<td>Breakfast</td>
<td>100 ± 19.8</td>
<td>92.7 ± 9.5</td>
</tr>
<tr>
<td>Lunch</td>
<td>100 ± 14.1</td>
<td>82.7 ± 11.2</td>
</tr>
<tr>
<td>Dinner</td>
<td>100 ± 11.8</td>
<td>104.6 ± 9.6</td>
</tr>
</tbody>
</table>

$^1$ All values are means ± SEMs; $n = 5$/group.

$^2$ Significantly different from dextrin maltose group, $P = 0.000001$ (Student’s $t$ test).

$^3$ Significantly different from dextrin maltose group, $P = 0.002$ (Student’s $t$ test).

$^4$ The percentage of dietary energy intake for dextrin maltose = [dextrin maltose (kcal)]/[dextrin maltose mean (kcal)] × 100. The percentage of dietary energy intake for prebiotics = [prebiotics (kcal)]/[dextrin maltose mean (kcal)] × 100.
Although we observed significant changes in VAS scores and plasma gut peptide concentration involved in appetite and body weight regulation, hunger and satiety VAS scores were not correlated with the gut peptide concentrations (GLP-1, PYY, and PP) or breath-hydrogen excretion at any time point or when expressed as the AUC (not shown).

**Plasma glucose and insulin**

Interestingly, we show that the AUC for plasma glucose concentration was significantly lower after the prebiotic treatment (Figure 5A). Neither the fasting glucose concentration nor the plasma insulin concentration was significantly affected by the prebiotic treatment (Figure 5, A and B). The glucose response was inversely correlated with the breath-hydrogen excretion areas under the curve (0–180 min; Pearson’s correlation $r = -0.73, P = 0.02$).

**DISCUSSION**

In the present study, we confirmed that supplementation with prebiotics for 2 wk is associated with lowered subjective hunger, as previously described (21). The mechanisms by which the nutritional modulation of gut microbiota fermentation affects the regulation of appetite sensation in humans are poorly understood. Evidence from animal studies has shown that changes in gut microbiota after fermentable dietary carbohydrate ingestion decrease food intake, body weight, and fat mass development by mechanisms associated with the upregulation of endogenous GLP-1 and PYY production (11, 14–18, 20, 29, 30). For the first time, we associated the appetite regulation effects of prebiotics with increased postprandial plasma gut peptide concentration responses after a standardized meal in healthy subjects. Together, these data confirm previous observations (21, 31) and support the role of the modulation of microbial activity (fermentation) by prebiotics in the control of appetite sensations. In agreement with this, Archer et al (31) have shown that prebiotics added to food as fat replacements were able to lower energy intake during a test day. However, acute treatment with prebiotics does not necessarily affect appetite sensation, which suggests that adaptive processes are necessary to observe the satietogenic effect of prebiotics (32). One could speculate that the modulation of the gut microbiota and the related endogenous gut hormone production might be part of this mechanism.

In addition, the present study provides evidence that the micromethod for blood sampling could be useful for measuring the plasma concentration of several gut peptides to correlate their secretion with appetite sensations and with glucose homeostasis. However, although the plasma gut peptide concentrations in our
study were comparable to those reported in the literature, it could be useful to validate this micromethod against the standard forearm venous sampling method.

In our study, the transient increase in the concentration of plasma gut peptides that regulate food intake (GLP-1 and PYY) was a phenomenon associated with changes in the appetite VAS score after a standardized test meal; however, we did not find significant correlations between these variables. The relevance of such an effect on a subsequent meal is an important phenomenon to take into account when assessing the relevance for energy intake control in humans (33). The persistence of the appetite regulation effect throughout the day was supported by our previous study. Indeed, by using a similar intervention protocol, we have shown that the decrease in hunger and the increase in satiety were also present in prebiotic-treated volunteers at dinner (21).

Interestingly, we show that prebiotic pretreatment also modulates the postprandial glucose response, and this is consistent with the increase of gut peptides related to glucose homeostasis.
or endocrine variable measured during our study. This suggests as well as the glucose response but not with any other metabolic persistent and independent of meal intake. We show a relation state suggests that colonic events related to fermentation are significant increase of breath-hydrogen excretion in the fasting prebiotic treatment than during dextrin maltose treatment. The evidence that prebiotic treatment increases colonic fermentation because breath-hydrogen excretion is significantly higher during fermentation, which was assessed through the hydrogen breath test, glycemic excursions and satiety through a mechanism involving the fermentation of nondigestible carbohydrate may affect the management of glucose homeostasis.

