# THE CONCEPTUAL APPROACH TO THE USE OF POSTBIOTICS BASED ON BACTERIAL MEMBRANE NANOVESICLES FOR PROPHYLAXIS OF ASTRONAUTS' HEALTH DISORDERS

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# THE CONCEPTUAL APPROACH TO THE USE OF POSTBIOTICS BASED ON BACTERIAL MEMBRANE NANOVESICLES FOR PROPHYLAXIS OF ASTRONAUTS' HEALTH DISORDERS

*The functional fermented foods containing live microorganisms and their components are necessary for the normal functioning of the human body as normal gut microbiota needs fuel from external microbial organisms and their nanostructures — membrane vesicles (MVs), excreting outside. The concept that MVs may contribute to astronauts' health probably to the same extent as their parental microbial cells do and be a temporary substitute for living microbial cells until we know more about the behavior of microbes in the space environment. The advantage of MVs is that they are not alive and cannot be changed under unfavorable conditions as microbial organisms may be. As the model, we selected MVs of a robust to environmental factors kombucha multimicrobial culture (KMC), known for its health-promoting characteristics for humans. We exposed KMC on the International Space Station in a hybrid space/Mars-like environment for an initial proof-of-concept stage. In the exposure study, KMC has survived a long-term period in harsh conditions, and the MVs generated by post-flight kombucha community members did not acquire toxicity, despite the changed membrane composition in the environment imitated conditions on the Mars surface. This observation, together with our KMC metagenomic and comparative genomic analyses of the dominant KMC bacterium Komagataeibacter oboediens, showed that the ground reference sample and space-exposed ones were similar in topology and maintained their stability. In the next stage, we assessed the fitness, safety, and biodistribution of MVs of post-flight K. oboediens and showed that they were altered, but the modifications in membrane structure did not result in toxicity acquisition. Our proof-of-concept strategy is discussed in this review in line with the literature.*

***Keywords**: postbiotics, extracellular membrane vesicles, kombucha multimicrobial culture, fermented food, health promotion.*

## 1. INTRODUCTION

In a strategy of the solar system exploration, outlined in the third edition of the Global Exploration Roadmap, it is imperative to find solutions for astronaut health protection, especially in long-distance missions, *e.g.,* to Mars. Space environment factors, such as microgravity and radiation, induce changes in a gut microbiome composition with adverse outcomes for the crew's health [56, 68]. The most adverse effects include neurological [36], immune disorders, and gastrointestinal complications [80]. Correction of astronauts' gut microbiome with microbial therapeutics and prebiotics may be recommended within the spaceflight [5, 17, 51, 52]. The current proposal for correcting the intestinal microbiota of crews includes using simple artificial compositions of defined bacteria and yeast and producing yogurt or kefir, which have not yet been tested on low Earth orbit (LEO) [5]. Other fermented products containing food-grade microbial organisms, *e.g.,* kombucha multimicrobial community (KMC) (composed of beneficial for human mutualistic bacteria and yeasts, which together organize a robust micro-ecosystem, producing health effects [1, 89], could be considered [50, 52].

On the eve of long-duration flights, space administrations have no safety protocols and guidelines for probiotics production on board and use for crew's health disorders prophylaxis. Nevertheless, therapeutic microbes, such as lactic acid and other bacteria or yeasts, need special consideration regarding their safety, efficiency, genetic and community stability, and economic reasonability. While some fundamental and regulatory barriers to using live microbial therapeutics remain, there is a workable solution. Postbiotics as non-alive biologics, which are overall

secreted by food-grade microorganisms, are considered safer than living microbial organisms and could be the interim stage of microbial usage in the prophylaxis of health disorders of crewmembers during spaceflights. A novel class of postbiotics represents extracellular bacterial membrane vesicles (BMV) [50, 53, 70]. Our idea is to temporarily use postbiotic BMVs as an alternative to probiotics based on evidence that BMVs interact with the same pathways in hosts as parental bacteria, influencing host physiology. Researches to date indicate that postbiotics/BMVs can have direct immunomodulatory effects, and evidence can be found for the use of postbiotics to improve overall health [26, 94]. They may reach the brain and interact directly with the central nervous system (CNS) [43]. Thus, BMVs can be essential in modulating human behavior and controlling mood disorders, including anxiety and depression [27, 68].

## 2. THE GUT MICROBIOME AS A MICRO-ECOSYSTEM IN DICTATING HUMAN HEALTH

Any microbiome is a characteristic microbial community (a microbiota, a collection of microorganisms) occupying a well-defined habitat with distinct physical and chemical properties and specific activity in this habitat [7]. The entities usually not considered living microorganisms — phages, viruses, plasmids, prions, viroids — do not belong to the microbiota. The intestinal microbiota of mammals mainly consists of bacteria, archaea, fungi, and protists [45] and complement the host genome with millions of genes, contributing to functions absent in hosts, e.g., decomposing cellulose [28, 37, 72]. Bacteria represent the biggest proportion of microbiota (99 %). The gut microbiota is considered a «hidden organ», and mammal organisms populated with microbiota can be accepted as «superorganisms» (summarized in [85]). In addition to its role in nutrition, metabolism, and energy production, the gut microbiota regulates immune homeostasis and responses against pathogens and physical and chemical stressors. It has been observed that alteration in human gut microbiota has resulted in various chronic and acute metabolic diseases (obesity, neurogenic diseases, diabetes, and others) [42]. As a micro-ecosystem (a subset of the biotic community and environmental factors), it is balanced by commensals, symbionts, and pathobionts that collectively benefit the host, so the disrupted balance in a steady-state microbiome leads to disease. The microbiome is an open system subjected to acquiring novel information from foreign ecosystems through external signals and invasion by outsider microbes [34]. Specific environmental factors induce changes in microbiome composition, which may alter host-microbe interactions and affect immune function, leading to health problems.

