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BRIEF REPORT

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Acute stressor alters inter-species microbial competition for resistant starch-supplemented medium

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ABSTRACT

Gut microbiome community dynamics are maintained by complex microbe-microbe and microbe-host interactions, which can be disturbed by stress. *In vivo* studies on the dynamics and manipulation of those interactions are costly and slow, but can be accelerated using *in vitro* fermentation. Herein, *in vitro* fermentation was used to determine how an acute stressor, a sudden change in diet, impacts inter-bacterial species competition for resistant starch-supplemented medium (RSM). Fermentation vessels were seeded with fecal samples collected from 10 individuals consuming a habitual diet or U.S. military rations for 21 days. *Lactobacillus spp*. growth in response to RSM was attenuated following ration consumption, whereas growth of *Ruminococcus bromii* was enhanced. These differences were not evident in the pre-fermentation samples. Findings demonstrate how incorporating *in vitro* fermentation into clinical studies can increase understanding of stress-induced changes in nutrient-microbiome dynamics, and suggest that sudden changes in diet may impact inter-species competition for substrates.

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Gut microbiome; *in vitro* fermentation; military; resistant starch; inter-species competition; microbial ecology

Introduction

Within the gut microbiota, synergistic relationships between cross-feeding microbial partners facilitate degradation of complex dietary substrates. This cooperation modulates the gastrointestinal environment and subsequently microbial community interactions. Gut microbiota structure can be disturbed by stressors impacting the community's ability to metabolize dietary compounds, and consequently host-microbiome dynamics.[1] While there is substantial interest in determining how stressors alter the structure and cooperative dynamics of the human gut microbiota, elucidating these relationships *in vivo* is time-consuming, expensive, and difficult due to uncontrolled factors. *In vitro* fermentation models represent a time- and cost-effective alternative that can complement human studies to increase understanding of nutrient-microbiome dynamics and interrogate stressor-induced perturbations to the competitive metabolic balance for substrates beyond what can be derived from analyses of human fecal samples.

Gut microbiota community dynamics are rapidly stressed by substantial changes in host diet.[2] U.S. military personnel and civilians living through humanitarian crises experience such stress when switching from habitual diets to military rations such as the U.S. Military Ration Meals Ready-to-Eat (MRE). Although the averaged macronutrient distribution of MREs (50% energy from carbohydrate, 13% energy from protein, and 37% energy from fat) is similar to a standard western diet, an MRE-only diet is unique in that it lacks fresh fruits and vegetables, has limited variety, and is sterile.

Herein, we utilized *in vitro* fermentation to determine the metabolic impact on the gut microbiota of a sudden change to an MRE-only diet by characterizing growth dynamics within a resistant starch-supplemented medium (RSM). Resistant starch (RS) type II is a non-digestible carbohydrate that is metabolized by specific gut microbes, which in turn generate intermediate products that influence community metabolism dynamics.[3,4] By using RS, competitive growth niches that arise within a microbial community

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that may change upon a sudden shift to an MRE-only diet can be explored.

Results

Fecal samples were collected during a parallel-arm, randomized controlled trial before and after a 21 day period during which participants consumed their self-selected habitual diets (HAB-diet, n = 5; 41% energy from carbohydrate, 20% energy from protein, and 37% energy from fat) or an MRE-only diet (MRE-diet, n = 5; 50% energy from carbohydrate, 13% energy from protein, and 36% energy from fat) (STable 1). Samples from each diet group on each study day were then pooled to increase microbial diversity in the inoculum, maximize low abundant species, and reduce differences within diet groups. The pooled inoculum was used to seed *in vitro* fermentations (n = 3) to explore alterations in microbial metabolism, represented by inter-species competition for RSM (Figure 1).

qPCR was employed to quantitate microbial abundances in response to RSM as a function of diet. The organisms targeted in this study represent keystone gut bacteria identified as important for microbiome and host health. The selected species possess essential metabolic functions related to inter-species competition for nutrients[5] and include: RS degrader *Ruminococcus bromii*;[6] beneficial saccharolytic taxa *Lactobacillus*[7,8] *and* Bifidobacterium;[9] butyrate producers *Roseburia spp.*, *Eubacterium rectale* and *Faecalibacterium prausnitzii*;[2] mucin-degrader *Akkermansia muciniphila*;[10]*Bacteroides/Prevotella* as the most dominant intestinal residents;[11,12] and the phylogenetically and metabolically diverse *Clostridium-Eubacterium* group[13,14] (STable 2).

