BioNutrients-3: Precision Fermentation, Pasteurization, and Pathogen Detection - Towards Safe Fermentation and Production of Nutrients in Space

Frances Donovan | Synthetic Biology Project | Game Changing Development Program, Space Technology Mission Directorate
American Society for Gravitational and Space Research December 7, 2024

Nutrition in Space – Risks and Mitigations

- Nutrients degrade with time in food and supplements
  - Enhanced nutritional needs in space travel
  - Radiation and microgravity countermeasures
  - Disease-specific concerns
- Psychology of food/isolation/repetitive menu
- **Ideally produce some foods *in situ***

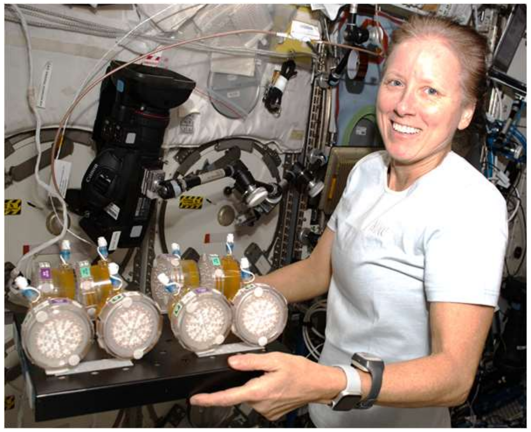
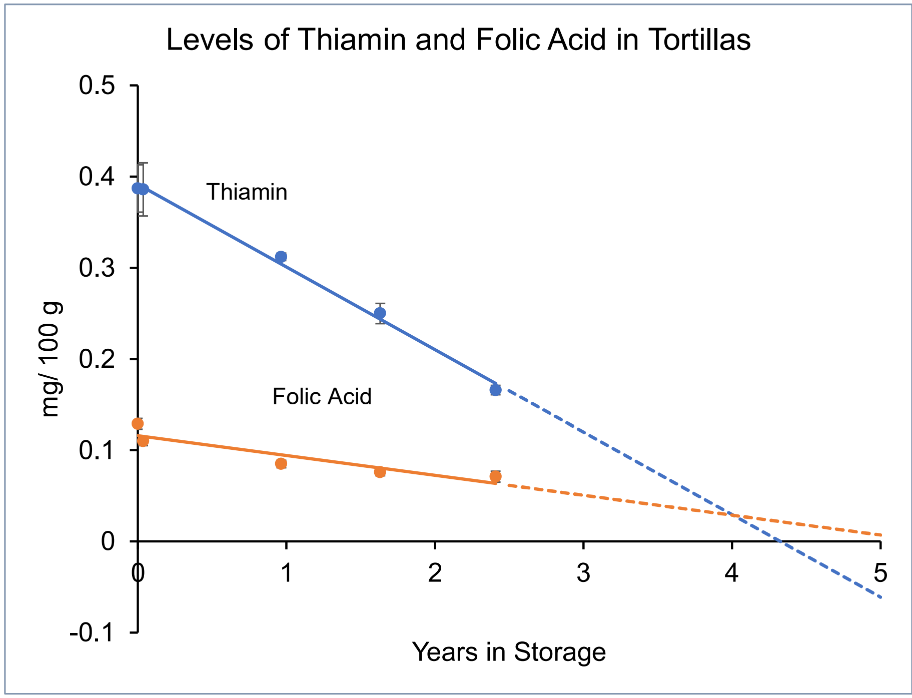


Photo Credits: NASA



Adapted from Zwart et al (2009) *Food Science* 74(7):H209-H217.
https://doi.org/10.1111/j.1750-3841.2009.01265.x

Fermented Foods and Genetic Engineering may provide a solution

Extensive history of production in non-sterile environments, amazing variety.

- Fermented foods ~5000 variations worldwide, (Ray, R.C. et al., 2024 https://doi.org/10.1007/978-3-031-72000-0_1)
- Earliest evidence for the deliberate application of fermentation circa 7000 BC, (McGovern 2004 https://doi.org/10.1073/pnas.040792110).
  - Inadvertent production and consumption of fermented foods is thought to predate this, as foods likely underwent spontaneous fermentation during storage, (Tamang 2020 https://doi.org/10.1111/1541-4337.12520)

Spaceflight compatible – Small resource requirements, long shelf life, and ease of genetic engineering.

- Fermented foods provide beneficial microbes, nutrients, bioactive compounds, flavors and food preservation.
- Genetic engineering can make fermented food products into a nutrient and therapeutic compound delivery system.

Photo credits creative commons

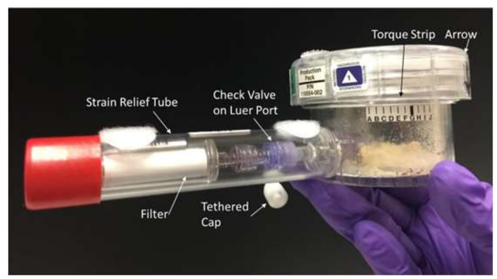
BioNutrients Overview

Aim: Develop and Demonstrate an on- demand nutrient production system for long duration missions to support nutrient degradation in stored foods.

Deliverables:

- Flight-tested, evolvable microbial production platform capable of producing many compounds (e.g., nutrients, medicines).
- Space-adapted microbial hosts for future generic use and genetic engineering.
- On-orbit safety and operational systems for future implementation.

Future Mission Implementation Concept



BioNutrients-1 Flight Production Pack Development for on-orbit testing

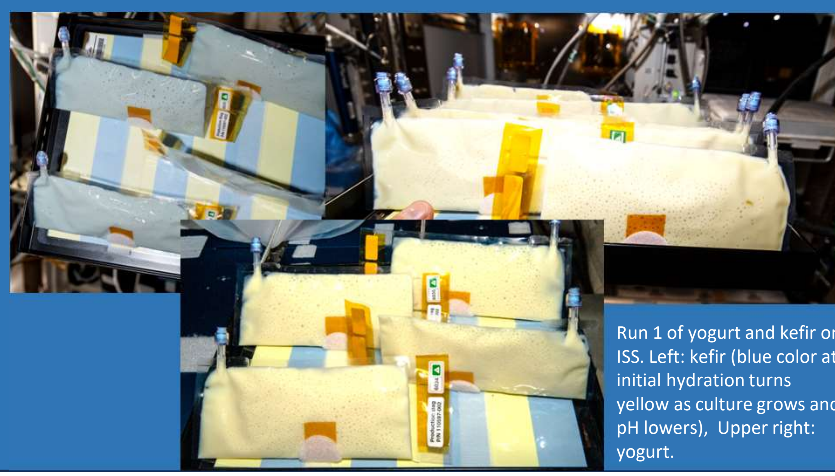
Engineering microbes to produce nutrients and medicines on-demand

Developing microbes adapted for use in space

BioNutrients-2/Gen-1 System: FEP bioreactor, new products

ISS

BN-2 yeast ISS production packs (*S. boulardii* producing beta-carotene, *S. cerevisiae* producing zeaxanthin, and *K. lactis* producing follistatin).