**Changes in gut microbiota composition under spaceflight.** During spaceflight, astronauts are exposed to multiple unique environmental factors, particularly microgravity and radiation, that also can induce changes in microbiome composition (*i.e.*, dysbiosis) (summarized in [85]) and cause a range of harmful health consequences. Exposure to external stressors, extreme hygiene, and psycho-emotional disorders in an aggressive environment can lead to dysbiosis and immune imbalance in astronauts. In addition, a diet that is insufficiently enriched with soluble fiber and avoids traditional probiotics may not be as effective as to keep the body and brain healthy. In previous times [57] and the last years [36, 62, 90], researchers on astronauts' gut microbiota mainly focused on microgravity's influence. It was discovered that alterations in the composition and functionality of the gut microbiome could be induced even by short-term space travel. Liu et al. [60] reported shifts between dominant genera in the microbiome during space missions of up to 35 days that led to an increased abundance of *Bacteroides* and a decrease of the probiotic taxa *Lactobacillus* and *Bifidobacterium* (the bacteria connected to immunomodulation). Under long-term space travel, the crew gut microbiome composition changed with a specific increase in the *Firmicutes*-to-*Bacteroidetes* ratio. It became more similar between astronauts and comparable to the microbiome composition of skin, nose, and tongue [90]. Moreover, the authors have reported the increase of genera associated with chronic intestinal inflammation and a reduction in the relative abundance of the genera with anti-inflammatory properties.

It may be suggested that supplementing live microbial foods may decrease space-related factors' impact on the gut microbiota. It may be a result of the flight's imposed conditions, including the decrease

in dietary fiber in the astronaut diet. Additionally, astronauts experienced spaceflight-related reductions in short-chain fatty acid (SCFA) — acetate, butyrate, and propionate, which fuel helpful intestinal bacteria in their gut microbiomes, such as *Pseudobutyrivibrio* and *Akkermansia* [90].

**Correction of dysbiosis and the usefulness of microbial therapeutics.** As the human gut microbiota is sensitive to orbital spaceflight, physiological adaptation to the new environment in future exploration missions will be more complicated than that in the ISS and lead to more changes in different body systems, including the gut microbiome. Recommendations have been formulated on correcting gut microbiota to keep the crew healthy and brains sharp [54, 85, 88]. We emphasize measures that include microbial therapeutics and postbiotics.

***Probiotics, fecal microbiota, and defined consortia perspectives on therapeutic microbes.*** Correction of astronauts' gut microbiome with microbial alive therapeutics could be appropriate in analogy to ground practice. However, considered a peculiarity of spaceflight missions, therapeutic microbes have limitations for use in spaceflight practice because of insufficient knowledge about their nature and safety or the reinsurance of officials responsible for flight safety. Among therapeutic microbial organisms, *probiotics* are considered «a live microorganism(s) which when administered in adequate amounts confer a health benefit on the host» (according to the Food and Agriculture Organization of the United Nations (Food and Agriculture Organization. 2006. Probiotics in food: health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization, Rome, Italy). The most studied probiotics are the lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacterium,* which are normal inhabitants of the human and animal intestine, and their presence is essential for maintaining the intestinal microbial ecosystem. Probiotics are necessary to maintain optimal immunity [63, 97]. Direct effects of probiotics on other microbes convey two primary mechanisms of action: inducing their antimicrobial effects by producing bacteriocins (peptides naturally synthesized by ribosomes) and secreting bile acids; both destroy pathogenic bacterial membranes — probiotics aid in decreasing the spread of antibiotic-resistant bacteria [29]. Maintaining a balanced microbiota by administering probiotics and probiotic-based foods and beverages will reduce antibiotic-resistant bacterial infections [86, 95]. *Lactobacillus* strains have been shown to produce neuroactive and neuroendocrine molecules to reduce stress-induced corticosterone and anxiety- and depression-related behavior [8]. In pain management, probiotics also have shown promising results [67].

It should be noted that probiotics are effective if there are indigestible foods in the diet. That is why *prebiotics* (food components that selectively stimulate the growth and/or activity of beneficial microorganisms directly in the human intestine) are recommended in the diet, and together with probiotics, these supplements form *synbiotics*. Synbiotics are often defined as 'synergistic mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and colonization of live beneficial microorganisms in the host's gastrointestinal tract' (FAO/WHO). Certain dietary fibers may preserve the probiotic efficacy by serving as the scaffold, *e.g.*, for probiotic Bacilli [83]. Synbiotics can modulate gut microbiota composition and microbial metabolite production [98].

