PERSPECTIVE

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Getting a grip on things: how do communities of bacterial symbionts become established in our intestine?

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The gut contains our largest collection of resident microorganisms. One obvious question is how microbial communities establish and maintain themselves within a perfused intestine. The answers, which may come in part from observations made by environmental engineers and glycobiologists, have important implications for immunologists who wish to understand how indigenous microbial communities are accommodated. Here we propose that the mucus gel layer overlying the intestinal epithelium is a key contributor to the structural and functional stability of this microbiota and its tolerance by the host.

A highly diversified community of microorganisms lives in our intestine with densities approaching 10^{12} microbes per milliliter of luminal contents in the distal gut[1]. This microbiota provides a variety of metabolic properties that we have not fully evolved, including the ability to abstract nutrients from the diet that would be otherwise indigestable (such as plant polysaccharides)[2]. The composition of this community of 10 trillion to 100 trillion members varies within the gut and between individuals[3,4]. Most members belong to the domain Bacteria, but there are also representatives from Archaea[5] and Eukarya, plus many viruses and bacteriophages[6]. The true extent of biodiversity remains unclear, as is the case in most natural ecosystems[7,8]. Most species seem to be refractory to cultivation using methods available now. Enumeration studies, based on culture-independent molecular techniques, such as 16S rDNA genotyping[4,9], have yet to be systematically applied to the length of the gastrointestinal tract.

'Back of the envelope' calculations suggest that there are at least 500–1,000 species residing in the adult human intestine, that the collective size of their genomes may be equivalent to the size of our own genome and that the number of genes in this 'microbiome' may exceed the number of our genes by as much as two orders of magnitude[10]. The high percentage of unique and uncharacterized proteins found by microbial community genome sequencing projects (ref. 11 and http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl, ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria and http://wit.integratedgenomics.com/GOLD/) indicate that 'mining' this microbiome may yield a rich load of previously unknown gene products that could have a variety of biomedical applications.

In considering how microbes and humans have coevolved in the intestine to forge symbiotic relationships, the dynamic nature of this ecosystem must be taken into account. Our intestine is lined with an epithelium that turns over rapidly and continuously throughout life: up to one billion to three billion cells are shed per hour in the small intestine, and approximately one tenth that number, in the colon[10]. Despite the rapid turnover of epithelial cells and the brisk rate of propulsion of food and water through the gut by peristalsis, some microbial species are able to establish themselves as entrenched residents of a given intestinal niche ('autochthonous' components[1]), whereas other species have a more nomadic existence ('allochthonous' components). This situation raises questions about what factors distinguish 'residents' from 'tourists', how microbial consortia establish themselves in different niches and how some species avoid washout. The answers have important implications for workers in many fields, ranging from those who study the foundations of syntrophy, which is an interaction that allows otherwise unavailable nutrients to be used by organisms with complementary metabolic capabilities, to ecogenomicists who study how the environment influences genome structure, function and evolution[7], to immunologists who seek to understand how a healthy host is able to accommodate a vast array of gut microbes and how this tolerance is lost in certain conditions, such as inflammatory bowel diseases[12].

Here we combine observations made by environmental engineers, glycobiologists and immunologists and propose that symbionts inhabiting the polysaccharide-rich mucus gel layer overlying the gut epithelium constitute a biofilm-like community and that retention in such a matrix benefits the host by promoting functions served by the microbiota, including digestion of luminal contents and fortification of host defenses. 'Biofilms' are broadly defined as dense cohesive communities of microbes that embed themselves within surface-associated matrices and resist hydrodynamic shear forces. The matrix often is composed of polysaccharides. Studies in other ecosystems indicate that when communities of microbes transition from a free-living to a sessile, matrix-encrusted biofilm state, they become persistent and resilient and show altered transcriptional profiles[13,14]. For example, compared with their planktonic forms, *Pseudomonas aeruginosa*

