Engineering sporulation and germination of *Anaerostips caccae* spores as health-promoting live material

Humans and microorganisms live in a continuous interaction. Each of us has around $10^{13}$ human cells and an equal number of bacteria, most of which are found in the intestinal tract. In the intestine, bacteria and host interact via many metabolic compounds, ranging from amino acid derivatives, bile acids, to short chain fatty acids (SCFAs). An archetypal compound is the SCFA butyrate that has anti-inflammatory effects and many other beneficial effects. The delivery of this beneficial compound can be orally via pill intake but this has a major downside being a strong off-odor compound and having to take many pills a day. An innovative approach involves identifying butyrate-producing microorganisms within the microbiota, incorporating them into synthetic microbial communities, and ensuring the availability of SCFA butyrate. However, challenges arise as many butyrate producers are anaerobic bacteria susceptible to oxygen exposure, complicating their supplementation as metabolically active bacteria. Notably, *Anaerostipes caccae*, a prominent butyrate producer, is an anaerobic spore-forming organism with spores exhibiting remarkable oxygen tolerance and extended viability. Hence *A. caccae* holds great promise as a novel live material to deliver health-promoting molecules. Leveraging this spore-based survival mechanism, there's potential to stabilize significant volumes of *A. caccae* biomass for the deployment under ambient temperature and air, contingent upon optimal germination conditions. Overcoming this challenge necessitates translating research on germinant receptor proteins from aerobic bacteria such as *B. subtilis* to their anaerobic counterparts present in the intestines. Under optimal germination conditions, this biomass could be supplemented with appropriate elicitors to facilitate optimal spore germination and outgrowth within the intestinal tract, as an integral component of analyzed and/or administered synthetic communities. The challenge here is to translate ongoing work on the Bacillus germinant receptor proteins present in germinosome complexes to the germination complexes as they are identified in anaerobic intestinal bacteria. As a team of expertise on spore formers (S. Brul), anaerobic gut bacteria (J. Zhang), and molecular simulation (J. Vreede), we are uniquely suited to do so through wet-lab single-cell spore germination experiments combined with molecular dynamics simulations to extend work on the alanine triggered spore germinant receptor GerA to predicted homologues in intestinal bacterium *A. caccae*. Knowledge based mutations will be generated and analyzed both in a microscopic tracking setting as well as the gut-microbiota interaction GuMI physiomimetic system. Molecular dynamics of several residues in *B. subtilis* predicts these to be highly important in regulating water permeation (Figure 1). The amino acid Y-97 is one of the prominent ones (manuscript in preparation). The plan is to identify/characterize the germination machinery in *A. caccae* using single-cell spore germination assays. Detailed investigation of the structure and function of the *A. caccae* germination proteins by alphafold structure prediction, molecular dynamics, and enhanced sampling will provide clues on how to optimize germination for specific conditions. We propose to use a combination of in silico, *in vitro* and *in vivo* characterization.

**Figure 1.** Simulation of water intake via spore protein (left panel) and spores under the microscope (right panel)
to promote germination of *A. caccae* spores, aiming to identify the best conditions that are ready to
further verify application of *A. caccae* spores in (pre)clinical trials. [500 words]

Refs:
10) Zhang, J., et al. (2024) NPJ Biofilms and Microbiomes 10, 31

Expected outcomes

The research is expected to **pioneer a groundbreaking live material in delivering beneficial butyrate**
by harnessing the unique capabilities of the anaerobic bacterium *A. caccae*, which possesses both
spore-forming and butyrate-producing abilities. By integrating experimental, analytical, and
molecular simulation methodologies, we expect to **unlock unprecedented insights into the**
**sporulation and germination processes of this anaerobic gut bacterium**. The anticipated outcomes
hold immense significance, as they will inform the development of optimized design for producing
spores as a long-lasting live material and initiating germination to confer health benefits within the
human gut. If successful, this innovative live material will mark a significant milestone as the first of
its kind to deliver beneficial molecules for health improvement. We expect to apply patents based on
the newly obtained knowledge and the know-hows of spore germination of *A. caccae*, which will
serve as a foundation to further development of this bacterium toward the bedside. Finally, there are
hundreds of culturable anaerobic bacterial species. The outcome of this project is expected to ignite
a new field to search for more live material delivering beneficial metabolic functions. [180 words].

Time Table/Planning

We will combine the applied funding from this call and our own in-cash contribution to form a 4-year
PhD project. To realize the project, we developed four major tasks (Table 1). The tasks are
independent but interconnected. We will first optimize the sporulation conditions (Task 1), as this
will provide more spores that provides more material to be tested in the Task 2. In addition, the in
silico molecular modeling of spore germination receptor (Task 3) will help facilitate the optimization
while independently obtain molecular insights of the novel germination mechanisms of *A. caccae*
spores. Finally, the spore germination of *A. caccae* spore and butyrate production in human intestine
will be tested in GuMI gut system (Task 4) to mimick the human intestinal environment.
Table 1. Tasks and timeline of the major tasks of the project.

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<tr>
<th>Tasks</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
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<td>Task 1: Sporulation of <em>A. caccae</em>: sporulation condition optimization, microscopic &amp; proteomic analysis of sporulation. (leading PI: S. Brul)</td>
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<td>Task 2: Germination of <em>A. caccae</em>: germination condition optimization, microscopic analysis, germination receptor expression, and beneficial metabolite production. (leading PI: S. Brul and J. Zhang)</td>
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<td>Task 3: Germination receptor molecular simulation of <em>A. caccae</em> spores (leading PI: J. Vreede)</td>
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<td>Task 4: Test of beneficial effects of <em>A. caccae</em>: germination and butyrate production of <em>A. caccae</em> spores in GuMI system mimicking the human gut environment (leading PI: J. Zhang)</td>
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<td>Patent, publication and thesis writing (leading PI: all)</td>
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