In addition to probiotics, fecal microbiota transplantation (FMT) is reasonable for reestablishing biodiversity in a dysbiotic gut microbiome [91]. The most common practice is to derive fecal samples from healthy donors and use them to inoculate the alimentary tracts of patients exhibiting dysbiosis. Several studies have shown that after antibiotic treatment, autologous (self) fecal microbiota transplantation could reconstitute the changed microbiome [82, 84]. While the FMT approach effectively demonstrates the therapeutic potential on Earth, this practice should be standardized for crews.

Another solution for correcting dysbiosis is a genome-guided designed microbial consortium (DMC), a rationally selected collection of known microbes for treating intestinal diseases. DMC anticipates the removal of potential pathogens or antibiotic-resistant organisms from the gut microbiome [9]. Selected microbes with specific genes present in DMC should have antagonistic activity toward target pathogens. Hence, DMC has a number of advantages over, *e.g.,* FMT because symbiotic interactions

between therapeutic microbes and host are challenging to characterize due to their complexity. Including other beneficial microbial organisms (*i.e.*, archaea, fungi) in DMC and understanding the mechanisms of action is the long way toward bringing DMC into actual astromedicine practice [2].

A new possibility for phage-based precise microbiome editing is on the horizon to modulate microbial activities important for public health [49]. Phage therapy is envisioned as the targeted elimination of pathogens and probably aids those who do not restrain but remove pathogens, although the process of pathogens' appearance is permanent, and only collective defense forces oppose this arms race [54]. However, this promising tool is still in its infancy and probably will be used in the distant future for crews.

To sum up, microbiome engineering is a rapidly evolving frontier for solutions to improve human health, and two general engineering strategies — to manipulate indigenous microbes or to introduce new members — will play a fundamental role in space exploration. Both strategies have been practiced crudely for thousands of years in human health. However, despite current technical advances, designed inoculants/transplants are not expected to establish or confer long-lasting (months to years) modifications to micro-ecosystem structure and functions in crews.

According to professionals and decision-makers, the use of alive microbes for prevention or correction of disorders in astronauts will require conducting research that meets all required norms known in national regulations for probiotic microbes design, including genetic level (genes for pathogenicity and toxicity, mobile genetic elements, viruses, and known molecular mechanisms that improve human health [18]). The next level is to screen the crew for immunological tolerance against the probiotics and to prove the predicted beneficial effects. Then, a viable probiotics library for the crew should be built, and a personalized in-fight probiotics administration protocol should be established [17, 54].

***Kombucha as a synbiotic and edible vaccine.*** A beverage and jellyfish-like zoogley (a cellulose-based pellicle film) of Kombucha can be a good form of a synbiotic for extreme expeditions for several reasons. First of all, kombucha culture is a source of probiotic bacteria and yeast, as well as nanocellulose fibers — a prebiotic [89]. Second, KMC provides SCFAs that increase immunity. In addition, KMC is a rich source of vitamins C and K2, a group of B vitamins, and other biologically active compounds and minerals necessary for digestion. Finally, *in situ* processed KMC zoogley, enriched with coarse microfiber, polysaccharides, and proteins will be a permanent source of food for the crew and animals [50]. The diversity and stability of natural mutualistic microorganisms and a wide range of activity is the advantage of KMC over probiotics, consisting of a single strain or an artificially composed mixture of beneficial strains of microorganisms. Numerous observations have shown that the kombucha culture regulates the activity of the gastrointestinal tract via normalizing microbiota, *e.g.,* suppressing putrefactive pathogenic microflora and improving the body's protective properties. These data of traditional medicine were confirmed by laboratory tests [16] and in the former U.S.S.R. clinical settings [6]. In particular, the consumption of kombucha culture by LPS-treated or the type-2-diabetes-mice promoted the diversity of their gut microbiota, activating the SCFA-producing bacteria and providing anti-inflammatory effects [92, 96]. The recovery of normal gut microbiota was also observed in mice with the nonalcoholic fatty liver disease treated with kombucha, *i.e.,* an increase in the proportion of *Bacteroidetes* and *Lactobacillus* and a decrease of pathogenic bacteria caused this disease [48].

A significant advantage of KMC over microorganisms of health-promoting/therapeutic and biotechnological value is waste-free production, ranging from small-scale KMC products manufactured within bioregenerative life support systems (a confined self-sustained artificial ecosystem for the growing plant and animal food and generation of $O_2$, water, other necessary consumables) to large-scale production of «by-product» — cellulose. The organization of the KMC as a micro-ecosystem provides another decisive advantage over most microorganisms: resistance to contamination, easy cultivation, and versatility. Our results show that although the diversity of the KMC members changes during the flight on the LEO, they maintain their function and tolerance to stressors [39, 70].

Thanks to the biofilm hub, kombucha culture is almost immortal and can be activated when needed;

this means that the KMC does not have an expiration date and is probably its main advantage. Slow-growing biofilms form many persisters (subpopulations of dormant cells) that tolerate adverse factors. Kombucha culture exhibits metabolic plasticity and can be adapted to various economically viable food sources. KMC can be easily, safely, inexpensively, and effortlessly reproduced *in situ* (during flights and outposts.). It is important to note that the final product of Kombucha, in addition to positive health effects, also creates positive emotions, and caring for Kombucha for making kraft foods is good for emotional balance.

