BioNutrients-1: Development of an On-Demand Nutrient Production System for Long-Duration Missions

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Future long-duration missions beyond low-Earth orbit will require advances in food technologies to address the documented problem of degradation of vitamins and nutrients in supplied foods stored long-term. To begin to address the issue of nutrient degradation, we are developing and flight-testing a platform biomanufacturing technology for *in situ* production of target nutrients. This technology is being tested over a five-year duration on the International Space Station (ISS). As part of the BioNutrients-1 project we have developed an on-demand system for the production of two carotenoids, β-carotene and zeaxanthin, by genetically engineering distinct strains of *Saccharomyces cerevisiae*, more commonly known as baker's yeast. The on-orbit nutrient production packs contain a desiccated yeast strain and edible growth substrate. Once hydrated, the contents of the production packs are intended to grow and produce a desired amount of ready-to-consume nutrients. In this current version the production packs will not be consumed and future missions will require an inactivation of microorganisms before consumption. In addition to the on-orbit hydration of the production packs a series of valuable microorganisms are currently being stored in stasis packs on the ISS including probiotics organisms, bacterial strains used in yogurt production, and organisms with potential use for future biomanufacturing. Analysis of returned ISS stasis packs and ground controls will include multi-omics studies and provide insight into long-term survival of organisms stored in a space environment. Both stasis packs and hydrated production packs will be intermittently returned to Earth for analysis. Preliminary data from long-term storage studies of stasis packs stored on the ISS for 47 days versus their ground control counterparts have shown no significant difference in viability. Currently no production packs have been processed.

Nomenclature

BN-1	=	BioNutrients-1
EPP	=	Earth Production Packs
EPPgc	=	Earth Production Packs ground control
COTS	=	Commercial Orbital Transportation Services
CAHS	=	Cytosolic abundant heat soluble
GRAS	=	Generally Regarded as Safe
HACCP	=	Hazard Analysis Critical Control Point
ISS	=	International Space Station
IPP	=	ISS Production Packs
IPPgc	=	ISS Production Packs ground control

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LEO = low earth orbit
MELFI = Minus Eighty-Degree Laboratory Freezer for the International Space Station
MRE = meals ready to eat
PWD = potable water dispenser
SABL = Space Automated Bioproduct Laboratory
SpX = Space X

I. Introduction

MANY of the current strategies developed for crewed missions within low Earth orbit (LEO) are impractical for future, long-duration lunar and Mars exploration missions due to a reliance on earth-based resupply. Shelf-life studies have indicated the existing NASA food system will not support long-duration missions that extend beyond two years.[1] Ongoing research shows that certain nutrients such as vitamin C, K, folate, and thiamin degrade to inadequate levels during processing or during storage[2]. To maintain crew health and safety the nutritional content of the food must be present in adequate quantities throughout the entire mission. Efforts to ameliorate the issue of food degradation in the NASA food system during storage include, temperature control, bioregenerative food systems including crop growth, enhanced storage techniques, and vitamin supplementation[2,3]. This study addresses mission needs by delivering an alternative approach to food production by supplying a specific quantity and quality of nutrients with substantially less time and infrastructure than other bioregenerative food systems, such as plant-based production methods. Current NASA food safety regulations also impose highly restricted microbial loads in foods. These strict regulations effectively prohibit the natural intake of microbes common in terrestrial diets, as well as the intentional supplementation of probiotic organisms for human microbiome management. The crew's microbiome status is of concern, as this may be a determinant of overall health.

To augment the current NASA food system we have developed a nutrient production pack system that contains an edible growth substrate, and food microbe engineered to produce target nutrient(s) for human consumption. This technology uses a single-species inoculum and single-use pack to ensure a safe food production environment. In this experiment, production packs are hydrated on the ISS and then frozen for return to Earth for analysis. Future applications will require a heat-deactivation step to kill the food microorganisms while maintaining nutrient quality before consumption by crew members. Ground controls are also included to determine the effects of storage and growth in the space environment. Our objective is to create a reliable, safe, and rapid production system for *in situ* production of necessary dietary nutrients using low mass, volume, and power. We are also beginning to address the feasibility of supplying probiotic organisms for maintenance of the gut microbiome. Long-duration viability testing at ambient temperatures of probiotic organisms is currently underway and are being stored in stasis packs on the ISS. Many of the microorganisms stored in the stasis packs were selected because they have been granted "Generally Regarded as Safe" (GRAS) status by the Food and Drug Administration and have potential use for biomanufacturing of useful products such as pharmaceuticals, vitamins, proteins, biofuels and/or bioplastics. Production packs and stasis packs were delivered to the ISS on April 17, 2019 on the Northrop Grumman-11 (NG-11) from Wallops Flight Facility. Recurrent revival testing and subsequent ground characterization of growth and expression of target nutrients will be analyzed at each return point for the duration of five years.