Run 1 of yogurt and kefir on ISS. Left: kefir (blue color at initial hydration turns yellow as culture grows and pH lowers), Upper right: yogurt.

Ground Control

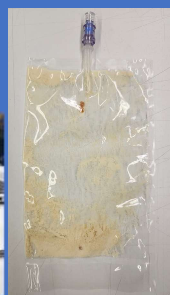
BN-2 yeast ground controls (*S. boulardii* producing beta-carotene, *S. cerevisiae* producing zeaxanthin, and *K. lactis* producing follistatin).

BN-2 ground controls yogurt top, kefir bottom.

Nicole Mann and Josh Casada hydrating and shaking the yeast and yogurt packs respectively. Featured in Space Station Science Highlights: Week of January 2, 2023 | NASA ISS Research on Twitter: "BioNutrients-2 tests an on-demand system to produce key nutrients from yogurt, kefir, and a yeast-based beverage. The study could help maintain the crew's health while reducing launch mass and volume requirements. https://t.co/fuxW39uTWd https://t.co/bzFZVYMIxS" / Twitter

BN-3 System and Experiment Overview

Improved Products and Designs


Crew access – attachable straw, same pinch closure as current food system.

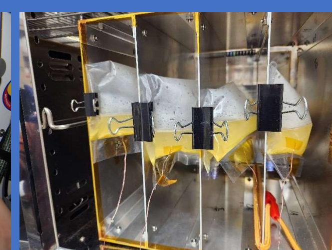
Products

- Yeast: carotenoids
- Yeast: carotenoids, riboflavin, folate (multi-nutrient)
- Yogurt – commercial
- Kefir – commercial
- Serial production - yogurt

E-Nose Compatible Design

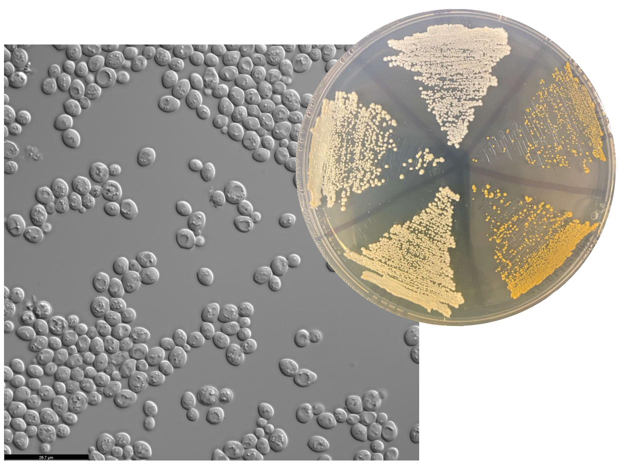

Pasteurization Capable


ISS GRFW


ARC test unit

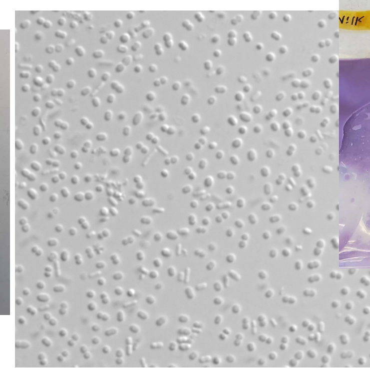
BN-3 Sample Types - Organisms

Flight Strain	**Product**	**Origin**	**Host Strain Designation**
Saccharomyces cerevisiae Y55 (Carotenoid strain)	Beta-carotene, zeaxanthin	NASA ARC, flown in BN-1	NCYC 3560
Saccharomyces cerevisiae Y55 (Multi-nutrient co-culture)	Beta-carotene, zeaxanthin, riboflavin, and folate	NASA ARC	NCYC 3560
Streptococcus thermophilus/ Lactobacillus bulgaricus	Yogurt	Commercial supplier	YO-MIX 151
Mixed organism culture	Kefir	Commercial supplier	C-FIR