To identify whether changes in growth profiles of individual species in response to RSM differed as a consequence of the MRE-diet relative to typical variation, as measured in the HAB-diet, 2-way ANOVA on qPCR products was employed to analyze the effects of day, diet and the interaction between the two (p-values reported in the text represent day-by-diet interactions unless otherwise noted). Samples collected immediately after inoculation (termed 0 hour) did not show differences in microbial species abundance (Table 1), with the exception of *Roseburia spp*. which increased within the MRE-diet group (p = 0.049). Conversely, the Principal Component Analysis (PCA) of qPCR products demonstrated a divergence of community composition during fermentation (Figure 2 (a)). Samples at inoculation (0 hour), which are akin to the respective microbial abundances seen within human fecal samples, displayed slight

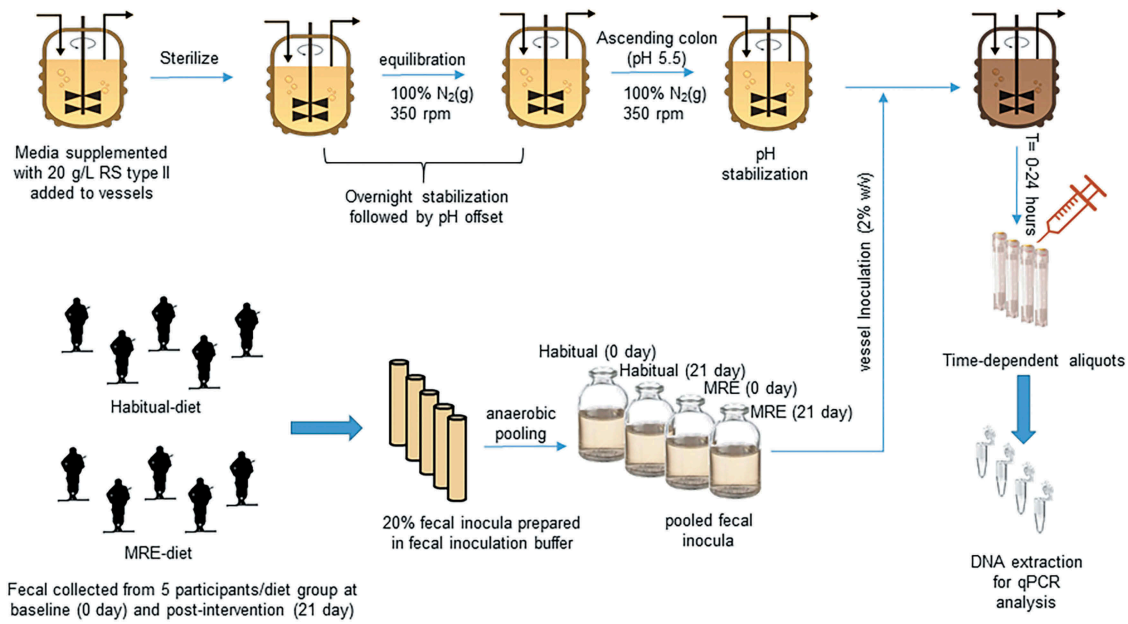

Figure 1. Schematic representation of the *in vitro* fermentation protocol.

Table 1. qPCR log copy numbers per mL culture at inoculation (0 hour fermentation).

