Probiotics maintain the intestinal microbiome homeostasis of the sailors during a long sea voyage

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ABSTRACT
The challenging conditions encountered during long sea voyages increase the risk of health-threatening physiological and psychological stress for sailors compared with land-based workers. However, how the intestinal microbiota responds to a long sea voyage and whether there is a feasible approach for protecting gut health during sea voyage are still unexplored. Here, we designed a 30-d longitudinal study including a placebo group (n = 42) and a probiotic group (n = 40) and used shotgun metagenomic sequencing to explore the impacts of sea voyage on the intestinal microbiome of sailors. By comparing the intestinal microbiome of subjects in the placebo group at baseline (d 0) and at the end of the sea voyage (d 30), we observed an alteration in the intestinal microbiome during the long sea voyage based on the microbial structure; the results revealed an increase in the species Streptococcus gordonii and Klebsiella pneumoniae as well as a decrease in some functional features. However, the change in the microbial structure of sailors in the probiotic group between d 0 and d 30 was limited, which indicated a maintenance effect of probiotics on intestinal microbiome homeostasis. At the metagenomic strain level, a generally positive correlation was observed between probiotics and the strains belonging to Bifidobacterium longum and Bifidobacterium animalis, whereas a common negative correlation was observed between probiotics and Clostridium leptum; this result revealed the potential mechanism of maintaining intestinal microbiome homeostasis by probiotics. The present study provided a feasible approach for protecting gut health during a long sea voyage.

Introduction
Seafaring has a long history and greatly promotes the circulation of goods, cultural communication and territorial defense.1 However, the challenging conditions encountered during long sea voyages increase the risk of disease and death for sailors compared with land-based workers. The ocean environment includes high humidity, high salinity, intense UV radiation, stormy waves, monotonous surroundings, altered circadian biorhythms, sleep deprivation, and an insufficient supply of fresh fruits and vegetables, causing significant health-threatening physiological and psychological stress.2–4 In addition to historically well-known septicemia caused by vitamin deficiency, chronic diseases of the immune system and digestive system have become the greatest risk to the health of sailors5 and have also become a potential threat to the development of seafaring.

The human intestinal microbiome is fundamental to the host immune system and is essential for maintaining human health.6 In recent decades, emerging evidence has highlighted the importance of the diversity of intestinal microbial species and functional genes in various chronic metabolic diseases. Although much attention has been paid to the health management of sailors during long sea voyages,5,7 the research in this area remains limited. For example, the isolation of a land-based environment and the insufficient supply of fresh fruits and vegetables during a long sea voyage were all able to influence the intestinal microbiome of sailors. However, how
the intestinal microbiota responds to a long sea voyage and how specific intestinal species or functional genes change are still unexplored. Probiotics are defined as live microorganisms that confer health benefits to the host when present in adequate amounts and commonly include bacterial strains of the genera *Lactobacillus* and *Bifidobacterium*. Currently, most probiotic-related studies have simply described fluctuations in intestinal microbiota profiles concluding that limited structural changes in the intestinal microbiota are a universal phenomenon that are accompanied by increased beneficial microbes and decreased pathogens after probiotic consumption. Some clinical studies have applied probiotics to treat a number of diseases, including liver disease, cardiovascular diseases, kidney disease and irritable bowel syndrome. Although not all of these studies were positive, we confirmed that probiotics exhibited common effects on intestinal microbiota regulation. Based on the consensus and the question we asked above, we wanted to know whether probiotics can maintain intestinal microbiome homeostasis in sailors during a long sea voyage. If so, how does this maintenance work?

Therefore, we designed a 30-d longitudinal study including two Chinese sailor cohorts (a placebo group, *n* = 42, and a probiotic group, *n* = 40) and used multi-probiotics, including *Lactobacillus casei* Zhang, *Lactobacillus plantarum* P-8, *Lactobacillus rhamnosus* M9, *Bifidobacterium lactis* V9 and *Bifidobacterium lactis* M8, which exhibited excellent probiotic characteristics in our previous studies, to explore the risk of a long sea voyage on the intestinal microbiome of sailors and reveal the potential mechanism of maintaining intestinal microbiome homeostasis by probiotics. The results of the present study will provide a feasible approach for protecting gut health during a long sea voyage and provide new insights into a personalized selection of probiotics based on population-level analyses.