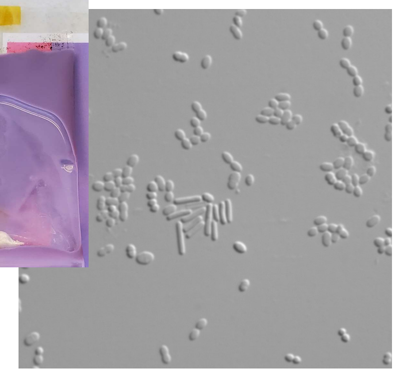


Riboflavin and folate overproducer

Carotenoid production in prototype bags

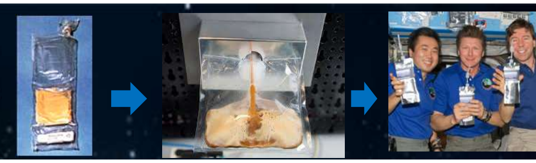


Kefir

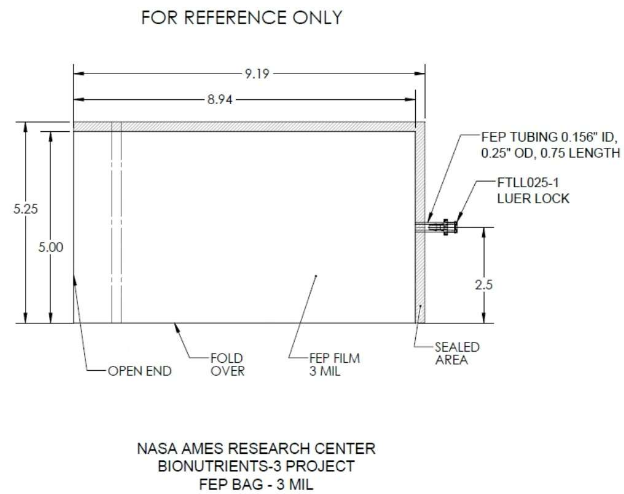


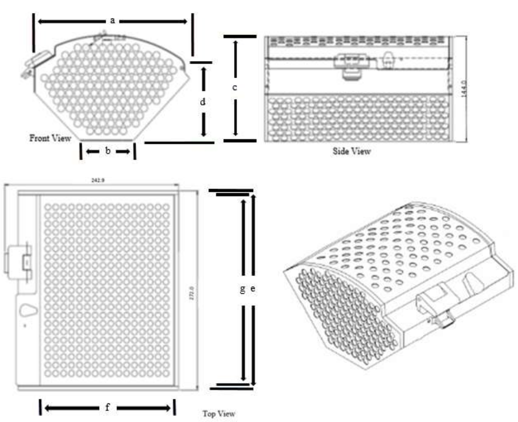
Yogurt

Gen-2 Bag Design Drivers



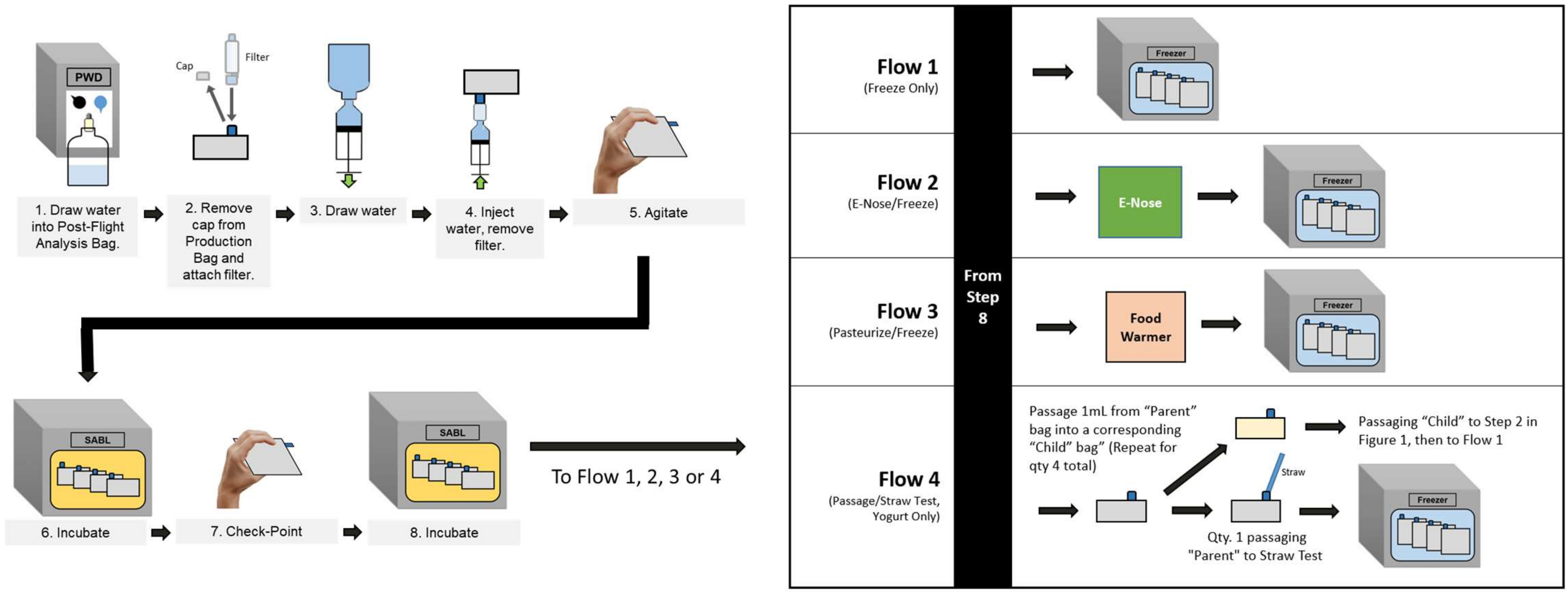
- Product volume
  - The various pathogen detection protocols and quantification of nutrients/production yield dictates a larger sample be produced per bag.
- Product yield
  - higher oxygen availability correlates with production in many aerobically growing yeast strains. PDMS has significantly higher gas permeability than FEP.
- Head space volume/gas expansion volume
  - Yeast cultures create CO_2 and ethanol as they grow, and this causes the bag to expand
  - E-nose testing requires creation of a head space and pumping in air to mix and sample from the gas content of the bag
- Ethanol production limits
  - Production of more than 10mg/day would require a category 1 Volatile Organic Compound Usage Agreements (VUA).
- Multifunctional port
  - Product access for crew, for E-Nose air sampling requires a port that allows open passage (the needle-less septa port must be removable)
- Fit into SABL, MELFI, POLAR
  - To grow and return





MELFI dimensions

Flight experiment design - On-orbit Operations



BioNutrients-3 Concept of Operations

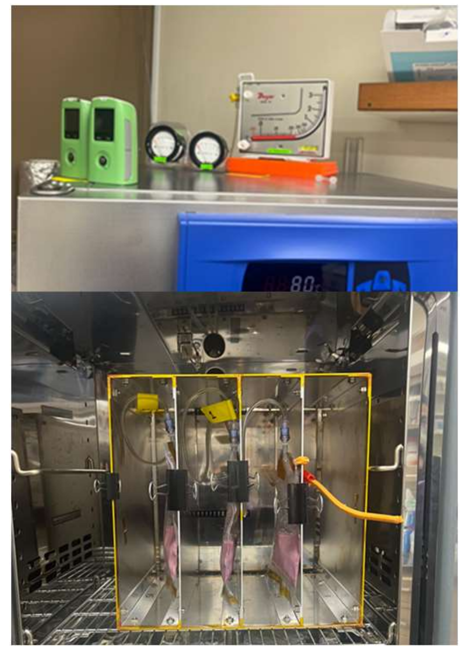
FEP bioreactor Design testing: Maximum Design Pressure

Objective: to determine the maximum pressure achieved in the FEP bags during the incubation and pasteurization phases of planned flight experiment. (Images below show Kefir experiment.)