II. BioNutrients-1 Concept of Operations

A. Production pack configuration and timeline

Production packs contain dehydrated yeast pellets, either zeaxanthin producing *S. cerevisiae* spores or β-carotene producing *S. boulardii* vegetative cells, and an edible substrate as a feedstock. The growth substrate consists of yeast extract, water-soluble protein powder, and dextrose optimized to increase overall biomass. Each production pack contains 2.1 g of growth media and either a vacuum dried *S. cerevisiae* spore pellet or five air-dried *S. boulardii* pellets. When the production packs are hydrated by the crew, the yeast become metabolically active and produce the product of interest. During fermentation *S. cerevisiae* produces carbon dioxide as a by-product. To inhibit the build-up of pressure inside the system the production pack lid includes three concentric ridges that compress a gas permeable membrane against a low-density polyethylene locking washer, forming three separate seals, to allow gaseous byproducts to escape. This configuration provides two levels of containment for liquid inside the production pack (see Fig. 1). The ISS Production Pack (IPP) itself is a hard shell made of high-density polycarbonate with a perforated

screw-top lid to allow gas to escape. The production pack includes a port with one-way check valve (with cap) and sterile 0.2 micron Sterivex filter to allow the crew to inject water sterilely from the potable water dispenser (PWD). A removable plastic fitting is also attached to the outside of the production pack to act as strain relief to ensure the microbial filter is not damaged during flight.

The hardware is then packaged inside a Meals Ready-to-Eat (MRE) type bag containing a Commercial Orbital Transportation Services (COTS) silica based desiccant bag and oxygen scavenger to assist in maintaining an oxygen and moisture free environment. These items were fastened into the bag with Velcro to keep them in place once the production packs are removed for hydration. The MRE type bags are vacuumed, purged with nitrogen, and heat-sealed. These precautions were taken because the presence of oxygen and water can be detrimental to the long-term viability of yeast and causes oxidative damage to the cells. Once the production packs were loaded with media and microorganisms and sealed in MRE type bags, they were stored at 4 °C prior to launch and during shipment. Production packs are delivered from the ISS intermittently over a five-year duration. At each timepoint, four *S. boulardii* and four *S. cerevisiae* production packs will be hydrated and grown. The configuration for the Earth Production Pack (EPP) and Earth Production Pack ground control (EPPgc) are mostly the same except the EPP and EPPgc do not include a microbial filter and strain relief fitting.

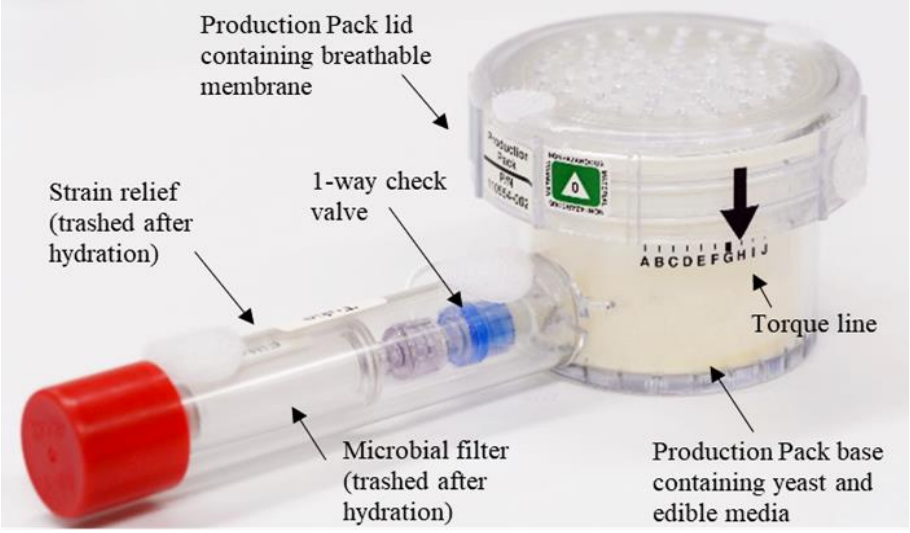
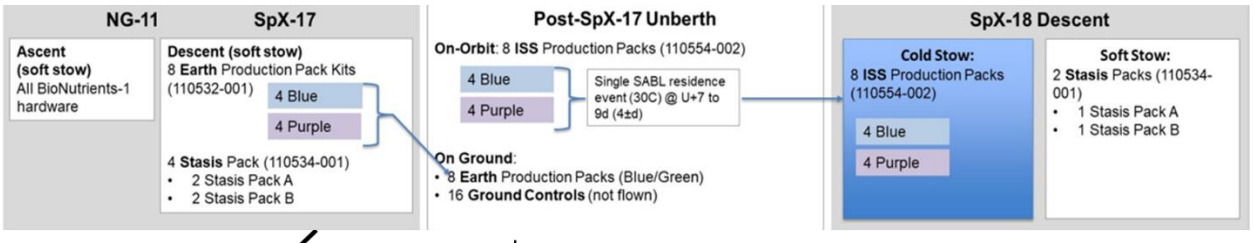


Figure 1. ISS Production Pack Configuration.