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biofilm communities persist within the respiratory tract of patients with cystic fibrosis and lead to reduced neutrophil oxidative bursts[15] and decreased complement activation[16] plus reduced recognition by opsonic antibodies[17]. Emerging evidence suggests that a dynamic interplay between the microbiota, the mucosal immune system and mucous gel layer may affect microbial community structure in the intestine. Multiple components of the mucosal barrier (dendritic cells[18], Paneth cells[19,20]) are capable of distinguishing and responding to specific microbial species. This species-specific recognition raises the question of how the mucosal immune system shapes and is shaped by microbial community composition and structure. For example, more than 80% of the mouse plasma cell population resides in the lamina propria of the intestine, with IgA being the predominant immunoglobulin isotype present in mucosal secretions. IgA responses in the gut arise mainly in its organized lymphoid structures (Peyer's patches, isolated lymphoid follicles), which contain conventional B2 cells, although B1 cells in the peritoneum also give rise to IgA-generating plasma cells[21]. *In situ* switching from IgM to IgA production by B1 and B2 cells is facilitated by dendritic cells[22]. Mucosal dendritic cells can harbor members of the microbiota for several days, allowing them to selectively induce localized IgA responses[23]. Studies of activation-induced cytidine deaminase–deficient mice indicate that IgA is prominent in regulation of the composition of the microbiota and attachment of its members to the epithelium[21]. IgA production is also regulated by mucosal-associated invariant T cells, which in turn are dependent on the microbiota for their proper development[24].

A subset of secretory IgA reacts with immunodeterminants expressed by members of the microbiota[25]. Together with mucus, secretory IgA can serve to anchor cultured human fecal bacteria on a human enterocyte-like cell line[26]. Although the mechanism underlying this anchorage is not known, partial deglycosylation of host glycans by the bacteria may expose core carbohydrate structures that serve as binding sites for IgA[26]. The importance of such anchorage in a polysaccharide-rich biofilm, within a perfused living intestinal 'bioreactor', is illustrated by observations made in mechanical bioreactors by environmental engineers.

Retention from the perspective of an environmental engineer

Engineers like to model the gut as a so-called 'plug-flow' reactor. An example of a plug-flow reactor is the horizontal flow, anaerobic, immobilized biomass (HAIB) reactor used for wastewater treatment (**Fig. 1a**). HAIB consists of microbial communities attached to an immobilized static carrier material that facilitates biofilm formation. These microbes process a unidirectional flow of wastewater within the anaerobic tube. As in the gut, microbial composition, substrate concentrations and pH vary along the length of the HAIB reactor[27].

The performance of the bioreactor is governed to a large extent by the retention time of its slowest-growing critical microbial component[28]. If the generation time of this component is longer than the wastewater (nutrient) retention time, there must be a mechanism to prevent washout. Formation of a biofilm on carrier material represents a key mechanism for retention of a mature microbiota, composed of members of Bacteria and Archaea, within the reactor vessel[27]. Effective retention leads to a coveted increase in the ratio of wastewater flux to reactor volume. Capital costs are reduced because of reductions in reactor size. By analogy, biofilm formation within the gastrointestinal tract would result in decreased gut size while maintaining efficiency, thereby conferring a distinct evolutionary advantage.

Five chemostats (bioreactors for growing bacteria in defined environmental conditions) were linked in series to create a simulator of the human intestinal microbial ecosystem[29]. Growth of fecal suspensions within this ecosystem results in homogeneous populations across the terminal three vessels, despite gradients of substrate concentration and pH[30]. This homogeneity reflects the inability of microbes to anchor themselves in the absence of a static carrier material. In contrast, gradients of substrate concentration and composition over the length of the biofilm-promoting HAIB reactor lead to regional differences in microbial ecology because a static carrier material is present.

Environmental engineers have found that biofilms can also form without carrier materials through a process known as 'self-immobilization'. Rapidly settling, dense, spherical biofilm aggregates were first identified in anaerobic upflow systems[31] (**Fig. 1b**). Formation of these granules