Procedure: Autoclave/dry, load with media and organisms, heat-seal, kit, hydrate, incubate, pasteurize, pressure test to 2.5X MDP.

Pre- and post- hydration, hand centrifugation, folding

Incubation



In food warmer mockup for pasteurization.

No loss of bag integrity observed following experiment and pressure tests.

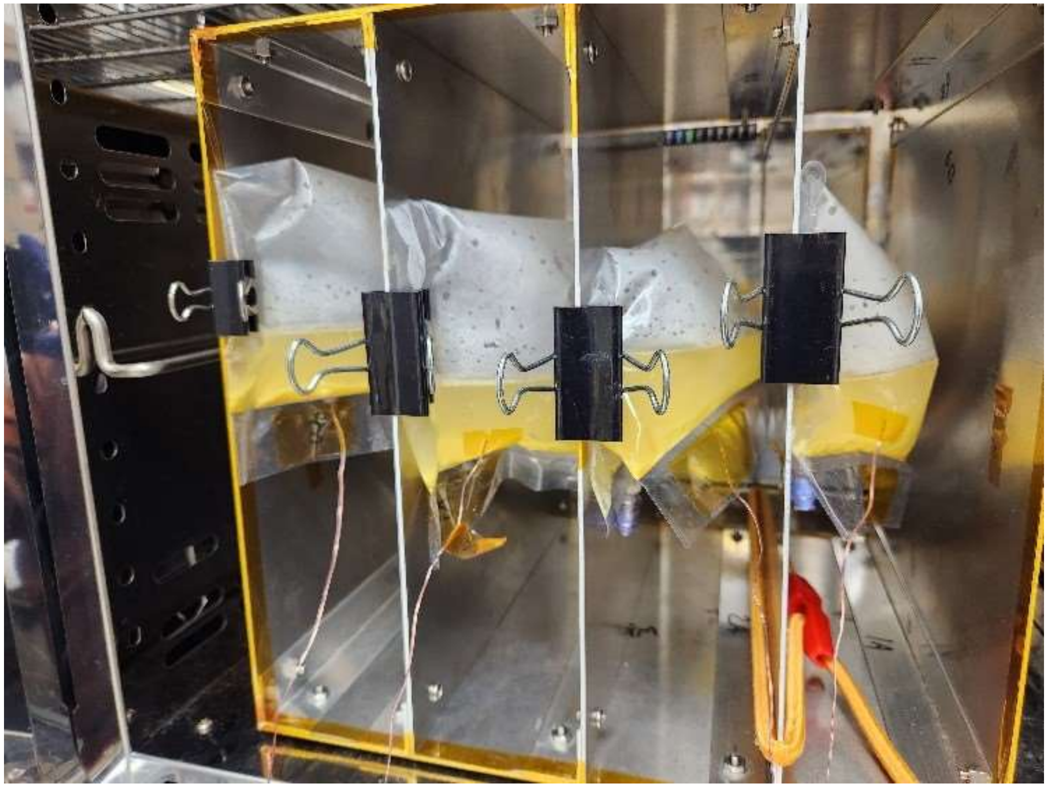
Pasteurization

Pasteurization in mock ISS Foodwarmer

- 4 sample bags prepared per experiment
  - 99.99% inactivation at 1 hour
  - 100% inactivation at 1.5 hours

- Galley Rack Food Warmer ground unit testing is planned to allow higher fidelity testing prior to launch.

- Bag integrity testing (pressure testing, handling post pasteurization) is critical to assess risks of implementing on the ISS

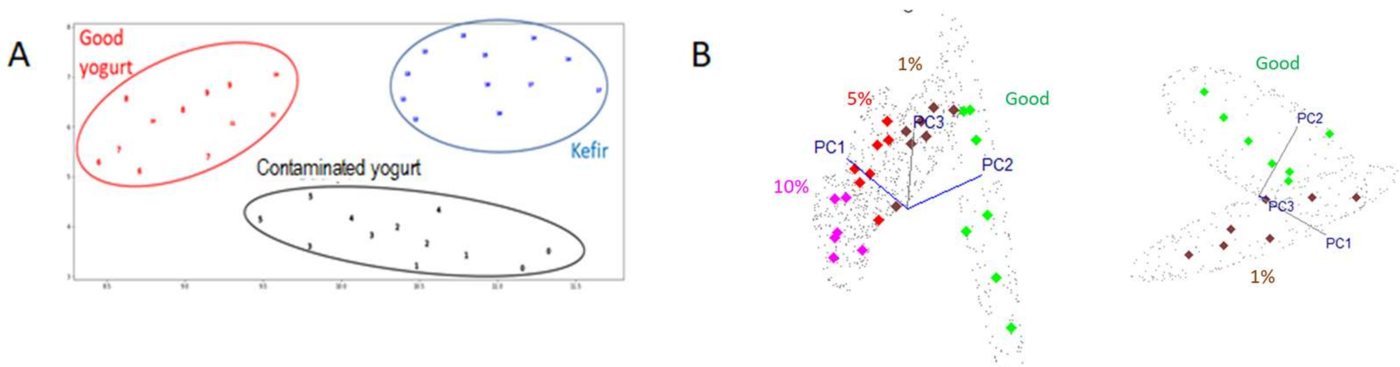


Mock ISS Food warmer – sample bags shown post-pasteurization

E-Nose food spoilage/pathogen detection in BioNutrients System

Spoilage and pathogen detection via E-Nose: successful discrimination between samples demonstrated.

- Three categories of milk products (good yogurt, *Kluyveromyces marxianus* contaminated yogurt, and kefir) were tested.
  Linear support-vector machine (SVM) cross-validation showed 100% identification accuracy.
- A total of 27 samples from four different categories (100% good, 1%, 5%, and 10% contaminated yogurt) were tested to learn E-Nose detection limits in identifying *K. marxianus* contaminated samples.
  - Linear SVM model showed 96% identification accuracy.
  - 37 CFU/ml (1.3 x 10^3 CFU/35 ml of bag) of the intentional contaminant was successfully detected in the 1% contaminated yogurt.



(A) Uniformed Manifold Approximation and Projection (UMAP) plot showing discrimination of three different groups: Good yogurt (red), *K. marxianus* contaminated yogurt (black), and Kefir (blue). (B) Principal Component (PC) analysis 3D scatterplots showing the first three PCs discriminate good yogurt (green) relative to contamined groups: 1% (brown), 5% (red), and 10% (purple).