The IPP are processed by the crew on-orbit while the IPP ground controls (IPPgc) are processed almost simultaneously on Earth. The IPP and IPPgc are executed following the same protocols. Additionally, the EPP have been passively launched as controls and passively returned at designated intervals for analysis on the ground. The EPP will not be processed on-orbit but will be stored on the ISS, while the EPPgc will remain on Earth. The EPP and EPPgc are therefore not hydrated until the EPP are returned to Earth. Table 1 exhibits the experimental design of all production packs.

Table 1. Experimental Design of Production Packs.

Handling	**ISS Production Pack (IPP)**	**ISS Production Pack ground control (IPPgc)**	**Earth Production Pack (EPP)**	**Earth Production Pack ground control (EPPgc)**
Storage	Stored on ISS at ambient temperature	Stored on Earth and maintained at 25 °C	Stored on ISS at ambient temperature	Stored on Earth and maintained at 25 °C
Hydration	Hydrated on ISS	Hydrated on Earth	Hydrated on Earth	Hydrated on Earth
State received from ISS	Hydrated and received frozen	N/A	Not hydrated and received at ambient temperature	N/A



Sample Return Time	1.6 M	4.4 M	8.8 M	11.9 M	19.8 M	24.7 M	30.4 M	32.4 M	36.5 M	40.5 M	60 M	Final Return	Spare	Total
Flight Return	SpX-17	SpX-18	SpX-19	SpX-20	SpX-21	SpX-22	SpX-23	SpX-24	SpX-25	SpX-26	SpX-27	SpX-28		
IPP Activated Samples	8 run1	0	8 run2	0	8 run3	0	8 run4	0	8 run5	0	8 run6	0	NA	NA
IPP Returned Samples	0	8	0	8	0	8	0	8	0	8	0	8	16	64
EPP Returned Samples	8	0	8	0	8	0	8	0	8	0	8	0	0	48
Stasis Packs A and B Returned	4	2	2	2	2	2	2	2	2	2	2	2	4	30

Figure 2. ISS Flown Returned and Activated Production Pack Timeline. *The Epp and IPP will be returned on alternating SpX descents over sixty months. The IPP are activated on the ISS directly after the return of the EPP to Earth, IPP are then returned on the next SpX flight. The upper portion of the figure provides additional detail of sample return. The grayed out portion of the lower half of the figure are samples that have already been returned or activated and returned to Earth for further analysis, the rest of the return times are estimates based on the SpX flight schedule. Stasis packs will be returned on all SpX flights returning either IPP or EPP*

The estimated timeline for the return of all sets of production packs from the ISS is shown in Figure 2. The EPP, IPP, and flight stasis packs were launched on NG-11. The first eight EPP, four *S. cerevisiae* and four *S. boulardii*, were returned on SpaceX-17 (SpX) after spending forty-seven days on the ISS. Once EPP were returned to Earth on-orbit hydration of the eight IPP occurred with near simultaneous processing of IPPgc. IPP were frozen and were intended to return on SpX-18 however, due to limitations in freezer space the IPP remained on the ISS until space became available for cold stowage return. The EPP and EPPgc were hydrated on Earth within fifteen days of processing the ISSgc. Production packs will be returned over a duration of five-years based off the SpX flight schedule. The timeline depicted in Figure 2 is an estimate and will be adapted based off the flight schedule and production pack performance.

B. On-orbit hydration of ISS production packs

The on-orbit hydration of the IPP requires the crew to unstow four zeaxanthin producing *S. cerevisiae* IPP and four β-carotene producing *S. boulardii* IPP per experiment. The crew member collects 250 mL of water from the ISS PWD adapter using a collection bag from the Johnson Space Center PWD group. The PWD adapter is trashed after the collection bag is filled; the water filled collection bag is used to hydrate the four IPP via a 60 mL syringe (provided by the on-orbit pantry). 33 mL of water is removed from the collection bag and injected into each of the IPP via the one-way check valve (Fig. 3). Once water is injected into all four IPP the crew member will remove the microbial filters and place a cap over the one-way check valve creating an additional seal. Near synchronous hydration of IPPgc occur on ground and are processed the same way as the IPP.