Results

Comparative analysis of health-related physiological and psychological indexes between the placebo and probiotic groups

Because of the special ocean environment, most sailors suffer physiological and psychological stress during long sea voyages. To evaluate the potential effects of probiotics on improving a sailor’s fitness, we designed a questionnaire including scores for bowel movement (hardness of feces, volume of feces, constipation, diarrhea, bloody stools and defecation frequency), pain (stomachache, headache, pectoralgia, backache and muscle soreness) and stress/anxiety (flustered, dizziness, amnesia, annoyance, low activity, mistrustful, poor appetite, self-accusation, loneliness, palpitation and insomnia) at the end of the trip (Table S1). We set each subject’s score for the questionnaire content to 10 as baseline. Therefore, a decreased score represented the unfitness of the subject in the corresponding questionnaire content during the voyage (Figure S1(a–c)). By comparing the scores between the two cohorts, significant differences in the decreased score of stress and anxiety were observed (Figure S1(c)) in the placebo group, which indicated the potential role of probiotics in preventing anxiety during a long sea voyage.

Alteration of the intestinal microbiome in sailors during a long sea voyage

To address our initial questions, how intestinal microbiota respond to a long sea voyage and how specific intestinal species and functional genes change, we compared the intestinal microbiome of subjects in the placebo group at baseline (d 0) and at the end of the sea voyage (d 30) by shotgun metagenomic sequencing (Table S3). A principal coordinate analysis (PCoA) was performed based on the Aitchison and Bray–Curtis distances of the metagenomic sequencing profiles at the species level (Figure 1(a, b)). The intestinal microbiota from the subjects in the placebo group on d 0 and d 30 were distinct with respect to organismal structure. To quantify the difference, we calculated the *P* values (Wilcoxon rank-sum tests) of PC1 based on Aitchison distances (*P* = .014) and Bray–Curtis (*P* = .058), which indicated that the impacts of a long sea voyage on the intestinal microbiota of sailors were significant. Meanwhile, it was observed that the microbial Bray–Curtis and Aitchison distances of sailors in the placebo group between d 0 and d 30 were significantly larger than those of sailors in the probiotic group (Figure 1(c)). The results were
confirmed by microbial alpha diversity analysis, in which we observed a sharp decrease in microbial alpha diversity between the subjects at baseline and at the end of the sea voyage (Figure 1(d), Table S4).

At the taxonomic level (Figures 2 and S2, Tables S5 and S6), we focused on the intestinal species that had no significant difference between the placebo and the probiotic groups at baseline but changed significantly at the end of the voyage (d 30) in the placebo group. To correct the potential bias introduced by the relative abundance matrix (Table S6), we transformed the relative abundance using a centered log-ratio (CLR) transformation of the relative abundance matrix (Table S6). By comparing the species abundance at baseline, we observed that the CLR abundances of the species *Bacteroides faecis* and *Roseburia hominis* decreased significantly in sailors of the placebo group at the end of the sea voyage, whereas the CLR abundances of the species *Streptococcus infantis*, *Streptococcus gordonii* and *Streptococcus salivarius* were enriched (Figure 2(b,c); Wilcoxon rank-sum tests). Meanwhile, we compared the difference in species abundance between the placebo and probiotic groups at the end of the voyage and found that the
Figure 2. The metagenomic species was not significantly different in the centered log-ratio (CLR)-transformed abundance at baseline between the placebo and probiotic groups but significantly changed at the end of the voyage (d 30) (Wilcoxon rank-sum tests). (a) The species had no significant difference at baseline between the placebo and probiotic groups but changed significantly only in the probiotic group during the sea voyage. (b) The species changed significantly only in the placebo group during the sea voyage. (c) The species changed significantly both in the placebo and probiotic groups during the sea voyage. (d) The species had no significant difference at baseline between the placebo and probiotic groups but changed significantly between the two groups at the end of the voyage.
species Bacteroides cacaee, Bacteroides eggerthii, Bacteroides intestinalis and Phascolarctobacterium succinatutens decreased sharply in the placebo group (Figure 2(d); Wilcoxon rank-sum tests).

To investigate the observed differences in the functional profiles of the intestinal microbiota during the long sea voyage, high-quality reads from all samples were assembled and annotated for protein-coding genes. Based on the results, a collective, non-redundant intestinal microbiota gene catalog was created. Next, for each sample, the reads were mapped to the collective gene catalog to reconstruct sample-specific gene profiles, and metabolic pathways were also generated with the Kyoto Encyclopedia of Genes and Genomes Orthology database (Tables S7 and S8). PCoA was performed based on the Bray–Curtis distances of the intestinal microbial functional gene profiles (Figure S3), and the specific changes in the microbial metabolic pathway were represented by a decreased toluene degradation ability and ubiquinol and glycogen synthesis ability (Table 1). Additionally, a significant shift from the intestinal microbial carbohydrate-active enzyme (CAZy) gene profile (Table S9) based on the Bray–Curtis distances (Figure 3(a)) and a sharp decline in the alpha diversity of microbial CAZy genes (Figure 3(b)) were observed at the end of the voyage in the placebo group, which were represented by a decrease in the relative abundance of the gene families glycoside hydrolases (GH), glycosyltransferases (GT) and polysaccharide lyases (PL) (Figure 3(c)). These results indicated that the long sea voyage not only disordered the balance of the intestinal microbiota of sailors but also reduced the diversity of functional features of intestinal microbiota.