E-Nose ground unit integrated design including pump

E-Nose Interface design for use with BioNutrients Bag designs

Summary BioNutrients-3/Gen-2 System

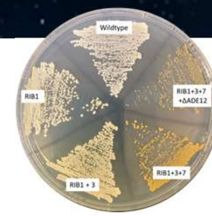


Figure 1: genetically engineered yeast strains developed to co-produce carotenoids, riboflavin and folate.

- **BN-3: on track to produce multiple nutrients in a single pack, to test pasteurization in micro gravity, compatibility with E-Nose devices, serial production, and crew access with a straw.**

- Gen 2 Critical Design Review completed April 23, 2024
  - **Engineering testing** including maximum design pressure (MDP) testing, static load test, and freezer thermal cycling testing (ARC) is complete with formal testing at JSC planned. Fit checks for SABL and MELFI complete.
  - **FEP and PDMS** bioreactors were developed. PDMS permeability results in excessive ethanol release (data not shown).
  - **Pasteurization** in mock ISS food warmer successful, testing in flight-like engineering unit at JSC planned for Fall 2024.
  - **New edible pH indicator dye** performance confirmed, (fig. 3).
  - **Product viability/biomass production**, nutrient production and volumes required for all testing and analysis confirmed in larger Gen-2 FEP bag design.

- Received guidance on process to apply for FDA approvals and crew testing from Human Research Program.

- HACCP baselined
  - Hazard Analysis Critical Control Point (HACCP) plan was reviewed with Advanced Food Systems lead, KSC Veggie team and Eurofins food safety consultant prior to baselining in April 2024.

Figure 2: photos from static load test and functional leak testing of FEP bags. Freezer test will be conducted at JSC cold stowage group.

Figure 3: New edible pH indicator dye confirmed (pre and post growth pictured above. Endpoint pH, syneresis (whey/yogurt), and yogurt viabilities show no significant differences with and without the pH indicator dye.

Synthetic Biology Team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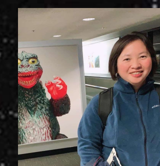
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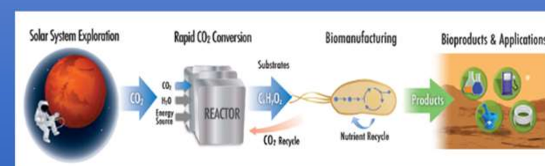
Synthetic Biology GCD project Elements



CO_2 based manufacturing

BioNutrients

Safety: Daniel Varnum-Lowry
Q/A: Leonard Hee
Logistics at KSC: Satro Narayan

Former team members and students: Aditya Hindupur, Amy Gresser, Aphrodite Kostakis, Asif Rahman, Ava Karanjia, Benjamin Alva, Eliza Zaroff, Eric Litwiller, Jason Samson, Jing Li, John Hogan, Jon Galazka, Julie Levri, Katherine Fisher, Leonard Lee, Matthew Kanan, Marilyn Murakami, Mathangi Soundararajan, Michael Dougherty, Paul Milazzo, William Tyukayev

Image Credit: NASA


Thank you

Fermented Foods and Genetic Engineering may provide a solution

Extensive history of production in non-sterile environments

- Fermented foods can be made with cereals (i.e. breads), legumes (i.e., miso, nato, soy sauce), vegetables (i.e., kimchi, sauerkraut, *kocho*), roots and tubers (*gari, fufu, lafun*), fermented fish sauce and pastes, sausage, milk (i.e., yogurt, cheese, kefir, levan), (Ray, R.C. et al., 2024 https://doi.org/10.1007/978-3-031-72000-0_1)
- Earliest evidence for the deliberate application of fermentation has been found in pottery vessels discovered in China dating from 7000 BC that were used to ferment rice, honey, and fruit, (McGovern 2004 https://doi.org/10.1073/pnas.040792110).
- Inadvertent production and consumption of fermented foods significantly predate this, as foods must have undergone spontaneous fermentation during storage, (Tamang 2020 https://doi.org/10.1111/1541-4337.12520)




Spaceflight compatible – mass, volume, resources, shelf life and ease of genetic engineering

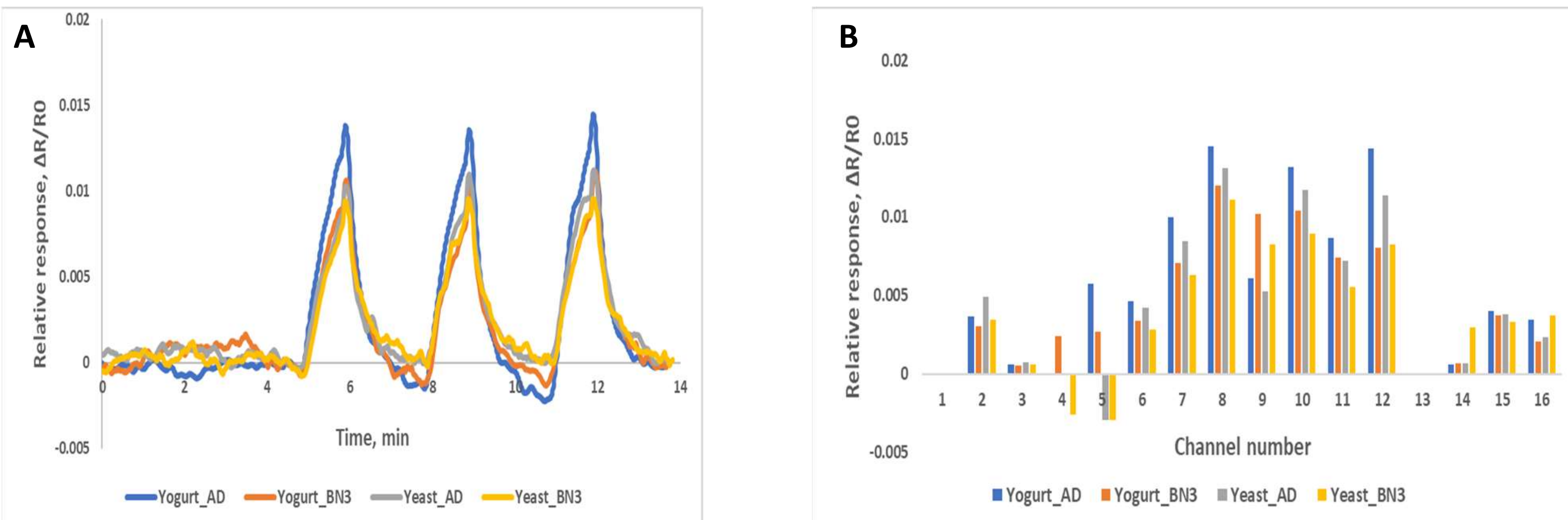
- Fermented foods provide beneficial microbes, nutrients, bioactive compounds, flavors and food preservation.
- Genetic engineering can make fermented food products into a nutrient and therapeutic compound delivery system.
- Small resource required, thermal and shelf-life stability, relatively low thermal, water and space requirement for growth, makes them ideal candidates for Space Flight.