Figure 3. Astronaut David Saint-Jacques Hydrating the IPP On-Orbit the ISS.

Once hydrated, the crew will shake the IPP for 60 seconds until the pellet is mostly dissolved. The IPP is then placed in the Space Automated Bioproduct Laboratory (SABL) incubator at 30 °C for a total of forty-eight hours. After six hours in the SABL, production packs are removed by a crew member and shaken again for another 60 seconds in order to fully dissolve the yeast pellet and to ensure mixing of the media and yeast. During the 48-hour duration the rehydrated yeast begin to grow and produce the product of interest. When the 48 hour incubation is

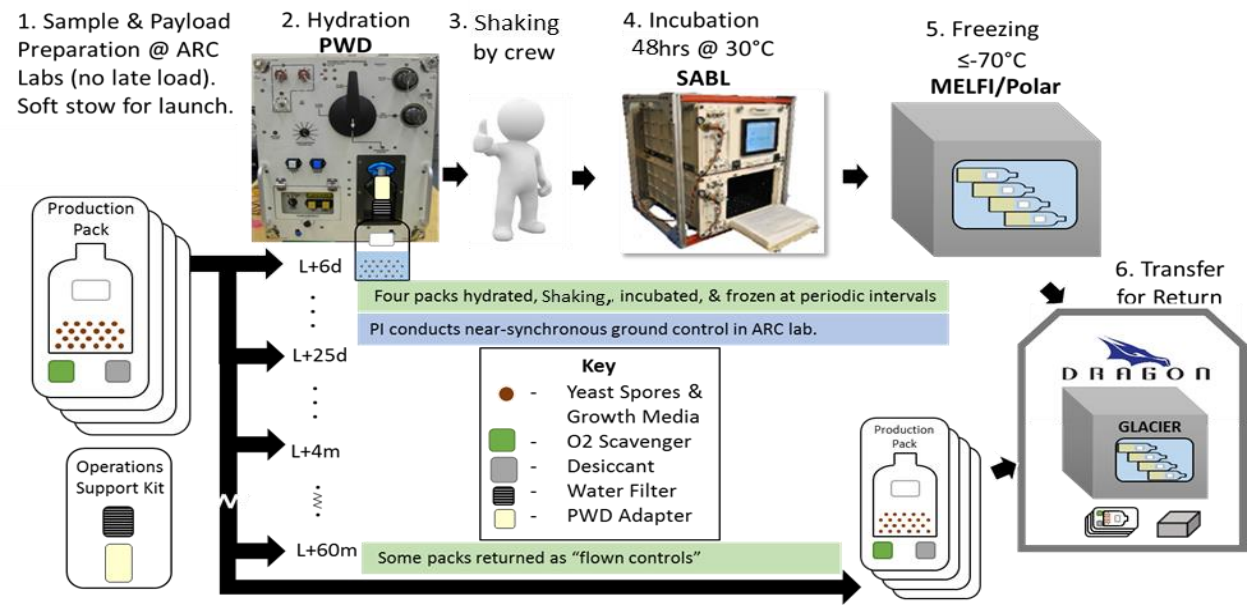


Figure 4. Concept of Operations for ISS hydrated Production Packs. *Production packs and stasis packs launched on NG-11 from Wallops Flight Facility on April 17, 2019.*

complete all four IPP will be placed in the Minus Eighty-Degree Laboratory Freezer for ISS (MELFI). Exposure to extremely cold temperatures will cause the yeast to go dormant and fermentation to stop. At the next available return, the frozen IPP will be stowed in the Dragon General Laboratory Active Cryogenic ISS Experiment Refrigerator for return to Earth where the IPP will remain frozen. The IPP's are transported back to the NASA Ames Research Center in a liquid nitrogen Dewar where they are placed in the -80 °C freezer for future analysis. Four non-activated EPP and stasis packs will also be returned at ambient temperatures for ground processing and analysis (Fig. 4).

C. Stasis Packs

Each set of stasis packs (A and B) include a series of microorganisms and media listed in Table 2 and 3. Stasis packs were prepared separately for flight as nominal and late load. Stasis pack B contains microorganisms that are more susceptible to degradation during long-duration storage and were prepared and loaded closer to time of flight. Stasis pack A contains anhydrobiotic yeast species and spores, which can survive in a desiccated dormant state for long periods, allowing for nominal loading for flight.