*Risk assessment of KMC members.* The US Food and Drug Administration (FDA, 1995) has concluded that Kombucha is safe for human consumption if adequately prepared. However, in extraterrestrial conditions, there is a risk of mutations that adapt microorganisms to new conditions and may harm human health. An experiment was conducted on the International Space Station (ISS) to prove or disprove this, where kombucha microorganisms were affected by space/Mars-like factors. The presence of humans on Mars is expected by the end of this decade; therefore, in research, it is vital to add to the factors of real space on the LEO characteristic Martian factors (ultraviolet light, atmosphere, and pressure). Simulated on the ISS experiments reduce the cost of deep space experiments that do not require getting to Mars. In the experiment BIOMEX we investigated the influence of Mars-like factors simulated on the ISS on the survival of the KMC IMBG-1, genetic stability, and toxicity of returned KMC samples [20, 70]. Dehydrated living cellulosic KMC films populated by pro- and eukaryotic microbial organisms that were latent within the cellulosic hub were used in a space experiment. After the period of 1.5 year-exposure, the KMC members recovered the kombucha culture [70].

***Study of the metagenome of the KMC and the genomes of Komagataeibacter spp. from the standpoint of biosafety.*** We used a metagenomic approach to determine the structure and functionality of the KMC community, previously influenced by $UV^+$ and $UV^-$ Mars-like conditions modeled on the LEO. After the action of hybrid space/Martian factors, there was a shift in the KMC structure. Ultraviolet-irradiated samples were mainly changed, and the dominant genus *Komagataeibacter* showed an increased number of species after the resuscitation. Functional profiling showed that the genes involved in the UvrABC system were most enriched in the metagenomes of KMC samples opened to UV. A comparison between post-flight and adapted during 2.5 years KMCs showed that the initial structure of the community was not restored completely.

This study demonstrates that the Kombucha complex communities can experience and resist severe stress, like that imposed by space and Mars-like conditions. Moreover, key KMC species have revived despite the harsh conditions and provided the community with the needed genes to form a three-dimensional cellulose-based hub [70]. These results encourage us to consider the KMC as a promising, robust micro-ecosystem that provides astronauts with probiotics, food, and biomaterials (cellulose) during long-distance space missions.

In the genome of *K. oboediens* IMBG180, a key bacterium of the KMC IMBG-1, islands of symbiosis and metabolic and virulence islands were found. However, they defected with any genes lost [78]. Again, the Rho transcription terminator gene was detected for prion-like proteins, but the Rho cPrD (prion-forming) domain was not found in the IMBG180 genome. This may mean that Rho does not behave like a prion in IMBG180. The genome has two plasmids that differ in sequence length. Two CRISPR-Cas systems were identified in the genome of IMBG strains of *K. oboediens* (both reference and post-flight), which were classified as CAS-VI-B and CAS-III-D. The only difference between the isolates was the position of some *cas* genes along the genome sequence. In the KMC metagenome, we found that the antibiotic resistome (total number of antibiotic-resistance genes) enriched in space-exposed samples, probably, in the same way as the genes involved in the UvrABC system because of the increased richness in bacterial community induced by spaceflight factors from latent state [74]. This finding indicates that we will pay more attention to mobile genetic elements and antibiotic resistance studies in bacteria from fermented foods.

The space-exposed bacteria's genome was compared with the ground-based reference genome to understand the genome stability of *K. oboediens* dur-

ing a long-time exposure under extraterrestrial conditions. Slight differences in size, total gene prediction, the total number of protein-coding genes, and other features between samples were determined. A remarkable similarity between strains in sequenced genomes was noticed. Results suggest that the genomes of *K. oboediens* IMBG180 (ground sample) and *K. oboediens* IMBG185 (space-exposed) are remarkably similar in topology. Nonetheless, there was a difference in the length of plasmids and the location of the *cas* genes. Despite these differences, they do not affect metabolic profiles. Minor changes were observed in central carbohydrate and energy metabolism pathways, gene numbers, or sequence completeness. These findings suggest that *K. oboediens* maintains its genome stability and functionality in the KMC exposed to the space/Mars-like environment.

***Limitations for therapeutic microbes. More questions than answers before traveling to Mars.*** The criteria used for probiotics selection are highly stringent: to be Generally Recognized As Safe (GRAS); to survive through the gastrointestinal tract and to colonize it, finally; to tolerate low pH and bile salts; to produce antimicrobials and inhibit pathogens; do not possess transferable antibiotic-resistance genes; to be of human origin (summarized in [26]). Some of these traits, *e.g.*, the efficacy of probiotics to integrate into the host gut ecosystem, is variable [44]. One of the shortcomings of wild-type probiotics is their non-specificity. However, a given probiotic could be engineered to exhibit species-specific inhibition of the pathogen and its associated infection [64]. The long-term stability of lactic acid bacteria starters for probiotics production during extended space missions could be problematic because of the impact of radiation [93]. Probably, spores of bacilli and cells of probiotics naturally incorporated into cellulose fibers might be used for in-flight probiotic/synbiotic food production [32, 50].