	MRE Day 0	MRE Day 21	HAB Day 0	HAB Day 21	Main effect (day)	Main effect (diet)	Interaction (day*diet)
C. coccoides	1.87 ± 0.21	1.98 ± 0.14	2.08 ± 0.02	1.93 ± 0.08	0.818	0.333	0.138
E. rectale	1.95 ± 0.20	2.13 ± 0.16	1.80 ± 0.02	1.68 ± 0.15	0.760	0.008	0.127
F. prausnitzii	1.72 ± 0.11	1.75 ± 0.17	1.59 ± 0.01	1.47 ± 0.12	0.553	0.016	0.288
R. bromii	1.04 ± 0.13	1.13 ± 0.15	1.18 ± 0.03	1.08 ± 0.08	0.925	0.492	0.160
A. muciniphila	1.74 ± 0.32	1.36 ± 0.13	1.88 ± 0.02	1.67 ± 0.04	0.019	0.053	0.450
Bifidobacterium spp.	3.26 ± 0.30	3.33 ± 0.12	3.10 ± 0.05	3.10 ± 0.07	0.726	0.073	0.714
Lactobacillus spp.	2.59 ± 0.29	2.83 ± 0.06	3.21 ± 0.08	3.09 ± 0.03	0.532	0.001	0.084
Roseburia spp.	1.53 ± 0.14	1.65 ± 0.12^	1.44 ± 0.03	1.28 ± 0.11	0.787	0.006	0.049
Bacteroides/Prevotella	3.64 ± 0.18	3.73 ± 0.14	3.61 ± 0.01	3.56 ± 0.08	0.719	0.203	0.329

Data are mean (n = 3) ± SD. ^$p \leq 0.05$ compared to HAB diet on the same day.

variability between MRE 0 and 21 day relative to HAB 0 and 21 day. However, a fermentation-induced convergence in the microbial communities after 24 hour exposure to RSM from individuals consuming habitual diets (i.e., MRE day 0, and HAB day 0 and 21) was evident, with distinct clustering in individuals consuming MREs (i.e., MRE day 21, PERMANOVA $p = 0.02$, Figure 2(a)). The strongest contributors to the variability in this response were *F. prausnitzii, A. muciniphila* and *Lactobacillus spp*. (Figure 2(b)).

Absolute changes in abundance of individual taxa, represented as change scores over the course of fermentation (0 to 24 hours), elucidated differential competition for RSM (Figure 2, Table 2). Change scores compensate for vessel-to-vessel and replicate variations within the fecal inoculum by representing abundance changes derived from qPCR products during the 24 hour exposure to RSM as a function of study diet. *Lactobacillus* proliferation in response to RSM was reduced following MRE consumption ($p < 0.001$, Figure 2(c)), while *R. bromii* proliferation, negligible at day 0 in the MRE-diet subjects, substantially increased after the 21 day intervention ($p = 0.022$, Figure 2(d)). *E. rectale* proliferation in response to RSM also differed between diet groups, with a significant change between the 0 and 21 day HAB diet that was not evident with the MRE-diet ($p = 0.015$).

Discussion

Diet change as a stressor has been shown to induce alterations in competitive microbial dynamics, resulting in microbiota compositional changes. Microbiota shifts have been shown in animal studies during acute and prolonged food restriction,[15,16] low non-digestible carbohydrate intake[17] or diets high in fat and protein.[18,19] Similar responses to acute changes have also been reported in a limited number of human studies.[2,20] In contrast, other human studies have shown that acute diet fluctuations have not impacted gut microbial composition.[21–23] Our findings similarly did not show compositional changes as a function of the MRE-diet perhaps due to the subtle nature of the perturbations. However, *in vitro* fermentation revealed changes in inter-species microbial competition dynamics involving taxa similar to those observed due to food deprivation, altered micronutrients levels and diet composition.

Lactobacillus and *Ruminococcus* are gram-positive *Firmicutes* that are specialists for the degradation of specific glycan structures, which allows them to dominate their niches.[24] *Lactobacillus spp*. in particular are key beneficial human gut organisms.[7,8] Here, we observed an attenuation of *Lactobacillus* competitive growth dynamics due to the MRE diet. Although *Lactobacillus* cannot directly utilize resistant starch (RS), they metabolize intermediate breakdown products, like monosaccharides and pyruvate produced by RS degraders.[24,25] In support, rodent studies have demonstrated *Lactobacillus* growth after RS supplementation.[26,27] Observed differences in *Lactobacillus* abundance following RSM fermentation may therefore have resulted from shifts in the abundance of unmeasured microbes that initiate RS degradation, increased competition for substrate, and/or alterations in metabolic pathways. In contrast, *R. bromii* exhibited greater growth following the MRE diet. As a keystone species for RS degradation,[6] increased growth of