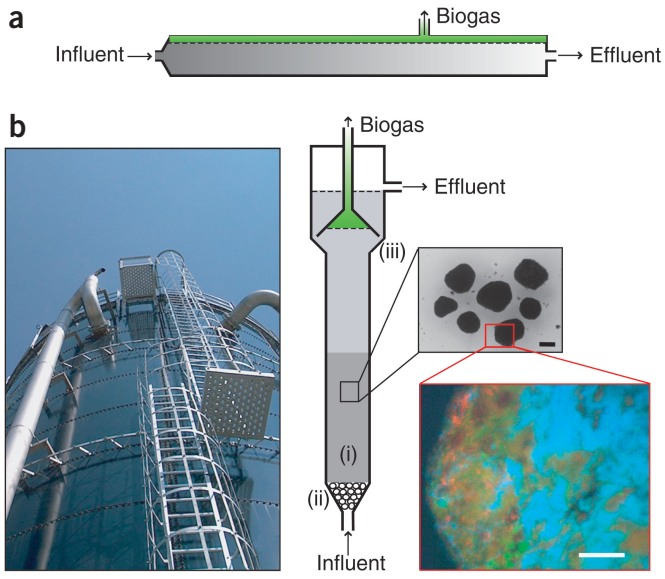

Figure 1 Engineered bioreactors provide clues as to how microbial communities are maintained. (**a**) HAIB bioreactor. This bioreactor requires a static carrier material (such as immobile polyurethane) that promotes formation of a dense biofilm. The bioreactor is fed from left (influent is wastewater) to right (effluent is the clear liquid). The microbial community structure changes over the length of the bioreactor, reflecting changes in substrate levels and pH. Biogas, a mixture of methane and carbon dioxide, is withdrawn from the top. (**b**) Anaerobic upflow bioreactor. The reactor is fed from the bottom. An upflow hydraulic pattern is maintained that keeps the granular blanket (i) in a 'fluidized' state. A feed distribution system on the bottom of the reactor (ii) is required to distribute the influent evenly over the plain of the reactor. A gas-solids separation system (iii) is required to provide internal settling of granules, thereby preventing them from washing out with the effluent because of mixing effects by biogas production. Dense, well settling, granular biofilms form spontaneously as selection pressures inherent to the system eliminate less cohesive community members. Top inset, view of granules by dissecting microscopy. Granules often appear black because of iron sulfide formation in the reduced anaerobic environment. Scale bar, 1 mm. Bottom inset, view of a sectioned granule (by epifluorescence microscopy) analyzed by fluorescence *in situ* hybridization analysis with rRNA probes (green, Archaea; red, Bacteria; blue, *Methnosaeta concilii*). *M. concilii* resides internally and provides the backbone to which other Bacteria and Archaea adhere. Scale bar, 50 μm. Fluorescence *in situ* hybridization photo courtesy of D. Zheng and L. Raskin (Department of Civil and Environmental Engineering, University of Illinois, at Urbana-Campaign, Urbana, Illinois). Full-scale reactor photograph courtesy of Biothane Corporation (Camden, New Jersey).

(known as granulation or self-immobilization) in the upflow system occurs as a result of hydraulic as well as microbial selection pressures. Systems that combine shear forces with a way of separating poorly settling from well settling biomass are able to select for granules, provided that the hydraulic selection pressure is moderate enough to develop a microbial community containing sufficient amounts of all the necessary components required to initiate granulation[32]. Environmental engineers have found that nuclei for granule formation consist entirely of microorganisms. Some evidence indicates filamentous methanogens (Archaea) serve this purpose (ref. 32 and D. Zheng, L.T.A. and L. Raskin, unpublished data). These methanogens function as the backbone of the aggregate on which other Bacteria and Archaea grow to form a dense granule. Microorganisms within the granule produce the matrix in which the community is embedded. The definition of the biofilm holds (discussed above), but the biofilm is not attached to a carrier material (hence the term self immobilization).

Retention as viewed by the glycobiologist

Given the observations and considerations described above, we propose that the following criteria need to be satisfied to identify a structure in the intestinal ecosystem as a biofilm: it must have a polymer-based matrix (such as polysaccharide); it must be capable of recognizing components evolved by microbes to mediate their attachment and thus oppose their washout; and it must facilitate nutrient harvest and exchange.

The mucus gel layer that overlies the gut epithelium satisfies these criteria. It is a dense matrix of polysaccharides (and proteins) derived mainly from the epithelium's goblet cell lineage. Its thickness and glycan composition vary along the length of the gut[33]. Mucin proteins, the main component of mucus, may be secreted or cell associated and contain regions rich in *O*-linked glycans. Analysis of carbohydrate structures along the length of the gastrointestinal tract of two humans showed that although their mucin-associated glycans were diverse, their region-specific glycosylation patterns were well conserved[34]. These conserved glycan structures may help direct members of the microbiota to distinct host niches by serving as nutrient sources for these organisms (discussed below)[35].