Despite progress in researching various microbiomes as candidates for microbial therapeutics, technologies exploiting a microbial structure and function remain limited and need to be enhanced by significant interdisciplinary collaborative efforts. The design of efficient living therapeutics is considered revolutionary [17]. However, for these discoveries, humankind will spend a long time in labs and debates, but before traveling to Mars, we have a shortage of time. Meantime, astronauts cannot be on a long-distance mission without the support of their gut microbiome by biologicals. Postbiotics as non-alive biologics can be considered the interim stage of probiotics usage in the prophylaxis of health disorders of crewmembers during spaceflights. The term «postbiotics» as a promising inanimate MV-based means for astronauts has been coined in our research [53, 70].

## 3. POSTBIOTICS BASED ON EXTRACELLULAR MEMBRANE VESICLES AS AN INTERIM SOLUTION BEFORE THE MICROBIAL THERAPY ERA IN ASTROMEDICINE

According to the International Scientific Association for Probiotics and Prebiotics, a postbiotic is «a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host» [76]. Postbiotics refer to probiotic-derived products obtained from food-grade microorganisms that confer health benefits when administered in adequate amounts [10]: secreted by viable cells metabolites, its by-products, the products of cell lysis [94], and cell nanostructures such as extracellular membrane vesicles [40, 70]. Research indicates that postbiotics can have direct immunomodulatory and clinically relevant effects [40, 70].

**Microbiota-secreted membrane vesicles are critical players in microbe-host communication.** Extracellular membrane vesicles are nanoscale structures formed by a living cell and released into the extracellular space to perform biological functions [33, 87] (Fig. 1A, B). MVs carry membrane and cytoplasmic proteins, DNA, various classes of RNA, lipids, ATP, and other bioactive molecules between cells of all three domains of life that secrete several types of membrane nanosized vesicles with different physiological properties. Although their biogenesis is different, they are all formed by phospholipid membranes and excreted by cells externally. Theoretically, all types of cells produce heterogeneous populations of MVs and are present in body fluids. MVs are incapable of self-reproduction and have no metabolism but transfer bioactive molecules from one cell to another, including over long distances, overcoming the blood-brain barrier. Due to their unique properties,

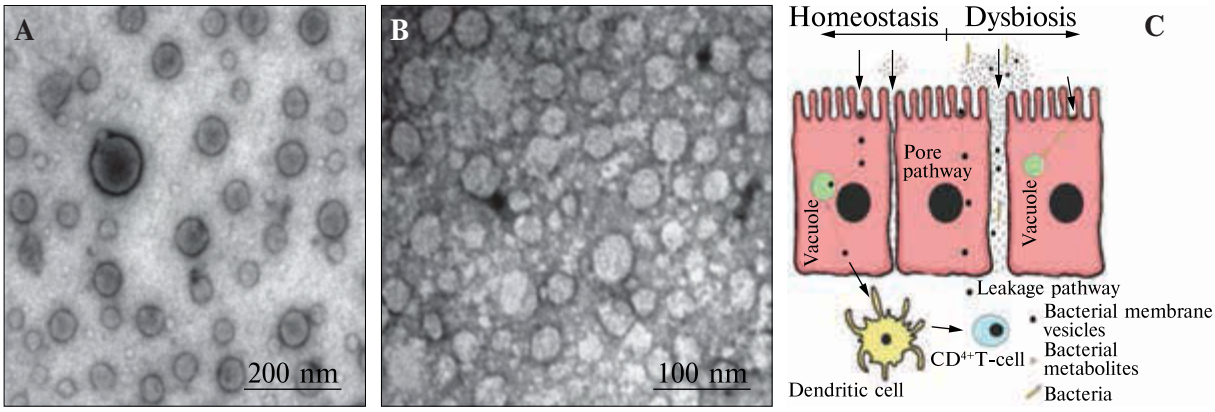


***Figure 1.*** Membrane vesicles (MV)s as a non-cell postbiotic or edible vaccine. A, B — morphology of extracellular membrane vesicles of kombucha community members: from bacteria *Komagataeibacter oboediens* and yeast *Pichia fermentas*, respectively. Transmission electron microscopy. Bars 200 and 100 nm. C — a graphical image of a fragment of the intestinal cell monolayer. MVs are transported into the gut by endocytosis or direct fusion with the cell membrane [13]. Paracellular transport is via either the pore pathway or the leakage pathway. MVs released by the gut bacteria restore the intestinal epithelial barrier function disrupted by enteric pathogens [31]. Local immune dendritic cells exposed to MVs activate naïve T-cells and trigger higher levels of cytokines and specific regulatory miRNAs [25]. The probiotic bacteria MVs facilitate the delivery of bacteriocins to resist pathogens in the host gut [21] and manipulate the host cellular activities via the small RNAs carried as cargo [81]

MVs have become an attractive object of host-microbial research. More and more evidence appears that host-microbial crosstalk is mediated by MVs released by gut microbiota [14, 23—25]. In regenerative medicine, MVs are a promising method of treating various diseases and non-healing wounds [4, 79].