### Probiotics maintained intestinal microbiome homeostasis during the long sea voyage

Since we observed that the long sea voyage had a significant impact on the intestinal microbiome, we further addressed our second question, whether probiotics can maintain intestinal microbiome homeostasis in sailors during a long trip. The structures of the intestinal microbiota of subjects in the probiotic group were compared (Aitchison and Bray–Curtis distances based on metagenomic species level, Figure 1(a,b)), but we did not observe any significant difference, and the P values (Wilcoxon rank-sum tests) of PCI based on Aitchison and Bray–Curtis distances were all greater than 0.05. Additionally, the microbial Aitchison and Bray–Curtis of sailors in the probiotic group between d 0 and d 30 were significantly less than those of sailors in the placebo group (Figure 1(c)). To confirm our observation, we further compared the microbial structure of sailors between the placebo group and the probiotic group on d 0 and d 30. Interestingly, no significant difference was found at baseline between the two groups, but the compositions of the intestinal microbiota of the two groups were highly distinct at the end of the sea voyage, which confirmed the positive impacts of the probiotics consumed. Even though the change in microbial structure was limited, we could also

### Table 1. The significantly different pathways in the placebo group between d 0 and d 30.

| Pathway description | Log2 FC | LfcSE | Stat   | P value | Pathway description | Log2 FC | LfcSE | Stat   | P value |
|---------------------|---------|-------|--------|---------|---------------------|---------|-------|--------|---------|
| 1,3-propanediol biosynthesis | 1.07    | 0.34  | 3.11   | 0.0019  | Ketoglucurate metabolism | -0.81   | 0.38  | -2.13  | 0.0329  |
| 4-methylcatheline degradation | -1.72  | 0.37  | -4.67  | 0.0000  | Fucose degradation | -0.59   | 0.26  | -2.30  | 0.0217  |
| Adenosine nucleotides degradation | -1.36  | 0.40  | -3.40  | 0.0007  | L-arginine biosynthesis | -0.50   | 0.20  | -2.55  | 0.0106  |
| Aerobic respiration | -1.41  | 0.42  | -3.34  | 0.0008  | L-citrulline metabolism | -1.96   | 0.42  | -4.62  | 0.0000  |
| Allantoin degradation in yeast | -1.12  | 0.36  | -3.13  | 0.0017  | L-glutamate degradation | -1.09   | 0.35  | -3.15  | 0.0016  |
| Anaerobic sucrose degradation | -1.17  | 0.54  | -2.16  | 0.0305  | L-phenylalanine degradation | -0.86   | 0.37  | -2.32  | 0.0204  |
| Aromatic compounds degradation | -2.17  | 0.38  | -5.73  | 0.0000  | Mevalonate pathway | 1.42    | 0.43  | 3.32   | 0.0009  |
| Catechol degradation | 2.17   | 0.38  | 5.73   | 0.0000  | Myo-inositol degradation | 1.18    | 0.34  | 3.49   | 0.0005  |
| Chorismate metabolism | -2.04  | 0.49  | -4.19  | 0.0000  | Peptidoglycan biosynthesis | -2.11   | 0.56  | -3.78  | 0.0002  |
| DOP-N-acetylysamine biosynthesis | 1.09   | 0.36  | 3.03   | 0.0025  | Phosphatidate metabolism | -1.34   | 0.42  | -3.21  | 0.0013  |
| Factor 420 biosynthesis | 1.24   | 0.40  | 3.07   | 0.0021  | Protocatechuate degradation | -1.14   | 0.37  | -3.08  | 0.0021  |
| L-1,2-propanediol degradation | -1.56  | 0.43  | -3.61  | 0.0003  | Isopropenyl biosynthesis | 0.97    | 0.33  | 2.93   | 0.0034  |
| Glycogen biosynthesis | 0.92   | 0.35  | 2.63   | 0.0086  | Sucrose degradation | -1.17   | 0.55  | -2.15  | 0.0317  |
| Heme biosynthesis from glycine | -0.90  | 0.38  | -2.39  | 0.0168  | Toluene degradation | 2.18    | 0.41  | 5.29   | 0.0000  |
| Purine nucleotides degradation | -0.28  | 0.13  | -2.05  | 0.0402  | Ubiquinol biosynthesis | 0.77    | 0.39  | 1.99   | 0.0468  |