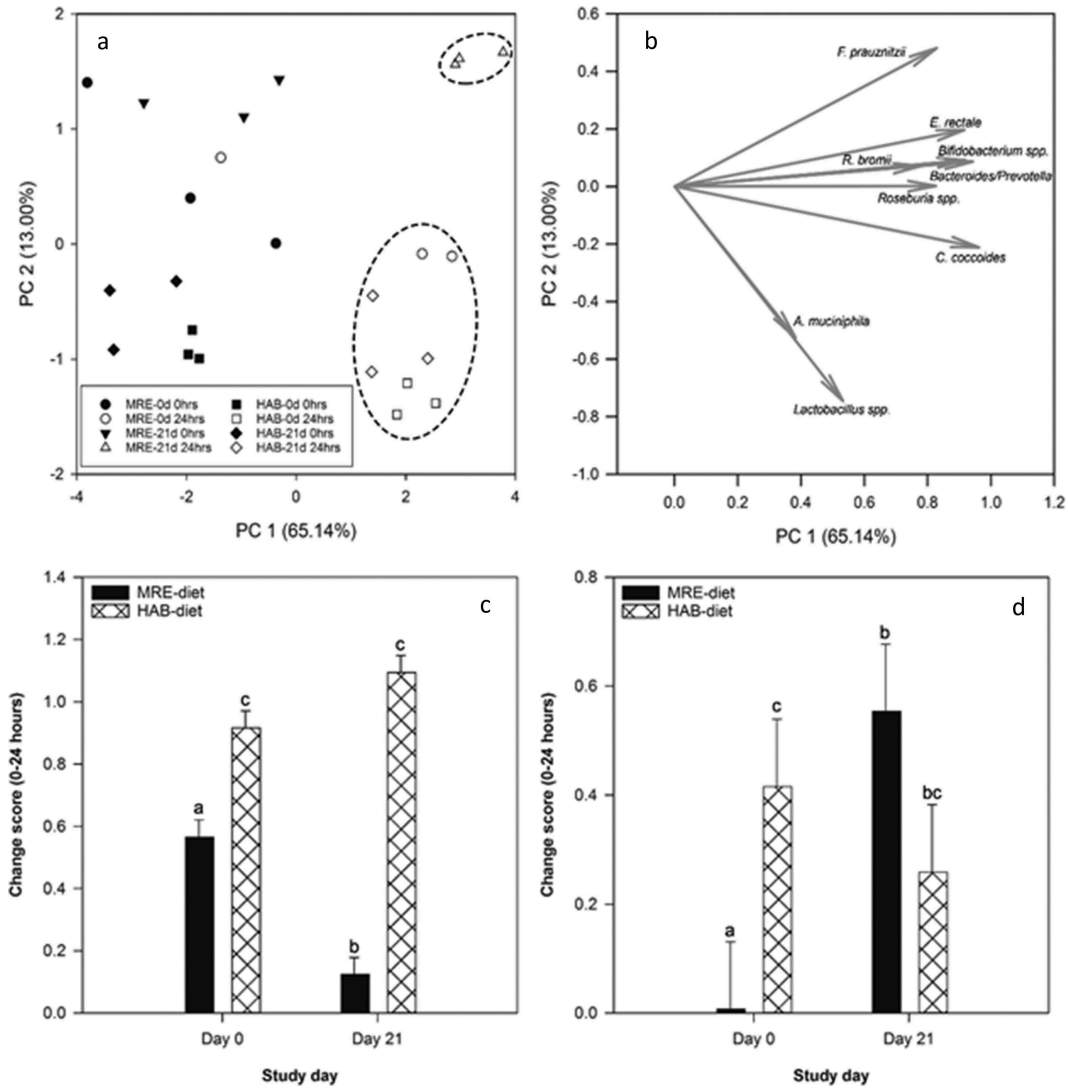

Figure 2. Inter-species competition for resistant starch supplemented medium (RSM) as a function of study diet. Microbial community variation for HAB and MRE diets (a) Principle component scores and (b) component loading plot from Principle Components Analysis (PCA) of qPCR products and absolute abundance changes (shown as 0–24 hour change scores) for *Lactobacillus spp*. (c) and *R. bromii* (d) in response to RSM (n = 3). Comparison of selected bacteria species and groups at 0 and 24 hours of fermentation demonstrates an effect of MRE consumption on inter-species competition for RSM (a, b). *Lactobacillus spp*. competitive growth dynamics was attenuated after 21 days of MRE intervention compared to 0 days; conversely, *R. bromii* exhibited an enhanced growth response to RSM after 21 days MRE intervention (c, d). Shared letters within each graph indicate no significant difference ($p > 0.05$). A small constant (0.015) was added to the *R. bromii* change scores to improve visibility of the bars; statistical analyses were performed on the original values.