The mucus gel layer also satisfies the second criterion: it can serve to oppose washout of microbes. Mucus turns over more rapidly than the underlying epithelium but more slowly than the transit time of food. Mucin-targeted microspheres have longer gastrointestinal transit times than those of uncoated microspheres[36]. Mucus promotes host-symbiont interactions in both invertebrate and vertebrate systems. For example, the Hawaiian squid *Euprymna scolopes* uses mucus secreted from cells near its light organ to selectively amass its bioluminescent symbiont, *Vibrio fischeri*, from the ocean[37].

Bacteroides thetaiotaomicron, the first prominent symbiotic bacterial species in the human distal intestine whose genome was sequenced[38], contains a 4,779-member proteome that lacks proteins with homology to known adhesins. However, it has evolved 163 paralogs of two outer membrane polysaccharide-binding proteins (SusC and SusD)[10,38]. It is likely that these families of proteins have been expanded to help this symbiont retrieve and use a variety of dietary carbohydrates. These proteins may also serve to mediate bacterial attachment to mucus glycans. DNA microarray–based profiling of the *B. thetaioatomicron* transcriptome has confirmed that these genes show notable differences in their expression patterns as a function of growth phase and nutrient availability (J.L.S. and J.I.G., unpublished data).

Mucus also satisfies the third criterion: it can serve to enhance nutrient harvest and exchange. *In vitro*, *E. coli* can grow rapidly in mucus obtained from the mouse intestine but not in intestinal luminal contents. Whole-genome transcriptional profiling of *E. coli* strain MG1655 during growth *ex vivo* on cecal mucus showed induction of genes involved in the use of several component sugars[39]. Mutants that lack the ability to penetrate mucus are unable to colonize the mouse gut; moreover, *in vivo* competition studies using wild-type MG1655 and isogenic mutants that lack the ability to catabolize various nutrients confirmed that carbohydrates are dominant in the initiation and maintenance of colonization of the intestines of conventionally raised mice[39]. The relative preference for seven sugars *in vitro* mirrored the effect of mutations affecting their utilization pathways on fitness *in vivo* (gluconate→*N*-acetylglucosamine→*N*-acetylneuraminic acid equivalent to glucuronate→mannose→fucose→ribose)[39]. *B. thetaiotaomicron* contains a large number (172) of predicted glycosylhydrolases, including those that process mucins[38]. Cocolonization studies of germ-free mice have demonstrated that wild-type *B. thetaiotamicron* 'outcompetes' an isogenic strain that lacks the capacity to use a subset of mucopolysaccharides[40,41]. Thus, this highly successful human gut symbiont has evolved mechanisms that allow it to attach to and graze on mucus-associated glycans. *Bacteroides* is a predominant genus in normal adult colon[4,9]. If members of *Bacteroides* are able to avoid washout from the ecosystem by attaching to mucus, other bacterial species stand to benefit: they can harvest oligosaccharides generated by the numerous glycosylhydrolases exported by *Bacteroides* spp. (ref. 38 and ftp://ftp.sanger.ac.uk/pub/pathogens/bf/) as in the case of *E. coli*, which depends on mucosal-derived monosaccharides for colonization, yet lacks of the requisite glycosylhydrolases for mucus degradation. Other microbes (methanogens) use the short-chain fatty acid products of microbial fermentation of carbohydrates. Removal of substrates from vicinity of *Bacteroides* spp. may also be advantageous for stoichiometric reasons, driving reactions that are otherwise energetically unfavorable. The net result is promotion of symbiotic and syntrophic relationships[42].