All microorganisms were placed in Cryotubes, sealed together with Kapton tape, and heat-sealed as a unit under nitrogen in MRE type bags. Stasis packs were prepared to investigate five-year stasis at ambient temperatures in a space environment. Thirty were delivered to the ISS (fifteen each of stasis packs A and B) and an additional thirty-four were kept on ground as controls. The four additional stasis packs were stored as spares or used for time zero testing (pre-launch). Intermittent viability testing as well as multi-omics studies will occur each time a stasis pack is retrieved from the ISS. DNA and RNA will be isolated from grown cultures depending on organism survival in order to assess the effect of long-term storage and the space environment on the genome. Organisms that exhibit enhanced survival during long-duration storage will be identified and characterized in order to identify a strain that is well adapted to the space environment. These space-adapted strains can be used in future engineering efforts for production of alternative products and/or used to identify genetic information that may elucidate mechanisms for survivability in alternative organisms.

1. Yogurt producing bacteria

This study addresses mission needs by investigating *in situ* production of a fresh probiotic yogurt that could potentially maintain the crew's microbiome health and generate target nutrients. The yogurt starter strains include, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, and can be engineered to produce vitamins, nutraceuticals, useful enzymes, and medicines within the yogurt. As a demonstration, green fluorescent protein (GFP) was expressed in *S. thermophilus*, to validate the feasibility of genetic modification. Yogurt starter strains are known to be vulnerable to non-refrigerated temperatures during long-duration storage, and were included in the stasis packs to determine shelf life at ambient temperatures. Additionally both strains were

stabilized by two different methods; encapsulated as sodium alginate beads and coated with dry milk ("encapsulated"), or lyophilized with trehalose in filter paper matrix ("non-capsulated"), and included in stasis pack A of this BN-1 flight experiment. Encapsulation of microorganisms has previously been shown to extend their shelf-life and increase survival in the gastrointestinal tract[4,5].

The spore forming and GRAS *Bacillus* species are also being investigated as another solution for facilitating milk coagulation for a fermented milk product. These species are capable of long-duration survival in a spore state and can withstand the space environment for years even when exposed to large doses of radiation[6]. To further enhance survival of *Bacillus subtilis* and *B. coagulans*, each were sporulated then encapsulated in sodium alginate beads or lyophilized and included in the stasis packs. Bacillus species also carry the additional advantages of availability of genetic engineering tools and innate ability to produce vitamin K, which is currently below the recommended daily intake in the NASA food system[2].

2. Yeast

S. cerevisiae spores and *S. boulardii* vegetative cells are currently being stored is stasis packs on the ISS to determine how well these organisms survive and how well carotenoids preserve in the cell over time. Carotenoids will be extracted and quantified using high performance liquid chromatography with each stasis pack that is delivered from the ISS as well as its associated ground control. To ensure long-term viability of vegetative cells, *S. boulardii* cells have been encapsulated in an alginate matrix with trehalose to determine if encapsulation can prolong viability during long-duration storage. To potentially further enhance long-duration storage, *S. boulardii* strain MYA-796, has been genetically modified for desiccation tolerance to increase trehalose stores in the cell. Trehalose is a commonly used disaccharide that naturally occurs in yeast, plants, and nematodes[7]. *S. cerevisiae* has previously been engineered to enhance endogenous trehalose stores during growth and has shown an increase in desiccation tolerance when stored long-term[8,9]. Integration of cytosolic abundant heat soluble (CAHS) proteins, from the tardigrade *Hypsibius dujardini*, have also been integrated into the genome of *S. boulardii*. CAHS proteins have been shown to increase desiccation tolerance in tardigrades as well as in yeast when expressed. The effect of CAHS proteins during desiccation tolerance is similar to the effect of trehalose on yeast cells, in that they form a non-crystalline amorphous solid (vitrify) when undergoing desiccation[10]. This effect may physically prevent protein denaturation and aggregation within the cell thereby increasing survivability during desiccation and storage. The heterozygous and homozygous *S. cerevisiae* genomic knockout strains are also included in the stasis packs and will be anaylzed throughout storage. Genomic analysis of these genetically modified organisms may help to elucidate genetic features that enable sustained viability in the space environment.

Kluyveromyces lactis and *Komagataella phaffii Kurtzman*, were also selected for storage in stasis packs. The aforementioned yeast are capable of growing on a wider range of temperatures and carbon sources than that of *S. cerevisiae*, and can perform important roles as future cell factories for bioproduction of biofuels, proteins, foods, and pharmaceuticals. Both organisms were also treated with ultraviolet radiation (UV) to increase genetic variability within the strain. Multi-omic analysis of surviving organisms will help to elucidate what, if any genetic variances increase long-duration survival in a desiccated state aboard the ISS.