Table 2. qPCR log copy numbers per mL culture (change scores 0–24 hour fermentation).

	MRE Day 0	MRE Day 21	HAB Day 0	HAB Day 21	Main effect (day)	Main effect (diet)	Interaction (day*diet)
C. coccoides	0.57 ± 0.10	0.61 ± 0.19	0.56 ± 0.07	0.56 ± 0.08	0.728	0.692	0.784
E. rectale	0.39 ± 0.13	0.35 ± 0.13^	0.40 ± 0.04	0.77 ± 0.12*	0.036	0.010	0.015
F. prausnitzii	0.13 ± 0.20	0.47 ± 0.23	0.19 ± 0.04	0.27 ± 0.05	0.046	0.451	0.166
R. bromii	0.00 ± 0.03^	0.54 ± 0.31*	0.40 ± 0.07	0.24 ± 0.10	0.154	0.658	0.022
A. muciniphila	0.09 ± 0.22	0.34 ± 0.14	0.00 ± 0.03	−0.06 ± 0.23	0.358	0.040	0.189
Bifidobacterium spp.	1.19 ± 0.14	1.13 ± 0.05	1.01 ± 0.08	0.96 ± 0.05	0.301	0.008	0.854
Lactobacillus spp.	0.57 ± 0.09^	0.12 ± 0.13*^	0.92 ± 0.11	1.09 ± 0.02	0.044	<0.001	<0.001
Roseburia spp.	0.04 ± 0.21	0.07 ± 0.18	0.28 ± 0.08	0.57 ± 0.09	0.103	0.003	0.171
Bacteroides/Prevotella	0.56 ± 0.18	0.61 ± 0.13	0.48 ± 0.05	0.46 ± 0.02	0.880	0.100	0.610

Data are mean (n = 3) ± SD. *$p \leq 0.05$ compared to Day 0 for same diet; ^$p \leq 0.05$ compared to HAB diet on the same day.

R. bromii after MRE exposure may indicate reduced competition for RS.

In this study, *in vitro* fermentation was used to demonstrate that an acute stressor alters *in vitro* competitive growth dynamics of individual taxa within fecal microbiota exposed to the same environmental conditions and nutrients. That the MRE-diet resulted in suppressed growth of *Lactobacillus* in response to RSM is of particular interest as this genera is known to enhance gut barrier integrity and immune function,[28,29] which are both compromised by military-relevant stressors.[30,31] This genus was also recently shown to be suppressed in the gut microbiota of Soldiers sojourning at high altitude.[23] More research is needed into the value of promoting *Lactobacillus*; however, this work indicates future studies should consider that the response of *Lactobacillus*, and potentially other beneficial microbes, to nutrient supplementation may differ as a function of diet or stress. The *in vitro* analysis revealed variable microbial growth dynamics that would not be apparent if solely examining changes within fecal microbial community compositions typically performed for human microbiome studies. Furthermore, *in vitro* fermentation may provide a time and cost efficient approach to disentangle that variability and identify candidate nutrients for favorably modulating the gut microbiota and serve as a complement to traditional genomic analyses.

Methods

Participants

Ten men (of n = 64 total participants; 18–62 years of age; BMI ≤30 kg/m^2) participating in a parallel-arm, randomized controlled trial conducted in Natick, MA between June 2015 and March 2017 were selected for this experiment. Exclusion criteria for the randomized trial can be found on the Online Supplemental Material.

Study design and diet

At enrollment participants were randomly assigned using computer-generated randomization to one of two study groups; the control group (HAB-diet) was instructed to maintain their normal diet and eating patterns throughout the study, and did not receive any study food or beverages. The intervention group (MRE-diet) was provided with 2–3 U.S. military ration meals/day (see supplementary information for description) and instructed to consume only those foods and beverages for 21 consecutive study days (see supplementary information for additional details).