**Note:** Log2 FC represents the log fold change, and a value>0 represents a related metabolic pathway enriched in the placebo group on d 0; a value<0 represents a related metabolic pathway enriched on d 30.
observe some specific changes at the microbial species level. The CLR abundances of the species *Bifidobacterium longum*, *Bifidobacterium animalis* and *Lactobacillus plantarum* increased significantly in sailors at the end of the sea voyage, whereas those of the species *Clostridium leptum*, *Klebsiella pneumoniae* and *Prevotella copri* decreased (Figure 2(a); Wilcoxon rank-sum tests). Similarly, the change in the intestinal microbial metabolic pathway was limited in the probiotic group during the sea voyage (Table 2), and no significant change was observed in the diversity of microbial CAZy genes (Figure 3(a,b)). Taken together, these results suggest that the probiotics maintained intestinal microbiome homeostasis during the long sea voyage.

The potential effective mechanism underlying the interaction between probiotics and intestinal microbes

After confirming that the probiotics were able to maintain intestinal microbiome homeostasis during the long sea voyage, we were eager to explore the third question: how do the probiotics work? To address this question, we had to elucidate the correlation and the interaction between the probiotics consumed and the host symbiotic intestinal microbes. Effective persistence in the host gut is crucial for the health-promoting characteristics of consumed probiotics. Accordingly, by mapping to the whole genomes of the consumed probiotics, we calculated the relative abundance of the five strains (Figure 4(a), Table S10), and the five probiotics exhibited excellent colonization ability. Then, we further explored the correlations between the consumed probiotics and the other intestinal microbes by constructing a bundle network based on the determined Spearman’s rank correlation coefficients (Figure 4(b), Table S11). As shown in Figure 4(b), a generally positive correlation was observed between the strains *Lactobacillus casei* Zhang, *Lactobacillus plantarum* P-8, *Lactobacillus rhamnosus* M9, *Bifidobacterium lactis* V9 and *Bifidobacterium lactis* M8 and the species *Bifidobacterium longum*, *Bifidobacterium animalis*, *Bacteroides eggertii*, *Clostridium butyricum*, *Eubacterium rectale*, *Enterococcus faecalis*, *Enterococcus faecium* and *Escherichia coli*. No significant negative correlation was observed. The heatmap revealed a significant decrease in the relative abundance of CAZy gene families, including glycoside hydrolases (GH), glycosyltransferases (GT) and polysaccharide lyases (PL), in the placebo group during the sea voyage.
Lactobacillus fermentum and Lactobacillus plantarum, whereas a common negative correlation was observed between the five probiotics and the species Clostridium leptum, Alistipes shahii, Klebsiella pneumoniae, Streptococcus salivarius and Prevotella copri.

Finally, since we observed the potential role of probiotics in stress and anxiety improvement during

Table 2. The significantly different pathways in the probiotic group between d 0 and d 30.