3. *One-Carbon (C1) utilizing bacteria*

Methylobacterium extorquens AM1 is a methylotrophic bacterium that can utilize simple carbon sources such as acetate, succinate, and methanol for use in biotechnological production of products. *Cupriavidus necator* is a hydrogen oxidizing bacterium that can utilize C1 substrates to produce novel products. *C. necator* also produces large amounts of the biodegradable storage polymer polyhydroxybutyrate (PHB), a material that resembles plastics such as polypropylene. PHB can potentially be used for three-dimensional printing to manufacture components in space. Five-year storage at ambient temperatures will help determine the feasibility of using these microorganisms for future bioproduction of useful products. Additionally, both strains were treated with UV radiation to increase genetic diversity, which may increase survivability among cells stored long-term.

4. *Media*

The growth media that is utilized in the production packs will also be stored in the stasis packs to determine if the potency of the media is affected during long-duration storage. The growth media powder is hydrated in water and the same yeast strain is grown from frozen stock in the hydrated media. Growth curves will be monitored to see if the overall growth of the yeast is affected negatively by storing growth media over five-years. The skim milk powder will also be tested on its ability to be used as a substrate for yogurt producing bacteria to make a fermented milk product after long-duration storage.

Table 2. **Table of Stasis Pack A samples.** *Stasis pack A, late load on NG-11.*

Quantity (Vials)	Vial Size	Contents	Additional treatment
2	2 mL	*Steptococcus thermophilus* (ATCC BAA-491) with GFP plasmid	Encapsulated
4	2 mL	*Streptococcus thermophilus* (ATCC BAA-491) with GFP plasmid	
4	2 mL	*Lactobacillus delbruekkii subsp. bulgaricus* (ATCC-BAA-365)	
2	2 mL	*Lactobacillus delbruekkii subsp. bulgaricus* (ATCC-BAA-365)	Encapsulated
4	2 mL	*Saccharomyces cerevisae* Y55 (NCYC 3560) spores engineered with genes for zeaxanthin production	
1	2 mL	*Methylobacterium extorquens* AM1 (ATCC 14718)	
2	2 mL	*Methylobacterium extorquens* AM1 (ATCC 14718)	UV treated
1	2 mL	*Cupriavidus necator* (ATCC 17699)	
2	2 mL	*Cupriavidus necator* (ATCC 17699)	UV treated
1	2 mL	*Kluyveromyces lactis* (ATCC 8585)	
2	2 mL	*Kluyveromyces lactis* (ATCC 8585)	UV treated
1	2 mL	*Komagataella phaffii Kurtzman* (ATCC 20864)	
2	2 mL	*Komagataella phaffii Kurtzman* (ATCC 20864)	UV treated
3	10 mL	Media A (28.6% Pepto-Pro protein powder, 42.6% dextrose, 28.6% yeast extract)	
3	10 mL	Media B (16.7% soy peptone, 11.1% yeast extract, 55.6% glycerol, 16.7% food grade agar)	

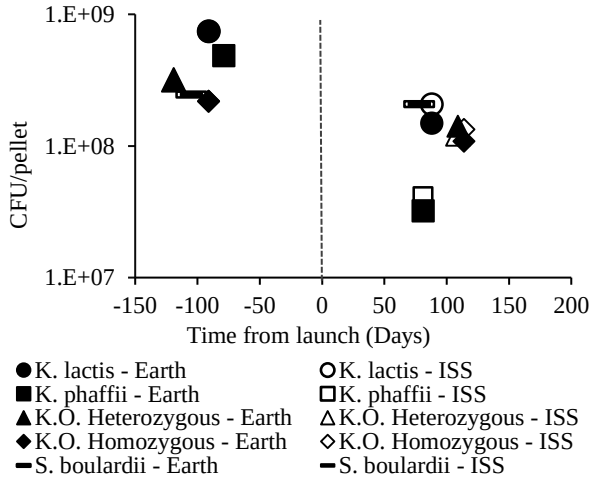
Table 3. Table of Stasis Pack B samples. *Stasis pack B, nominal load on NG-11.*

Quantity	Vial Size	Contents	Additional treatment
4	2 mL	*Saccharomyces cerevisiae subsp. boulardii* (ATCC MYA-796) engineered with genes for β-carotene production and desiccation tolerance	
4	2 mL	*Saccharomyces boulardii* (ATCC MYA-796) engineered with genes for β-carotene production	
4	2 mL	*Saccharomyces boulardii* (ATCC MYA-796) engineered with genes for β-carotene production	Encapsulated
4	2 mL	*Bacillus subtilis* (ATCC 6633)	
2	2 mL	*Bacillus subtilis* (ATCC 6633)	Encapsulated
2	2 mL	*Bacillus coagulans* (ATCC 7050)	Encapsulated
4	2 mL	*Saccharomyces cerevisiae* (ATCC BY4743) Homozygous knockout strain	
4	2 mL	*Saccharomyces cerevisiae* (ATCC BY4743) Heterozygous knockout strain	
3	10 mL	Media A (28.6% pepto-pro protein powder, 42.6% dextrose, 28.6% yeast extract)	
3	10 mL	Skim milk powder	