Photo credit creative commons

E-Nose is compatible with BN-3 bag design and interfaces


Ami Hannon

- Preliminary testing with the new BN-3 prototype bags generated similar responses to those observed using the COTS American Durafilm bags or BN-2 bags, demonstrating that the interface is functional for E-Nose sampling.
  - Three clear response peaks suggesting that the sample was extracted properly, and the results were reproducible (A)
  - All 64 channels of the sensor array successfully contributed to generate “fingerprints” for both BN3 bag-based yogurt and yeast samples (B, 16 channels)



E-Nose results comparing yogurt and yeast samples from COTS American Durafilm (AD) or BN-2 bags vs BN-3 bags (A) Time-varying responses as change in resistance (B) Response of first 16 sensors.

Potential of Synthetic Biology in Space

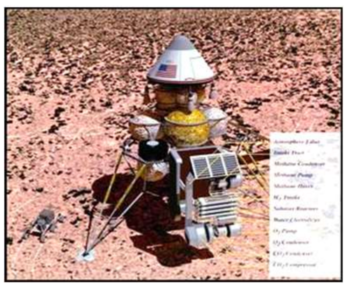
Biological systems are:

- Scalable
- Programmable
- Precise (pure isomers)/selective
- The only route of production in some cases (protein therapeutics)
- Low T^o and pressure
- Regenerable

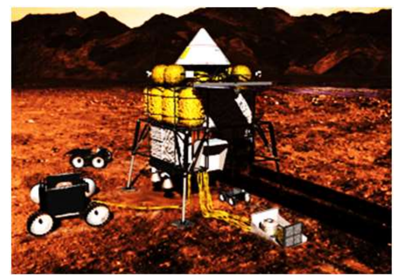
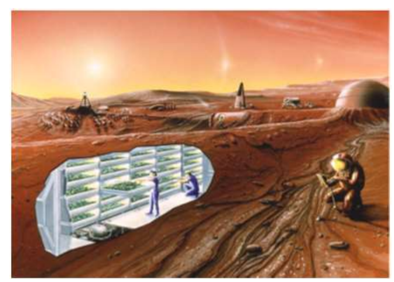
In Space Production of Mission Products

A wide array of different mission resources will eventually need to be produced on-site for mission sustainability/cost effectiveness

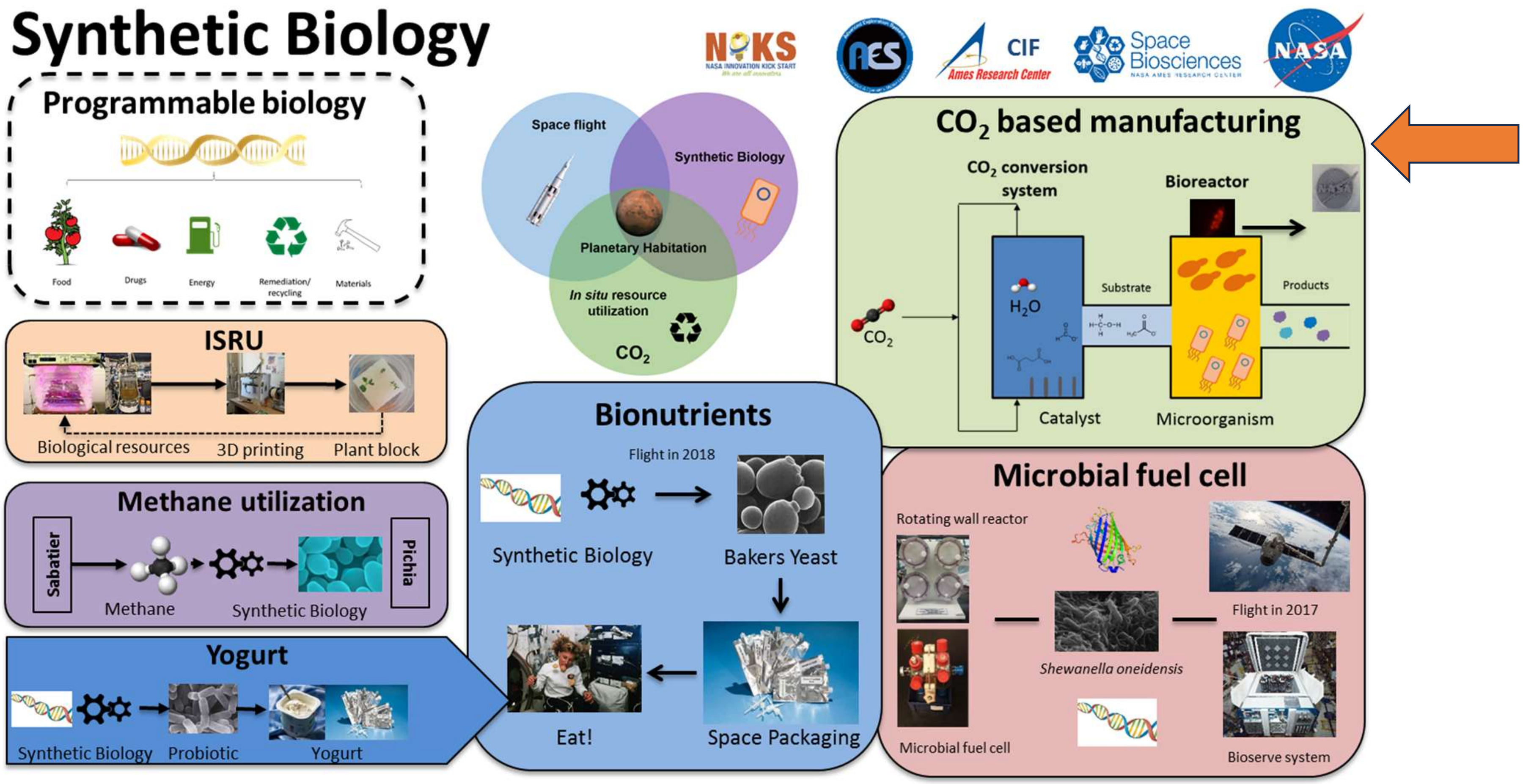
- Fuels, foods/nutrients, chemicals, plastics, binders, medicine
- Biomanufacturing can provide compounds abiotic systems cannot
- Biomining – highly selective trace element sourcing