| Pathway description                                      | Log2 FC | LfcSE | Stat  | P value | Pathway description                                      | Log2 FC | LfcSE | Stat  | P value |
|----------------------------------------------------------|---------|-------|-------|---------|----------------------------------------------------------|---------|-------|-------|---------|
| 1,4-dihydroxy-6-naphthoate biosynthesis                  | 0.71    | 0.34  | 2.11  | 0.0352  | Isoprene biosynthesis                                    | 0.76    | 0.32  | 2.33  | 0.0197  |
| 4-deoxy-L-threo-hex-4-enopyranurate degradation          | −0.33   | 0.13  | −2.57 | 0.0101  | L-glutamate degradation                                   | −0.89   | 0.40  | −2.24 | 0.0252  |
| 4-methylcatechol degradation                             | −0.84   | 0.38  | −2.22 | 0.0263  | L-histidine degradation                                   | −0.40   | 0.19  | −2.09 | 0.0364  |
| 5-aminomimidazole ribonucleotide biosynthesis            | −0.19   | 0.08  | −2.26 | 0.0238  | L-isoelucine biosynthesis                                 | −0.24   | 0.09  | −2.57 | 0.0101  |
| Adenosine nucleotides degradation                         | 0.87    | 0.35  | 2.51  | 0.0121  | L-lysine biosynthesis                                     | −0.24   | 0.09  | −2.60 | 0.0094  |
| Adenosine ribonucleotides de novo biosynthesis           | −0.20   | 0.09  | −2.38 | 0.0172  | L-valine biosynthesis                                     | −0.24   | 0.09  | −2.57 | 0.0101  |
| Adenosylcobalamin salvage from cobinamide                | −0.32   | 0.14  | −2.33 | 0.0197  | Ectoine biosynthesis                                     | 0.91    | 0.34  | 2.68  | 0.0074  |
| Aerobic respiration                                       | 0.95    | 0.43  | 2.19  | 0.0284  | Ethylene biosynthesis                                    | 0.69    | 0.34  | 2.03  | 0.0426  |
| Branched amino acid biosynthesis                          | −0.24   | 0.10  | −2.47 | 0.0134  | Flavin biosynthesis                                     | 1.25    | 0.40  | 3.16  | 0.0016  |
| Pyruvate fermentation to acetate and lactate             | −0.27   | 0.12  | −2.36 | 0.0185  | Queuosine biosynthesis                                   | −0.30   | 0.11  | −2.80 | 0.0051  |
| CDP-diacylglycerol biosynthesis                          | −0.16   | 0.08  | −2.00 | 0.0452  | Starch degradation                                      | −0.24   | 0.10  | −2.29 | 0.0221  |
| Chorismate biosynthesis                                   | −0.19   | 0.09  | −2.17 | 0.0297  | Sucrose degradation                                     | 1.51    | 0.59  | 2.56  | 0.0071  |
| Coenzyme A biosynthesis                                   | −0.27   | 0.12  | −2.26 | 0.0240  | Sulfoglycolysis                                          | 1.27    | 0.47  | 2.69  | 0.0071  |
| D-fructurionate degradation                               | −0.36   | 0.12  | −2.94 | 0.0033  | Pentose phosphate pathway                                | −0.35   | 0.15  | −2.31 | 0.0210  |
| D-galacturonte degradation                                | −0.33   | 0.12  | −2.69 | 0.0072  | Peptidoglycan biosynthesis                               | −1.82   | 0.54  | −3.36 | 0.0008  |
| D-glucuronide and D-glucuronate degradation                | −0.36   | 0.12  | −3.03 | 0.0024  | Phosphatidate metabolism                                 | −1.33   | 0.39  | −3.42 | 0.0006  |
| dTDP-N-acetylglucosamine biosynthesis                    | 1.69    | 0.37  | 4.55  | 0.0000  | Glutaryl-CoA degradation                                 | −0.31   | 0.13  | −2.30 | 0.0216  |
| Thiamin diphosphate biosynthesis                          | −0.26   | 0.11  | −2.34 | 0.0191  | PreQ0 biosynthesis                                       | −0.22   | 0.10  | −2.23 | 0.0254  |
| Methylylerythritol phosphate pathway                     | −0.21   | 0.09  | −2.40 | 0.0162  | Ubiquinol-6 biosynthesis                                 | −1.77   | 0.37  | −4.81 | 0.0000  |
| Pantotenate and coenzyme A biosynthesis                   | −0.26   | 0.10  | −2.54 | 0.0110  | Catechol degradation                                     | −1.28   | 0.41  | −3.13 | 0.0018  |

Note: Log2 FC represents the log fold change, and a value>0 represents a related metabolic pathway enriched in the probiotic group on d 0; a value<0 represents a related metabolic pathway enriched on d 30.
a long sea voyage as well as the correlation between the consumed probiotic and specific intestinal microbes, we further explored the relationship between the probiotic-derived changes in intestinal bacterial populations and the anxiety index of the questionnaire results. Using a co-abundance algorithm, we assembled all the shotgun metagenomic sequencing data into 1498 metagenome-assembled genomes (MAGs) (Table S12), assigned specific taxonomic levels and constructed a phylogenetic tree (Figure 5(a)). Spearman’s rank correlation coefficient was calculated to quantify the correlation. We observed a significant positive correlation (R > 0.4) between the metagenomic species *Klebsiella pneumoniae*, *Streptococcus salivarius* and *Clostridium leptum* and the anxiety index, whereas the metagenomic species *Bifidobacterium longum*, *Bifidobacterium animalis*, *Bacteroides eggerthii*, *Bacteroides caccae* and *Bacteroides faecis* were significantly negatively correlated with the anxiety index (R > 0.4). In summary, these results indicated that probiotic consumption maintained intestinal microbiome homeostasis and introduced some positive changes in intestinal species, which also prevented sailor anxiety during a long sea voyage.