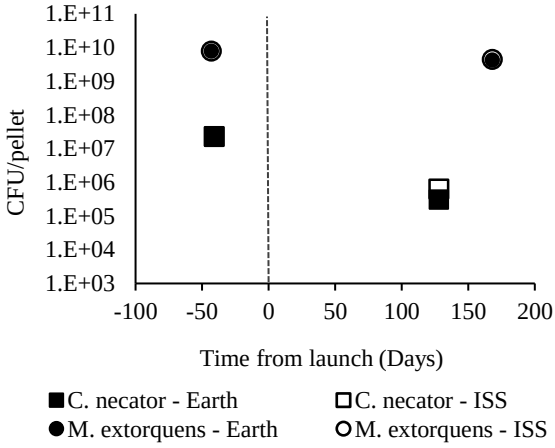
III. Preliminary Results and Discussion

Stasis packs, IPP, and EPP are currently being stored on the ISS for intermittent testing over a five-year duration. The IPPgc, EPPgc, and stasis pack controls are stored in a 25 °C incubator on Earth to maintain a constant temperature over the five-year duration. The storage temperature was chosen based off average temperature data logged from the ISS. All organisms within the stasis packs have been stored in a desiccated state by either lyophilization, vacuum drying, or air-drying depending on the organisms survivability during drying. Once stasis packs are returned they are initially stored at 25 °C, repackaged, and stored at 4 °C until all samples are analyzed. Viability testing and multi-omics analysis will occur as necessary, when samples are returned from the ISS.

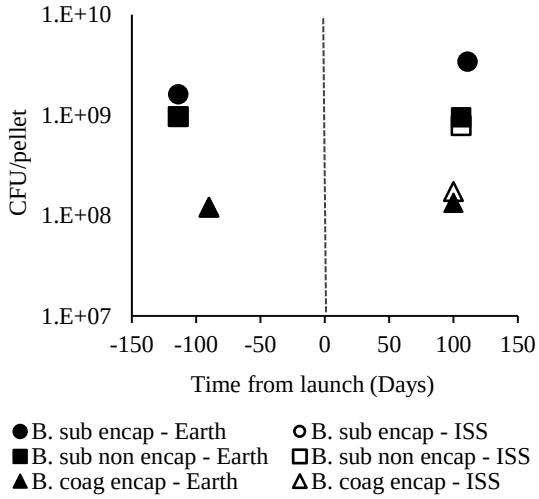
Preliminary stasis data show that after forty-seven days on the ISS there is no significant difference in viability between organisms stored on the ISS versus ground controls. Loss in viability appears to be dependent on storage at ambient temperatures rather than exposure to ISS environmental conditions. Many of the yeast strains have exhibited a minimal loss in viability (Fig. 5A). However many yeast are anhydrobiotes and are capable of surviving for years in a desiccated state. Data suggests these yeast strains may survive the full five-years on the ISS.



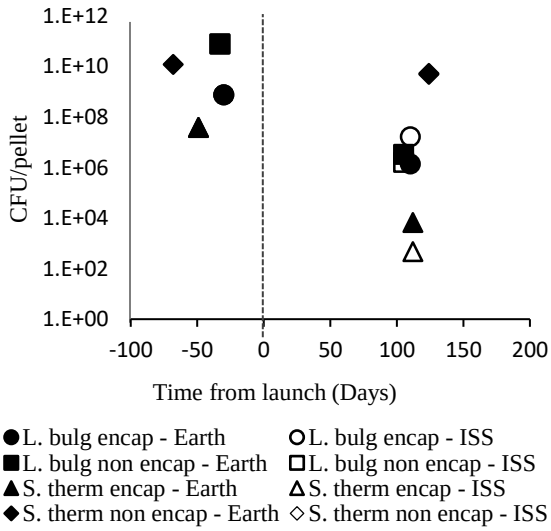
A)



B)



C)



D)

Figure 5. Viability of Stasis Pack Organisms after Forty-Seven Days on the ISS. *Viability for all microorganisms was tested at time of manufacture (directly after cells were dried and prepared for shipment on NG-11) and again when samples were returned on SpaceX-17 on June 3rd 2019, after spending a total of 47 days on the ISS. Viability is determined by plate counts and is expressed as colony forming units per pellet.*

The C1 utilizing bacteria demonstrated high variability in viability directly after desiccation and during storage. *C. necator* was air-dried and their survival was tested directly after cells were dried by plate count method. *C. necator* resulted in a 90% decline in viability upon drying (data not shown). This strain continued to decline in viability over time and was almost entirely dead by the first flight return (Fig. 5B). Optimization of survival during desiccation and long-duration storage will need to occur to increase viability of *C. necator* to be a space relevant microogranism. *M.*

extroquens maintained high viability directly after lyophilzation and continues to survive well during storage. There is no significant difference in viability between ISS stored samples and their ground control counterparts (Fig. 5B).