In gram-negative bacteria, MVs are formed by extrusion of outer cell membrane fragments and have been characterized as containing their components: lipopolysaccharides, enzymes, and other proteins, as well as DNA molecules and RNA species [11, 23, 24]. In gram-positive bacteria, vesicles with cytoplasmic contents are extruded through gaps in the cell wall [61] or formed due to prophage activation. Archaea also produce single membrane MVs through a flexible external cell wall-like structure. Unicellular fungi such as yeasts are protected by a thick, well-built cell wall composed of glycoproteins and polysaccharide chitin. This barrier protects them from the external environment and limits the exchange and communication in both directions, including via MVs. A cell wall must be remodeled before MVs cross, so fungal MVs have been reported to contain the cell wall remodeling enzymes [59, 77].

In the gut ecosystem, bidirectional microbiota-host communication does not always depend on direct cellular contact and is performed by secreted microbial factors, *e.g.*, MVs, that can penetrate the mucous layer and gain access to cells of the intestinal mucosa [46, 55] (Fig. 1C).

***Biological functions of bacterial extracellular vesicles in host-microbe interactions.*** The functions and effects of BMVs on host physiology depend on the diversity of their cargo, and the latter is influenced by bacterial species, growth, and environmental factors. Bacteria package small molecules, proteins, and genetic material into BMVs to provide a supportive environment under interaction with the host [3], *e.g.*, enzymes that aid polysaccharide digestion in the gut or host-indigestible glycans and host mucins [41]. BMVs released by *L. acidophilus* deliver bacteriocins and thus kill pathogenic bacteria [21]. A study shows that gram-negative and gram-positive-derived BMVs deliver DNA into other bacterial cells, mediating horizontal gene transfer. It also facilitates the interaction of transported nucleic acids with their specific intracellular receptors in host cells, triggering the modulation of host immune responses [38]. Besides DNA, some studies have shown the presence of RNAs in BMVs, which protect these fragile molecules. Non-coding small RNAs (sRNA) have important implications for regulating the host immune

system and other cellular processes [47]. Bacterial pathogens translocate bacterial sRNAs packed into BMVs into host cells and use them to affect host defense signaling pathways [75], therefore, mimicking eukaryotic miRNAs that regulate the expression of key sensors and regulators of host immunity. At the same time, host extracellular vesicles use to transfer host miRNA molecules to microbes [11].

BMVs, as mediators of probiotic beneficial effects, are known for their role against pathogens by transferring antimicrobials and modulating host innate and adaptive immunity (summarized in [26]). For example, BMVs produced by probiotic bacteria reduce the increased expression of pro-inflammatory cytokines and down-regulate enzymes associated with injury and inflammation [23, 24, 73]. BMVs from *Escherichia coli* activate dendritic cells and subsequent T-cell responses and protect the intestinal epithelial barrier function [31]. The BMVs from the gut bacteria mediate modulation of dendritic immune cells to coordinate suitable T-cell responses through several mechanisms and influence the release of immune mediators through exosomes, *i.e.,* modulate the exosome cargo [25].

***Vesicles from the probiotic bacteria could be a safe free of bacteria strategy to preserve astronauts' health.*** A new generation of postbiotics, the BMV-based products, are expected to avoid the risks associated with the administration of living bacteria. BMVs play a role in many of the same activities as parent bacteria, but they have the advantage of access to the blood circulation and the CNS. BMVs so far facilitate the delivery of signaling and fragile molecules, such as sRNA, that could not survive under transportation being unprotected. BMVs from beneficial bacteria can independently influence the host [43] and could be used with concurrent administration with antibiotics and other antimicrobial agents. If oral consumption of live bacteria rapidly leads to circulation of their metabolites or constituents, *e.g.*, bacteriophages, in blood, so far demonstrating the putative effect of their hosts [14], consumption of the BMV-based postbiotics could prevent unwanted elements from entering the bloodstream; it is crucial for astronauts with affected intestines and immunocompromised patients. BMVs have also a set of advantages over both alive and non-viable bacterial cells: they are quite safe, with reduced risk for adverse effects in vulnerable individuals with an impaired immune system (1); they have no risk for mutations (2); BMVs are not alive and cannot divide (3).