**Discussion**

In our present research, we found that the intestinal microbiome of sailors in the placebo group was seriously disordered during a long sea voyage, as indicated by the changes in some important intestinal species and the significant decrease in carbohydrate-active enzyme genes. The rich diversity of host intestinal microbes as well as their CAZy-represented functional genes is the foundation for maintaining intestinal microbiome homeostasis and host fitness. It is well known that most of the fiber, polysaccharide and polyphenol in our daily diet cannot be degraded and utilized by our own cells. Carbohydrate-active enzymes encoded by the human gut microbiome catalyze the breakdown of glycoconjugates, oligosaccharides and polysaccharides to fermentable monosaccharides. Therefore, the vast number of CAZy genes in intestinal microbes, which outnumbers that of humans by 100 times, helps us degrade these complex carbohydrates into short chain fatty acids (SCFAs) directly or by a cross-feeding mechanism, which improves our gut health fitness. In contrast, a low diversity of intestinal microbes with CAZy-represented functional genes is

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**Figure 5.** (a) Phylogenetic tree of the metagenomic assembled genome (MAG) from metagenomic samples. The heatmap of the tree shows the log2-fold change in the relative abundance of the MAGs during the long sea voyage (outer, probiotic group; inner, placebo group). (b) Spearman’s rank correlation coefficient between the probiotic-correlated species and the anxiety index. An R-value greater than 0.4 or less than −0.4 indicated that the correlation was significant.
closely related to the proliferation of pathogenic and conditional pathogens, which leads to various chronic diseases.

We supposed that the human intestinal microbiome was determined mainly by the factors of host genotype, environmental microbes and daily diet.\textsuperscript{21} The environment and diet, but not the host genotype, changed during the long sea voyage. It has been widely reported that the microbes in the soil are extremely abundant and that the environmental microbes in mainland areas come mainly from the soil,\textsuperscript{22} but the diversity of microbes at the surface of the sea is great.\textsuperscript{23} Accordingly, sailors are unable to interact with diverse microbes during sea voyages as they would on the mainland, which may greatly impact the intestinal microbiota of sailors. At the same time, diverse fresh vegetables and fruits are limited during long sea voyages, which may be a second factor explaining the reduction in the diversity of the intestinal microbes.\textsuperscript{24} Additionally, some studies also reported that gut microbial communities are bidirectionally plastic and resilient across a long stay in the space situational environment or with multiple dietary shifts\textsuperscript{25,26} but these studies lasted at least one half year, and they also highlighted significant changes in intestinal microbiota within months.

In the probiotic group, by supplying a mixed probiotic product containing \textit{Lactobacillus casei} Zhang, \textit{Lactobacillus plantarum} P-8, \textit{Lactobacillus rhamnosus} M9, \textit{Bifidobacterium lactis} V9 and \textit{Bifidobacterium lactis} M8, we observed that the CLR abundances of the species \textit{Bifidobacterium longum}, \textit{Bifidobacterium animalis} and some species belonging to \textit{Bacteroides} increased significantly in sailors at the end of the sea voyage; however, the CLR abundances of the species \textit{Clostridium leptum}, \textit{Klebsiella pneumoniae} and \textit{Prevotella copri} decreased (Figure 2(a); Wilcoxon rank-sum tests). The species \textit{Bifidobacterium} and \textit{Bacteroides} encode various CAZy genes that are able to degrade complex carbohydrates into SCFAs.\textsuperscript{27} The species \textit{Bifidobacterium longum} carries key genes encoding glycoside hydrolases and carbohydrate transport systems involved in the metabolism of 4-galactosyl-kojibiose and lactulosucrose, and acetic acid is the main metabolic end product followed by lactic and formic acids.\textsuperscript{28} SCFAs are the main energy source of cells. They can regulate epithelial barrier function and reduce the translocation of bacterial endotoxins across it\textsuperscript{29} and promote the secretion of mucin, defensin and antibacterial peptides. Accordingly, the consumed probiotic promoted the growth of beneficial microbes and inhibited the proliferation of pathogenic and conditional pathogens, which were essential in maintaining host health by stimulating natural immunity and contributing to the balance of the microbiota.\textsuperscript{30}