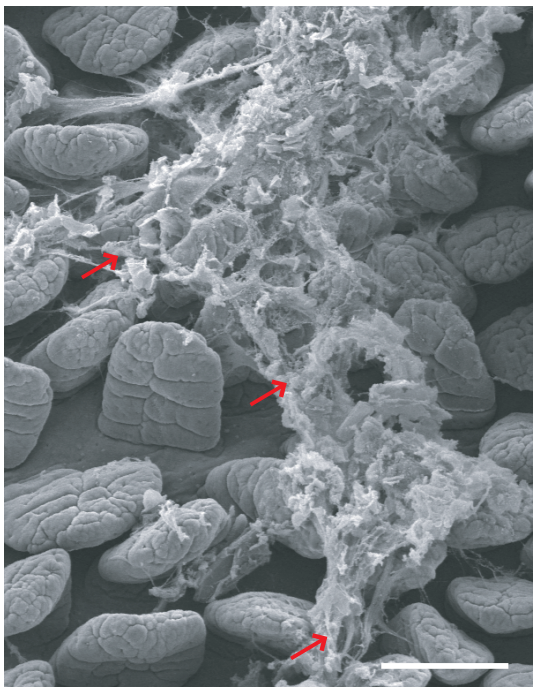

Figure 2 View of the distal small intestine of a mouse by scanning electron microscopy. The fingerlike projections are villi. Most of the mucus gel layer that normally overlies the villus epithelium has been lost during sample processing; arrows indicate remnants. Scale bar, 100 μm.

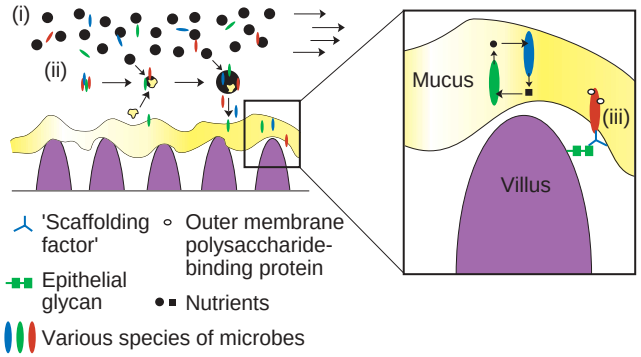

Figure 3 Proposed mechanism for microbial retention in the gut. By analogy to anaerobic upflow bioreactors that lack static carrier material, biofilm formation and retention of autochthonous components of the microbiota is made possible in part by use of mucus as a key element in the self-immobilization process. (i) Poorly settling materials (undigested food particles, planktonic bacteria) are rapidly washed out. (ii) Dense aggregates are formed by microbes themselves, undigested food, shed elements of the mucus gel and/or exfoliated epithelial cells. Aggregates serve as a scaffold for assembling microbial consortia, may be further transformed through microbial or mechanical processing and may have dynamic interactions with other granules and/or with the mucus gel layer. (iii) Aggregates and mucus promote nutrient harvest and metabolic exchange. Outer membrane polysaccharide-binding proteins may facilitate attachment of some species, such as members of *Bacteroides*, to mucus glycans. These interactions can be regulated by 'scaffolding factors', such as host or microbial lectins, and host IgA. Regional variation in the glycan composition and thickness of the mucus biofilm could serve as a 'molecular zip code' that helps promote niche-specific interactions and nutrient harvest. The sum of all interactions (i–iii) helps define the propensity for washout within a given niche.

These ideas can be tested experimentally using mice with reduced colonic mucin due to an engineered ablation of their goblet cells[43] and/or by assaying the effects of knocking out specific components of the host glycobiome and examining the effects on microbial ecology ('glycobiome' refers to the repertoire of genes involved in acquisition, biosynthesis and degradation of carbohydrates; http://afmb.cnrs-mrs.fr/CAZY/ contains a list of members of the human and mouse glycobiome). It will also be useful to determine whether partitioning of members of the intestinal microbiota to, and within, a mucus gel can be correlated with mucus glycan composition and features of the glycobiomes of embedded versus nonembedded species. The distribution of viable microbes in the (gastric) mucus of mice has been defined by micromanipulator-directed harvests[44]. Fluorescence *in situ* hybridization–secondary ion mass spectrometry represents a new tool for direct *in situ* identification of individual microbes and their metabolites[45] and may yield high-resolution views of microbial distribution within mucus gels and their physiological responses to this milieu.