In addition, we show a negative correlation between a gut microbial fermentation activity marker (breath-hydrogen excretion) and the glucose response AUC.

We show that GLP-1 plasma concentration and the glucose response at the standardized breakfast were inversely associated. We observed changes in plasma insulin concentrations in prebiotic-treated volunteers compared with dextrin maltose, but due to the large variation of this variable, we could not establish a significant correlation between insulin and serum incretin concentration. We have previously shown in animals that processes beginning with the gut microbiota and ending with colonic fermentation may have implications for glucose tolerance through several mechanisms, including GLP-1 secretion and the microbial-related modulation of systemic inflammation and endotoxemia (6, 8, 34, 35). Future studies in diabetic patients are required to investigate the relevance of the prebiotic approach for the management of glucose homeostasis.

Consistent with our results, Nilsson et al (5) have shown that the fermentation of nondigestible carbohydrate may affect the glycemic excursions and satiety through a mechanism involving colonic fermentation. The relation between the kinetics of fermentation, which was assessed through the hydrogen breath test, and GLP-1 production has already been suggested previously in patients with gastrointestinal reflux (4). In our study, we provide evidence that prebiotic treatment increases colonic fermentation because breath-hydrogen excretion is significantly higher during prebiotic treatment than during dextrin maltose treatment. The significant increase of breath-hydrogen excretion in the fasting state suggests that colonic events related to fermentation are persistent and independent of meal intake. We show a relation between postprandial hydrogen production and plasma GLP-1 as well as the glucose response but not with any other metabolic or endocrine variable measured during our study. This suggests that the extent of fermentation per se does not explain all of the changes in gut peptides that are observed with prebiotic treatment.

Interestingly, the relevance of gut hormone modulation by prebiotics in the management of obesity and metabolic syndrome in humans is supported by some authors. A recent clinical trial supports the evidence that prebiotics decrease food intake, body weight gain, and fat mass development in obese subjects (23). As with our research, the authors show higher plasma PYY concentration after a meal whereas, conversely, they failed to find increased GLP-1 plasma concentrations during a 6-h meal tolerance test (23). However, one cannot rule out that the lack of change in plasma GLP-1 concentrations is not involved in the apparent improved glucose tolerance and body weight regulation. Indeed, GLP-1 is secreted after a meal in the portal vein, where it exerts its physiologic role (36) and is rapidly degraded by the DPPIV enzyme within 90 s (37). Accordingly, it has been proposed that obese and type 2 diabetic subjects exhibit a higher DPPIV activity (38), a phenomenon that might be involved in the absence or the difficulty of measuring peripheral GLP-1 plasma concentration in this study. Another study also supports a weight-reducing effect of prebiotics in adolescents (10).

We provide support that the prebiotic treatment is associated with lowered subjective hunger ratings and a tendency to increase satiety ratings; this is linked to increased postprandial plasma gut peptide concentration (GLP-1 and PYY) responses after a meal in healthy subjects. Nevertheless, further investigations are needed to delineate the contribution of the specific changes in gut microbiota composition that are observed with prebiotic feeding [eg, Bifidobacterium spp (39), Lactobacillus spp, Roseburia spp (40), and Faecalibacterium prausnitzii (41)] compared with the “fiber effect” of prebiotics via the production of short-chain fatty acids on their bacterial fermentation. Collectively, our previous
and present data, together with recent findings by others (23), provide evidence that prebiotics could be a useful tool for controlling food intake and glucose homeostasis and promising agents for maintaining or restoring both glucose and energy homeostasis.

We thank R Verbeeck for English editing and A Guillot for statistical analyses.

The authors’ responsibilities were as follows—PDC: designed the experiments, collected the data, analyzed the data, wrote the manuscript, and supervised the project; EL, EMD, FMS, BDP, DN, FdB, and AMN: assisted with the data collection and participated in revising the manuscript; and NMD: contributed to the design of the experiment, the interpretation of the data, and the review of the manuscript and supervised the project. PDC is a research associate for FRS-FNRS Belgium. None of the authors had a conflict of interest.

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