**Extracellular membrane vesicles as a component of the KMC secretome.** Production of spherical protein-lipid MVs (20-500 nm) by KMC microbial cells is observed in bacteria and yeasts, covering both prokaryotes (gram-negative and gram-positive bacteria) and eukaryotes (yeasts). Fig. 1 shows MVs of bacteria and yeast originating from the kombucha community. The KMC produces six-seven MV populations composed of 50 % of metavesicleome, with two significant populations of MVs of a diameter of 141 and 164 nm, and the rest populations deviate in size. After the revival, laboratory control KMC samples produced MV populations that resembled the initial KMC, the MVs of exposed KMCs were characterized by different sizes and numbers of fractions, depending on the nature of stressors influenced. For example, MVs from post-UV-protected KMC samples did not possess small-size fraction vesicles but contained two significant populations with MVs diameters of 164 and 190 nm, demonstrating a shift in the MV average sizes. In contrast, MVs from unprotected samples produced more smaller-size MV populations. Most tested vesicle populations appeared to be of a single membrane; however, outer-inner bacterial vesicles were also detected. Some deformations in vesicles and their aggregations were observed in the KMC-exposed samples, exhibiting changes in the membrane lipidome. Membrane lipids such as sterols, fatty acids (FAs), and phospholipids (PLs) were modulated under the Mars-like stressors, and a level of saturated FAs increased, as well as both short-chain saturated and trans FAs appeared in the membranes of MVs shed by both post-UV-illuminated and protected on the ISS bacteria. The relative content of zwitterionic and anionic PLs changed, producing a change in surface properties of outer membranes, thereby resulting in a loss of interaction capability with polynucleotides. The changed composition of membranes promoted changes in MV fitness. Biochemical characterization of the membrane-associated enzymes revealed increased activity (DNAses, dehydrogenases) compared to wild type. Other functional membrane-associated capabilities

of MVs (*e.g.*, proton accumulation) were also altered after exposure to the spaceflight stressors.

***KMC MVs do not acquire the toxicity under Mars-like stressors simulated on the LEO.*** Changes in microbial membranes inevitably affect their communication with hosts. Both commensal and pathogenic gram-negative bacteria have developed various interaction mechanisms with host cells, such as the formation of BMVs, which can interact with host cell receptors and deliver cargo to their remote targets, *e.g.*, intestinal DNA-containing BMVs readily pass through obese gut barrier and deliver microbial DNAs into β cells, resulting in elevated inflammation and impaired insulin secretion [35]. The study of the impact of altered bionanostructures on the host *in vitro* and *in vivo* scenarios will be a vital task of Astromedicine in the development of new solutions for the intestinal microbiota health of astronauts. In our project, we show that, despite the change in the structure of cell membranes, MVs isolated from the irradiated on LEO KMCs in the BIOMEX did not show endotoxicity, cytotoxicity, and possibly neuromodulation [71]. Treatment of murine embryonal fibroblasts and macrophages by MVs/KMC at concentrations of 0.05—50.0 μg/L did not affect cell growth (BMVs act in femtomole concentrations, $10^{-15}$ of a mole) [71]. Incubation of BMV/*K. oboediens* with human cell culture COLO205 also did not show cytotoxicity, both from spaceflight and control samples of KMC [71]. MVs isolated from the ground control KMC showed a 50-times lower level of endotoxin activity in the endotoxin test compared to *E. coli*, which was used as a positive control. The level of metavesiculome endotoxin activity from post-flight unprotected KMC samples was 6 times lower than *E. coli*. The levels of endotoxin activity of BMV/*K. oboediens* from both protected and unprotected KMC were almost the same as the activity of BMV of the reference KMC. MVs of post-flight KMCs did not increase the level of L-[$^{14}$C] glutamate neurotransmitter in synaptosome suspensions in the nerve terminals of the rat brains, *i.e.*, do not acquire neuromodulation capacity. Moreover, MVs/UV-unprotected KMCs even reduced the content of L-[$^{14}$C] glutamate, but MVs from the protected samples of KMC did not differ from MVs of reference KMC [70].

Altogether, our results show that MVs, originating from nonpathogenic gram-negative bacteria might be considered candidates in the design of postbiotics or edible mucosal vaccines for *in situ* production in the extreme environment. Furthermore, these MVs could also be used as suitable drug/gene delivery vectors for applications in Astromedicine.

## FUTURE PERSPECTIVES

The crewed missions to Mars and extensive colonization of the red planet are the near future for humankind. Advanced biotechnologies could provide exhausting life-supporting materials aboard spacecraft and in extraterrestrial colonies, as they are based on the rational use of resources and energy, re-utilization, and contribute to health protection [12]. The bioregenerative life support system seeded by small-scale necessities brought from our planet is addressed to deploy the production of fresh life-supporting foods and materials for crews [60, 66]. The current space food system, which uses pre-packaged meals and beverages, has been considered sufficient for a future mission to Mars. Nevertheless, fresh-prepared food in the diet would be reasonable. Fermented foods containing alive microbial cells could solve many problems, such as the protection of normal gut microbiota and a fresh supply of antioxidants, enzymes, radioprotective agents, SCFAs, and vitamins packaged on Earth, which cannot be preserved within long-duration travels [15]. By producing kefir, growing plants and edible flowers, and taking care of Kombucha, crewmembers could get psychological comfort and sustain mental health. NASA is making experiments on the study of the crop functioning for developing new products [22] and producing probiotics [5]. Prospective applications of 3D food printing will play a pivotal role in health improvement and personalized nutrition during space missions [30]: it is believed that fungi, algae, and insects grown in the bioregenerative life support system should be introduced into the astronaut's diet [58].