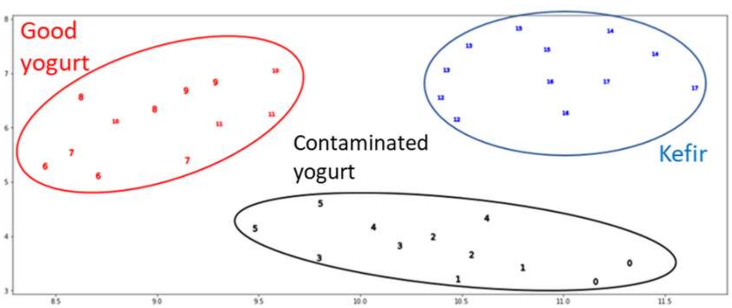
Overview of Projects



E-Nose Preliminary Results and compatibility testing

- Three categories of milk products (good yogurt, *Kluyveromyces marxianus* contaminated yogurt, and kefir) were tested with six (6) samples/category. For each sample, two (2) technical replicate sensor response peaks were used for linear support-vector machine (SVM) modeling.
- Leave-one-out cross-validation of individual technical replicates showed 100% identification accuracy.
- A total of 27 samples from four different categories (100% good, 1%, 5%, and 10% contaminated yogurt) were tested to learn e-nose detection limits in identifying *K. marxianus* contaminated samples. Linear svm leave-one-out identification showed 100% sensitivity for all samples, and 100% specificity accuracy for 5% and 10% contaminated samples.
- One out of seven good yogurt was misidentified as 1% contaminated yogurt which resulted in 86% specificity (37 cfu/ml (1.3x103cfu/ 35ml of bag) of the intentional contaminant was successfully detected in the 1% contaminated yogurt).

A



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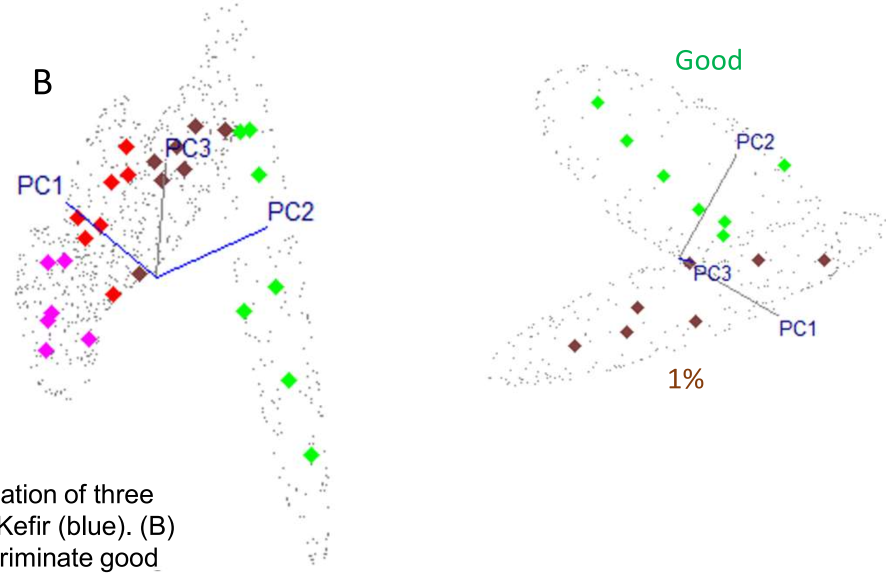
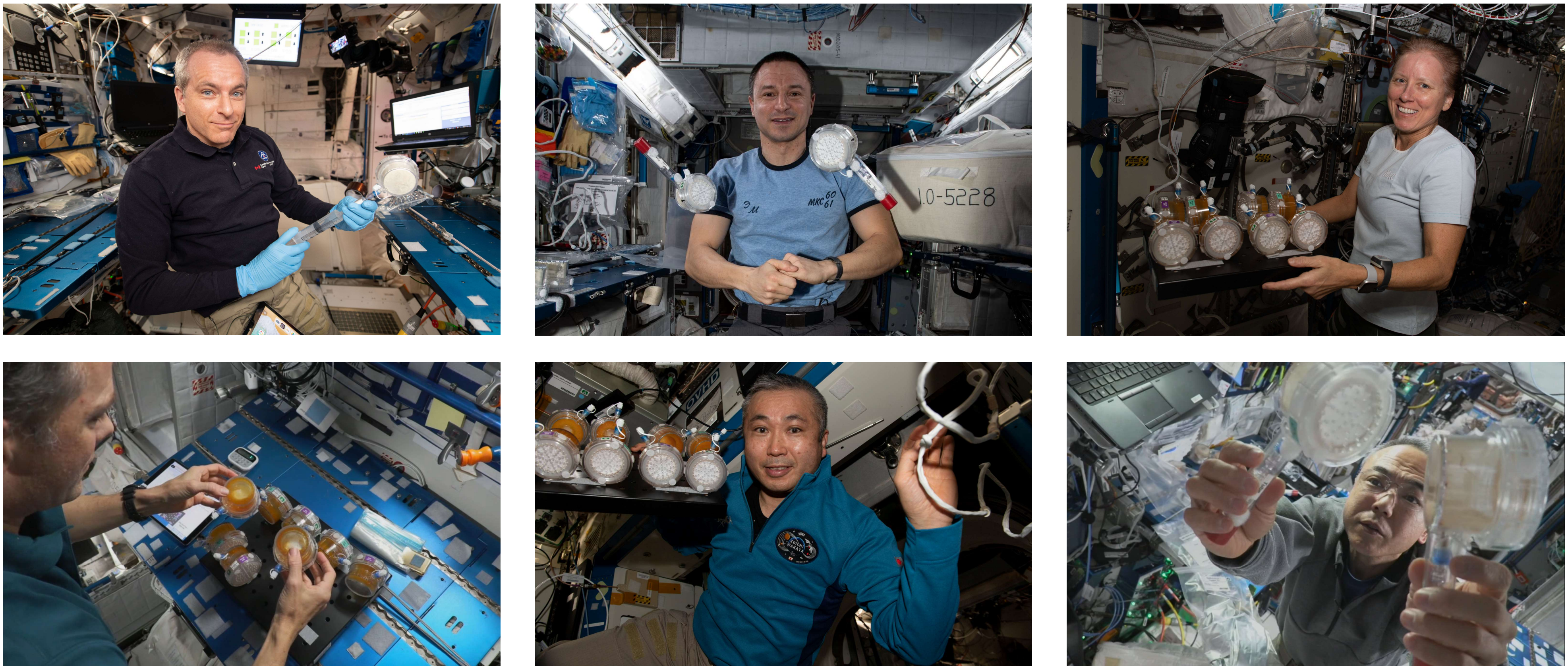