Batch fecal fermentation

All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. Fermentations were conducted using an HEL BioXplorer 100 (HEL Group, Borehamwood, United Kingdom) (Figure 1). A single fecal sample was collected during baseline (study days −10 to 0) and again during study days 20–21 from each subject. For additional information about sample collection refer to the Online Supplemental Material. Fecal fermentation medium was prepared based on Macfarlane et al.[32] with the following modifications: addition of resazarin (1 ug/L) and supplemented with potato starch (15 g/L). After mixing well, the nutrient-rich medium was added to fermentation vessels (125 mL/vessel) equipped with oxidation-reduction potential and pH probes (Applikon Biotechnologies, Foster City, CA), autoclaved for 35 minutes at 120 psig, and equilibrated overnight under constant headspace flush with oxygen-free N_2 (20 psig, 5 mL/minute) without pH adjustment. Calibration drift within pH probes was corrected by manual verification of pH. Vessels were adjusted and maintained to emulate the ascending colon (pH 5.5) by addition of 1N NaOH and 0.2N HCl.

For both days 0 and 21, equal proportions of fecal slurry aliquots from HAB-diet subjects (n = 5) and MRE-diet subjects (n = 5) were thawed and pooled separately in serum bottles in an anaerobic chamber (Coy Labs, Grass Lake, MI) just prior to inoculation. After pH equilibration, fermentation vessels were inoculated through the headplate septum using an 18 gauge syringe while under continuous gas flush. Vessels were inoculated with 10% ($^{v}/_{v}$) fecal slurry from 0 and 21 day HAB and MRE subjects. Parallel control vessels were inoculated with cell-free phosphate

buffer/glycerol. Single aliquots were removed from each vessel at 0 hour (inoculation) and after 24 hour incubation and stored at −80°C for DNA extraction and qPCR analysis. Fermentations were run in triplicate as experimental replicates.

qPCR

DNA from fecal samples was extracted using the QIAMP Power Fecal DNA Extraction Kit, QIAGEN, Inc. (Germantown, MD). DNA concentration (ng/uL) was quantified using Nanodrop (ThermoFisher Scientific, Inc., Waltham, MA). For absolute abundance qPCR analysis, standard curves were constructed using pure culture DNA from representative gut species purchased from ATCC (Manassas, VA): *Bacteroides thetaiotaomicron* 29148, *Bifidobacterium animalis subsp. lactis* 700541 and *Lactobacillus reuteri* 23272. For the remaining six organisms, a pool of 3 different fecal samples were used as a starting material[33,34] (SFig. 1). 10-fold serial dilutions were prepared in DNAse and RNAse free water. qPCR efficiency and quality control parameters ranged between 80–100%. Specific sets of primers were used to quantify each bacterial group (STable 2). qPCR reactions were carried out using the 2X Forget-Me-Not qPCR Master Mix (Biotium, Hayward, CA) and the iCycler iQ Optical module (Bio-Rad Laboratories, Hercules, CA). Genome size for each microorganism was used to calculate the copy number (http://cels.uri.edu/gsc/cndna.html).

Statistical analysis

Copy number/mL from qPCR were log-transformed and underwent Principal Component Analysis (PCA) with PERMANOVA to determine sample clustering. Fermentation change scores for each organism were calculated by subtracting copy number/mL at the time of inoculation (0 hour) from copy number/mL measured at 24 hours. Both change scores and 0 hour copy number/mL were subjected to 2-way ANOVA, with diet, study day and day-by-diet interaction as fixed factors and using Tukey's Least Significant Difference test for multiple comparisons. In models where fermentation change score was the dependent variable, a significant day-by-diet interaction indicated that the change in the growth of that organism during the 24 hour fermentation from 0 to day 21 differed as a function of the diet. PERMANOVA was performed using the R package Adonis2; all other statistical analyses utilized SigmaStat 4.0 (Systat, San Jose, CA). Significance was set at $p \leq 0.05$.

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Disclosure of Potential Conflicts of Interests

The authors report no disclosures of interest.

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