The emergence of a microbiota-gut-brain axis to describe the complex networks and relationship between the gastrointestinal microbiota and host reflects the major influence this environment may have in brain health and disorders of the central nervous system (CNS).\textsuperscript{31} Similar to our finding of intestinal microbial disorder in the placebo group at the end of the voyage, recent evidence also suggested that perturbations of the gut microbial community may play a key role in neuropsychiatric disorders, such as depression and anxiety. However, the potential intestinal species correlated with depression and anxiety reported in different studies were not consistent. For instance, we highlighted the beneficial species \textit{Bifidobacterium longum} and \textit{Bifidobacterium animalis} in our research. However, a recent intestinal microbiome study of a generalized anxiety disorder cohort focused on the low prevalence of five genera: \textit{Faecalibacterium}, \textit{Eubacterium rectale}, \textit{Lachnospira}, \textit{Butyrivibrio} and \textit{Sutterella}.\textsuperscript{32} Although we highlighted the different microbes in the intestine, the disorder in intestinal microbiome homeostasis (especially the microbial metabolites) was consistent for individuals who suffered from anxiety. The author also suggested that these genera could be relevant to mental health due to their documented production of short-chain fatty acid compounds.\textsuperscript{32} Accordingly, the reduced SCFA production in the anxiety population could result in intestinal barrier dysfunction,\textsuperscript{33} which could compromise proper immune responses and ultimately contribute to brain dysfunction. Meanwhile, we found that probiotic consumption prevented the potential anxiety of sailors during the long sea voyage. Currently, there is considerable evidence suggesting that the intake of probiotics may contribute to decreased anxiety and depression-like behaviors in both human and mouse models. For instance, a probiotic mix of \textit{Lactobacillus helveticus} and \textit{Bifidobacterium longum} given to healthy human
volunteers for 1 month alleviated psychological distress in comparison to a control group, consistent with the positive results seen in rats. This result indicated that there is significant potential for probiotic treatment to maintain intestinal microbiome homeostasis and to prevent anxiety and depression in sailors during long sea voyages.

By performing a longitudinal study containing two cohorts, we draw two main conclusions from the present research. The first is that the long sea voyage not only disordered the balance of the intestinal microbiota of sailors but also reduced the diversity of functional features of intestinal microbiota and increased the stress and anxiety of sailors. The second conclusion was that the probiotics maintained intestinal microbiome homeostasis and further prevented anxiety during the long sea voyage. The results of the present study will provide a feasible approach for protecting gut health during long sea voyages and provide new insights into a personalized selection of probiotics based on population-level analyses.

Materials and methods

Experimental design and subject recruitment

We designed a 30-d longitudinal experiment that included 2 Chinese sailor cohorts in the same warship: a placebo cohort (\(n = 42\)) and a probiotic-consuming cohort (\(n = 40\)). During the trial, the sailors in the two groups were performing a 30-d cruising task in the Yellow Sea. After being informed of the experimental guidelines and details, the volunteers agreed to participate in the subsequent experiment. All the subjects in the probiotic group were asked to consume a 2 g package containing mixed probiotics including 9.70 Log\(_{10}\) CFU of *Lactobacillus casei* Zhang, 9.70 Log\(_{10}\) CFU of *Lactobacillus plantarum* P-8, 9.70 Log\(_{10}\) CFU of *Lactobacillus rhamnosus* M9, 9.88 Log\(_{10}\) CFU of *Bifidobacterium lactis* V9 and 9.88 Log\(_{10}\) CFU of *Bifidobacterium lactis* M8 once daily for 30 d. Meanwhile, the subjects in the placebo group were asked to consume a placebo of the same weight and the same taste once daily for 30 d. The probiotic and the placebo formulations (lyophilized cells) were provided by the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University. Fecal samples were collected at baseline (d 0) and at the end of the long sea voyage (d 30). An individual health-related questionnaire that included physiological and psychological stress was obtained from every subject at the end of the sea voyage (Table S2). We set each subject’s score for the questionnaire content to a baseline of 10. Therefore, a decreased score represented the unfitness of the subject in the corresponding questionnaire during the voyage. Meanwhile, we recorded the weekly food supply on the cruise, and the menu from Sunday to Saturday was not the same but was repeated every week. The food supply on the cruise was in buffet form. Therefore, the subjects in the placebo and probiotic groups had the same choice of food in every meal. The subjects could eat according to their willingness, but they were suggested to eat at least two kinds of staple food, five kinds of entrées and two to three kinds of fruit every day for their nutritional needs. The study was reviewed and approved by the Ethics Committee of the Inner Mongolia Agricultural University (IMAU-EC-2018017, Hohhot, China), and informed consent was obtained from all volunteers before they enrolled in the study. The participants provided written informed consent to participate in the study. Sampling and all described subsequent steps were conducted in accordance with the approved guidelines. Fecal samples were collected from each subject in the morning before the first meal. After the weight of the fecal samples was determined, a sample protector (CW0592M, CWBIO, China) was added at a ratio of one-part fecal sample to five-part sample protectors, after which the samples were stored at \(-20^\circ C\) until further processing.