The microbial production system on board should meet NASA safety guidelines that prescribe no live microorganisms in the product (although fresh surface-sterilized carrots and celeries permitted for use by crews [65] probably contain endophytic bacteria). Spaceflight BioNutrients-1, BioNutrients-2 projects

(NASA) show that microbial cultures may be used safely for fermented foods production, including yogurt and kefir, for crew health [5]. Until now, food safety guidelines do not allow the crew to consume high amounts of live microorganisms, *i.e.*, fermented foods, during spaceflight. Obviously, this should be changed to nowadays trends in science. A set of criteria for the scientific substantiation of health claims on fermented food for production and consumption beyond Earth remain to be addressed. So, new safety protocols (and standards) must be developed for the production and storage of fermented foods for use by crewmembers. It will include a food safety real-time monitoring system designed to identify and control potential hazards during all phases of production and storage to ensure that the fermented food product is free of contamination. The change of a paradigm of acceptance of microorganisms as enemies on the principle of coexistence with them would greatly benefit the creation of a healthy biosphere in alien habitats. Humans are entirely dependent on microbial life and doomed to co-exist with various microbial organisms, therefore, in interplanetary missions, crews will carry hundreds of microbial species associated with their bodies. Around half of the microbial signatures on their habitation surfaces will be represented by crew members' microbiota. In addition, microbes on/inside plants and microbial components of bioregenerative life support systems will create a microbial background aboard spacecraft. In other words, its microbiome is built. The more diverse the microbiota, the more substantial balance of valuable and opportunistic/detrimental counterparts, and the healthier the built microbiome and ecosystem from the standpoint of human health will be established. It would be reasonable to choose beneficial microflora, which is self-organized as a microecosystem based on metabolic symbiosis, cooperation, and competitiveness and which co-evolved for centuries under the assistance of humans and proved a safe mutualistic coexistence with hosts to diversify the microbiota, *i.e.*, to modulate the microbiome in the artificial ecosystem. Applying competitive exclusion principles may counter-select pathogenic species and prevent further colonization, stably ecologically modulating the microbiome. Some examples show that probiotic-based strategies can rebalance the hospital microbiome, leading to a stable reduction of pathogen contamination and the associated infections (summarized in [19].

While the use of alive microbial organisms is being debated, microbial MVs from probiotic and gut commensal bacteria or probiotic communities like KMC could be efficient in protective mucosal immunization through oral, nasal, rectal, or vaginal routes. Also, the restoration of normal immune response could be facilitated by mucosal vaccination, using MVs of safe, rationally selected microbial organisms for microbiome-targeting produced *in situ*. Studies with lactic acid bacteria, propionibacteria, and akkermansia have shown that there is reason to believe that some exposure to their MVs might be beneficial in training the nascent immune system and down-regulating inflammations. MV-based products are expected to avoid the risks associated with the administration of live bacteria and are considered the interim stage of probiotic use to prevent crewmembers' health disorders until their safety is proven. Future research on GRAS microbes-derived MVs is needed to open new possibilities for using bioactive therapeutic molecules for crews in space missions.

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## КОНЦЕПТУАЛЬНИЙ ПІДХІД ДО ВИКОРИСТАННЯ ПОСТБІОТИКІВ НА ОСНОВІ БАКТЕРІЙНИХ МЕМБРАННИХ НАНОВЕЗИКУЛ ДЛЯ ПРОФІЛАКТИКИ РОЗЛАДІВ ЗДОРОВ'Я КОСМОНАВТІВ

Функціональні продукти, що містять живі мікроорганізми та їхні компоненти, необхідні для нормального функціонування організму людини, оскільки нормальна мікробіота кишечника потребує підживлення від інших мікробних організмів та їхніх наноструктур — мембранних везикул (МВ), що виділяються назовні. Це дослідження було започатковано концепцією, що МВ можуть робити свій внесок у здоров'я астронавтів так само, як і їхні батьківські

клітини, і стати тимчасовою заміною живих мікробних клітин, поки не стане відомо більше про поведінку мікробів у космічному середовищі. Перевага МВ полягає в тому, що вони є неживими і не зазнають змін за несприятливих умов, як це може відбуватись із мікробними організмами. Як модель ми вибрали МВ стійкої до факторів навколишнього середовища мультимікробної культури комбучі (МКК), відомої своїми оздоровчими властивостями для людини. На початковому етапі перевірки концепції ми експонували МКК на Міжнародній космічній станції в гібридному космічному/марсоподібному середовищі. Культура комбучі пережила тривалий період експонування, а МВ, утворені членами угруповання МКК після польоту, не набули токсичності, незважаючи на змінений склад їхніх мембран після перебування в середовищі, що імітувало умови поверхні Марса. Це спостереження разом з нашими метагеномним та порівняльними геномними аналізами МКК та домінантної бактерії угруповання комбучі — *Komagataeibacter oboediens* — показали, що геноми наземного та експонованого в космосі зразків були подібними за топологією та стабільністю. На наступному етапі ми розпочали оцінку придатності та безпечності МВ післяпольотного *K. oboediens* і показали, що вони були змінені, але зміни в структурі їхніх мембран не призвели до набуття ними цитотоксичності. Наша стратегія підтвердження концепції обговорюється в цьому огляді відповідно до даних літератури.

***Ключові слова***: постбіотики, екстрацеллюлярні мембранні везикули, мультимікробна культура комбуча, функціональне харчування, зміцнення здоров'я.