Yogurt producing strains have shown mixed results in viability when comparing encapsulated cells versus non-encapsulated. *S. thermophilus*, non-encapsulated cells, have shown higher survivability compared to its encapsulated counterpart however, in the case of *L. bulgaricus* the reverse is true. Viability of non-encapsulated *L. bulgaricus* cells are declining at a faster rate (Fig. 5D). *B. subtilis* and *B. coagulans* are spore formers and have been stored in a sporulated state within the stasis packs. Spore formers can survive in a quiescent state indefinitely and are capable of surviving extremes of temperature and radiation, making them ideal candidates for engineering space relevant products. There has been no apparent decline in viability from time of manufacture to the first return of ISS stasis pack samples (Fig. 5C). There has also been no significant difference in viability of ground controls compared to ISS stored yogurt producing strains.

To date, sixteen IPP have been hydrated and returned to Earth and are awaiting analysis. While no production packs have been processed at the time of writing this paper the following assays will be tested, multi-omic analysis will include genomics and transcriptomics of grown IPP and IPPgc, in order to better understand the effects of microgravity and long-duration storage on growth and on the genome. Assessment of carotenoid production and biomass will also occur to determine growth and production yields of all production packs. Analysis of the media will elucidate the yeast's ability to utilize available carbon through a glucose assay. Residual carbon in the media will be indicative of incomplete growth by the yeast after forty-eight hours. An ethanol assay will also be conducted to determine complete fermentation and growth. Safety is a critical element of this nutrient production platform due to the incubation step required for yeast growth and product production. Any contamination present could be hazardous to the astronaut's health. We will be testing for contamination in the IPP and IPPgc through microscopy and a standard series of food industry Petrifilm tests. This panel of analysis includes, total aerobic counts, yeasts and molds, total coliforms, staphylococcus, and salmonella. We expect the yeast and molds Petrifilm test to fail due to live yeast inside the production packs. Future production packs will require an inactivation step by heat kill prior to consumption by crew members.

IV. Future Work

The current design and concept of operations for BN-1 revolves around a hard-based pack, which was originally designed to accommodate build and safety concerns within a strenuous flight development schedule. Future BN projects will utilize a bag-based system for nutrient production, thereby reducing volume and mass. To further enhance the feasibility of the BN's concept for mission use, production packs will be designed to produce more than a single nutrient per pack, by either utilizing multiple organisms or using one organism to produce multiple compounds per bag. Production of multiple compounds simultaneously in a single bag will help ensure reduced consumables for use in long-duration missions. Further optimization of the media may help to reduce total mass of the production packs as well as enhance nutrient production yields and will be utilized in future testing.

Reducing the likelihood of potential contamination will be a vital requirement to ensure safe production of the product of interest. *In situ* production of products with high microbial loads demand new food safety procedures to be developed for space flight. A Hazard Analysis Critical Control Point (HACCP) plan will be designed and utilized for future BN projects and will identify and control any potential hazards during production. A HACCP plan is a food safety monitoring system utilized to control biological, physical, and chemical hazards during preparation, storage, and transportation. Critical control points throughout growth, packaging, storage, assembly and operations, and post processing will be identified and monitored via microbiological testing. Procedures will be developed for live cultures as well as a Pasteurized version (in order to satisfy total aerobic count standard) to satisfy NASA's food safety requirements. During development of the HACCP plan, personnel from the Human Research Program will be consulted to ensure NASA regulations are followed. The incubation period required for growth and production of nutrients within the Production Pack presents a novel risk for contamination not previously encountered in the NASA food system. Precautions must be taken to ensure that the contents of the Production Pack are safe for consumption.

V. Conclusion

The BioNutrients project seeks to provide advances of in-space biomanufacturing by addressing specific mission needs for future exploration efforts. Production of space relevant products is reliant on the organism's ability to produce products over an extended period. Returned stasis packs have shown that many of the yeast strains that have flown have maintained a high viability over time at ambient temperatures, and may survive for several years. Bacterial strains for yogurt production have mixed results and are impacted by how samples are desiccated and stored. The first forty-seven days on the ISS have shown no statistical difference in viability of microorganisms stored on the ISS versus their ground control counterparts. Future experiments will require radiation testing to determine the effects of radiation on survival outside low-earth orbit. To date, no data from Production Pack performance have been generated.

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