Shotgun metagenomic sequencing and quality control

All the samples were subjected to shotgun metagenomic sequencing by using an Illumina HiSeq 2500 instrument. Libraries were prepared with a fragment length of approximately 300 bp. Paired-end reads were generated using 100 bp in the forward and reverse directions. The reads were trimmed using Sickle and were subsequently aligned to the human genome to remove the host DNA fragments. An average of 7.51 gigabases (Gb) of high-quality paired-end reads was obtained for each sample, totaling 1232.42 Gb of high-quality data that were free of human DNA and adaptor contaminants (Table S3).
Non-redundant gene catalog construction and calculation of gene abundance

The shotgun reads were assembled into contigs and scaffolds using IDBA-UD, and then the contigs were used to predict the functional genes with MetaGeneMark. Finally, a non-redundant gene catalog was constructed using CD-HIT.

The abundances of genes were determined by aligning the reads to the gene catalog using Bowtie2. Subsequently, for any sample N, we calculated the abundance as follows:

Step 1: Calculation of the copy number of each gene:

\[ b_i = \frac{x_i}{L_i} \]  

Step 2: Calculation of the relative abundance of gene \( i \)

\[ a_i = \frac{b_i}{\sum_i b_i} \]  

\( a_i \): the relative abundance of gene \( i \)  
\( b_i \): the copy number of gene \( i \) from sample \( N \)  
\( L_i \): the length of gene \( i \)  
\( x_i \): the number of mapped reads

Metagenomic assembled genome (MAG)

The microbial taxonomic profile was constructed by the software MetaPhlAn2. Moreover, because the whole genomes of the 5 probiotics in the present study were sequenced, we could easily annotate and calculate the relative abundances of these probiotics in samples on d 30 by bowtie2. For metagenomic species analysis, the software MetaBAT was applied, and the co-abundance principle and canopy clustering algorithm were performed to generate MAGs by binning shotgun reads. After reassembling, the MAGs were assigned to a given genome when more than 80% of the subgene matched the same genome using BLASTn at a threshold of 95% identity over 90% of the gene length. If greater than 80% of the genes from an MAG had the same taxonomic level of assignment, then MGS was identified as the same microbe.

The centered log-ratio (CLR) of microbial relative abundance and the microbial Aitchison distance

It was debatable by using the microbial relative abundance in microbiome research. The total microbial load or absolute copy gene number of the whole microbes should be the efficient methods. Here, we performed a widely used centered log ratio of microbial relative abundance to verify our analysis results. The “composition” package in the R program was used to resolve the value of 0, and the “composition” package in R was applied to realize the relative abundance profile CLR transformation. The Aitchison distance could be directly calculated from the Euclidean distance of the CLR-transformed data.

Functional annotation and metabolic pathway analysis

The annotated amino acid sequences were aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLASTp (e-value ≤ 1e-5 with a bit-score higher than 60). The annotated sequences were assigned to the KEGG orthologue group (KO) according to the highest score. CAZymes (carbohydrate-active enzymes) were predicted from amino acid sequences by generating alignments with family-specific HMMs of CAZymes in the dbCAN database using the program Hmmscan in the HMMER 3.0 package. Reporter Z-scores were calculated to reveal the differences in enriched metabolic pathways between the control and PCOS groups, as previously described. Accordingly, a reporter score of > 2.3 (90% confidence according to the normal distribution) was used as a detection threshold to significantly differentiate between pathways.

Statistical analysis

All statistical analyses were performed using R software. PCA analysis was performed in R using the ade4 package. The differential abundances of genera, genes and KOs were tested with the Wilcoxon rank-sum test and were considered significantly different at \( p < .05 \). For boxplot construction, the package ggpubr was used. The
heatmap was constructed using the “pheatmap” package, and the Sankey diagram was built using the “riverplot” package. The CLR transformation was realized using the “composition” package. The networks were calculated by the Spearman rank correlation coefficient and visualized by networks in Cytoscape (Version 3.7.1).

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Data and code availability

The sequence data reported in this paper have been deposited in the NCBI database (metagenomic sequencing data: PRJNA554501). All analyses can be found under https://github.com/zhjch321123/long-sea-voyage-program.git.

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of the Inner Mongolia Agricultural University (IMAU-EC-2018017, Hohhot, China), and informed consent was obtained from all volunteers before they enrolled in the study.

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