The distribution of microbes within this gel may have an effect on their ability to be recognized by the host immune system. *B. thetaiotaomicron* and *Bacteroides fragilis* each contain eight conserved capsular polysaccharide synthesis loci. Elegant studies have shown that *B. fragilis* is able to modulate the character of its surface glycan landscape[46]. Modulation occurs through a serine site-specific type of DNA invertase (Mpi) that targets at least 13 loci distributed throughout the genome, including the promoter regions of seven capsular polysaccharide synthesis loci and a polysaccharide biosynthetic locus[47]. Modulation of carbohydrate structures on the bacterial cell surface may be a conserved mechanism for microbial adaptation to changes in the host glycan landscape and a way for autochthonous species to evade detection by the immune system. The signals that direct these changes remain to be defined. Intimate exposure to mucus components could be instructive, regulating expression of bacterial glycosyltransferases in addition to glycosylhydrolases.

Attachment without carrier material

Given that biofilm formation and biomass retention occurs in engineered anaerobic upflow reactors that lack a carrier material, mucus might be involved in self-immobilization. Dense aggregates could be formed by microbes themselves, by undigested food particles and/or by sloughed mucus and epithelial cells. These aggregates could serve as a scaffold for the assembly of microbial consortia and promotion of their syntrophic relationships. The aggregates could be further transformed through microbial or mechanical processing and have dynamic interactions with other aggregates and/or with the mucus gel layer (**Fig. 2**). Interactions could be influenced by 'scaffolding factors', such as secretory IgA[25], or host and microbial lectins (**Fig. 3**). The sum of these interactions would define the predilection of community members for washout from a given intestinal niche, as well as expression of the metabolic activities by niche-associated consortia.

Experimental support has been provided for some of these ideas[48,49]. Particulate-associated bacteria isolated from human fecal samples were capable of degrading insoluble plant cell wall polysaccharides (such as xylan or arabinogalactan), unlike organisms recovered from the nonparticulate fraction. Culture-based enumeration studies demonstrated considerable overlap between these fractions, although some genera seemed to be excluded from the particulate component. Thus, differences in the observed metabolic activities of the two fractions could reflect variations in their microbial species composition and/or the availability of substrates that induce specific microbial polysaccharide degradation pathways.

Prospectus

The gut microbiota epitomizes features found in all living systems: physical and functional compartmentalization, structural stability, selective signaling among component parts, the ability to adapt and a capacity for self-reproduction. The robustness of the system undoubtedly reflects very highly (co)evolved feedback and 'feed-forward' controls involving both microbes and host. The challenges for understanding how the parts in this living system work together on a micro and a macro scale are great. For example, in addition to *in vivo* genetic screens for microbial genes that are essential for survival within the host[50–52], it will be important to develop methods to accurately measure the rates of replication of autochthonous versus allochthonous species within given gut niches[53]. At the same time, we must further define the composition of key materials such as the mucus gel layer along the length of the gut, their rates of turnover, the mechanisms by which they promote nutrient and metabolic exchange, the nature and degree of their alterations in response to genetic or environmental diversity (diet), and the effects of their perturbations on gut microbial ecology.

An understanding of the adaptations of symbionts versus pathogens that allow them to be included in or excluded from mucus may be an important step in gaining further knowledge of the origins of the factors that prevent or promote mobilization of immune responses to microbial epitopes. For example, *B. thetaiotaomicron* attenuates production of proinflammatory cytokines by a human enterocytic cell line[20]. In addition, colonization of germ-free mice with *B. thetaiotoamicron* induces expression of an endogenous protein with species-selective bactericidal activity that is considerably greater for Gram-positive pathogens such as *Listeria monocytogenes*

than for Gram-negative members of the normal gut microbiota (*Escherichia coli* and *B. thetaiotaomicron*). Induction of this antibacterial protein by *B. thetaiotaomicron* occurs in the Paneth cells, an epithelial lineage that functions as a key component of the gut's innate immune system. These findings suggest that the microbiota may influence its own species composition through regulation of expression of endogenous host antibiotics[19].

The challenge now is to develop and deploy a range of *ex vivo* and *in vivo* experimental systems to explore the issue of how inclusion in or exclusion from matrices such as mucus affect host responses to the microbiota. These systems may range from man-made (mechanical) bioreactors to normal or genetically engineered germ-free model organisms that are colonized with defined collections of microbial species.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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