A. Uniformed Manifold Approximation and Projection (UMAP) plot showing discrimination of three different groups: Good yogurt (red), *K. marxianus* contaminated yogurt (black), and Kefir (blue). (B) Principal Component (PC) analysis 3D scatter plots showing the first three PCs discriminate good yogurt (green) relative to contaminated groups: 1% (brown), 5% (red), and 10% (purple).

Five Years and Six Runs Complete!



BioNutrients-1 Crew Members (left to right, top to bottom): David Saint-Jacques (Run 1, June 2019), Drew Morgan (Run 2, January 2020), Shannon Walker (Run 3, January 2021), Thomas Marshburn (Run 4, January 2022), Koichi Wakata (Run 5, January 2023), Satoshi Furukawa (Run 6, January 2024)

BN-2: Flat Pack FEP system, adds Yogurt and Kefir

Yeast carotenoids and Follistatin (protein therapeutic) production Yogurt and Kefir production (vitamin K, gfp demo product)

ISS

BN-2 yeast ISS production packs (*S. boulardii* producing beta-carotene, *S. cerevisiae* producing zeaxanthin, and *K. lactis* producing follistatin).

Run 1 of yogurt and kefir on ISS. Left: kefir (blue color at initial hydration turns yellow as culture grows and pH lowers), Upper right: yogurt.

- 90% reduction in mass
- Flat pack system
- 2 successful runs
- No contamination

Nicole Mann and Josh Casada hydrating and shaking the yeast and yogurt packs respectively. Featured in Space Station Science Highlights: Week of January 2, 2023 | NASA ISS Research on Twitter: "BioNutrients-2 tests an on-demand system to produce key nutrients from yogurt, kefir, and a yeast-based beverage. 🥛 The study could help maintain the crew's health while reducing launch mass and volume requirements. https://t.co/fuxW39uTWd https://t.co/bzFZVYMIxS" / Twitter

Nutrition in Space

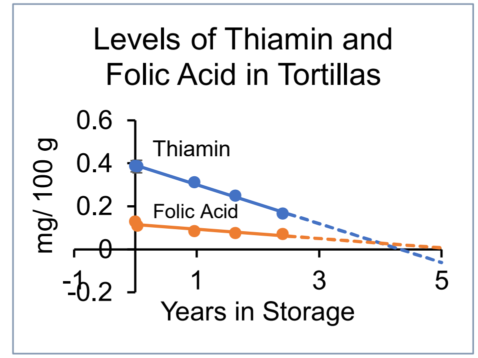
Nutrients degrade with time in food and supplements.

- Enhanced nutritional needs in space travel.
- Radiation and microgravity countermeasures.
- Critical in fighting stress and disease-specific concerns.

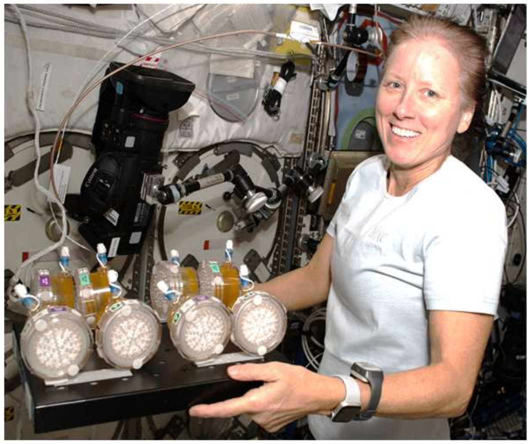
Psychology of food/isolation.

Production of some foods and supplements *in situ* as mitigation.

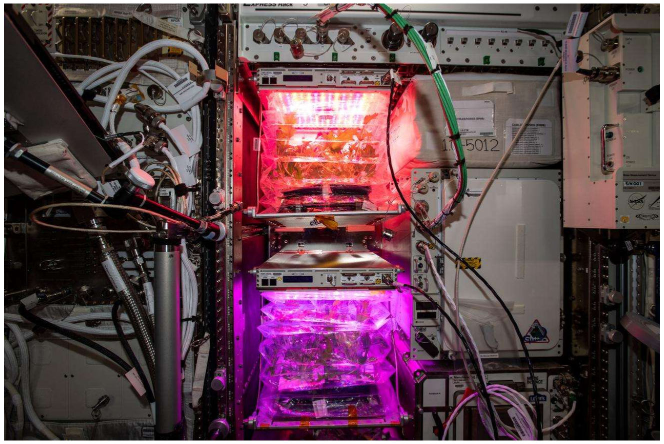
STS-127 meal-time. Clockwise from bottom left: ESA astronaut Frank De Winne, astronauts Christopher Cassidy, Mike Barratt and Tim Kopra, CSA astronaut Robert Thirsk, and astronaut Mark Polansky. From left to right at top: JAXA astronaut Koichi Wakata and Russian Federal Space Agency cosmonauts Roman Romanenko and Gennady Padalka.



Adapted from Zwart et al (2009) *Food Science* 74(7):H209-H217. https://doi.org/10.1111/j.1750-3841.2009.01265.x



Astronaut Shannon Walker with the BioNutrients (BN-1) hardware.



NASA Vegetable Production System (Veggie) during Veg-04B experiment.

Astronaut Peggy Whitson working on the Veg-03 experiment.

Image credits: NASA