R&D COLLABORATIVE PROPOSAL / COMPANY PARTNER SEARCH

The information you are about to provide in this form will be distributed among Spanish companies matching your company profile and that might be interested in the proposal of collaborative R&D project that you will be describing in this form. (Please use English language for filling in the document)

In the case that your company will establish a R&D project in collaboration with a Spanish company, you could present a Joint Project Proposal to the CHINEKA Program**.**

YOUR ENTITY PROFILE

Name:Jinan Daxin Biotechnology Co., Ltd

Number of employees: 12 people

Annual turnover: 2 million yuan

Balance Total: -20000 yuan

Year of latest financial report: 2 million yuan

Address: Room 1-1109, building 1, No. 15612, Century Avenue, high tech Zone, Jinan **City:** Jinan **Province:** Shandong **Postal Code:** 250101	**Telephone:** 15194117594 **Fax:** **Email:** papers@sinovita.com.cn **WEB site:** http://www.sinovita.com.cn/

Contact: Chao Huang **Position:general manager** **Telephone:** 15194117594 **Email:** papers@sinovita.com.cn	**Additional Contact:** **Position:** **Telephone:** **Email:**

COLLABORATIVE R&D PROJECT PROPOSAL

(Describe as precisely as possible the technology cooperation proposal. Describe what you have to offer and what you expect from your potential partner) Include: Sector Group; Abstract of Project; Innovations Offered; and Current State of Development

Title	(Do not exceed 120 characters) CHARACTERIZATION OF NEXT-GENERATION PROBIOTICS TO REDUCE FATIGUE VIA LACTATE METABOLISM		
Duration (YM- YM)			
Budget(1,000 Euro)	SPAIN	CHINA	TOTAL
	6137144.7275	6137144.7275	12274289.455
Technology Field (Click a box)	☐ Smart Cities ☐ Production Technologies, ☒ Biomedicine and Technologies for Health, ☐ Environment technologies ☐ Clean Technologies ☐ Modern Agriculture ☐ Others		
Summary	**1. KEY SCIENTIFIC ISSUES AND RESEARCH CONTENT** Fatigue is a common non-specific symptom experienced by many people and is associated with many health conditions. Often defined as an overwhelming sense of tiredness, lack of energy and feeling of exhaustion, fatigue relates to a difficulty in performing voluntary tasks. Fatigue accumulation, if not resolved, leads to overwork, chronic fatigue syndrome, overtraining syndrome, and even endocrine disorders, immunity dysfunction, organic diseases and a threat to human health. Muscle contractions activate ATPases and promote glycolysis which leads to the production of pyruvate that feeds into the TCA cycle for oxidation. If pyruvate production exceeds, excess pyruvate is converted into lactic acid (Wan et al., 2017). Lactic acid quickly dissociates to lactate and hydrogen ions promoting acidosis. Acidosis in working muscle causes inhibition of contractile processes, either directly or via metabolism, resulting in diminished exercise performance, thus, acidosis strongly contributes to fatigue (Theofilidis et al., 2018). Higher lactate level in the body can also alter the gut microbiota. The studies show that exercise is associated with changes in microbiome composition (Petersen et al., 2017; Clarke et al., 2014). A recent study proposed that the high-lactate environment of the athlete provides a selective advantage for colonization by lactate-metabolizing organisms such as *Veillonella*. Genes involved in a major pathway metabolizing lactate to propionate is at higher relative abundance in athletes postexercise. The study further testified that systemic lactate		

	resulting from muscle activity during exercise can cross the epithelial barrier into the lumen of the gut where *Veillonella* metabolises it into propionate leading to enhances athletic performance. *Veillonella* species metabolize lactate into acetate and propionate via the methylmalonyl-CoA pathway and this pathway is overrepresented in *Veillonella* of athlete metagenomic samples postexercise (Scheiman et al., 2019). These findings suggest that lactate generated during sustained bouts of exercise could be accessible to the microbiome and converted to the short chin fatty acids that can help to reduce fatigue and improve athletic performance. From this information we can anticipate that development of lactate utilizing next-generation probiotic strains may be beneficial to prevent acidosis and improve athletic performance. In recent years, sports nutrition supplements are considered as necessary for increasing exercise performance or reducing post-exercise fatigue and have played an important role in scientific exercise training. In addition to traditional herbs, proteins and probiotics have also received increasing attention as dietary supplements (Lee et al., 2020). Probiotic bacteria are the primary colonizers of the gastrointestinal tract, and they elicit numerous health benefits on their host, through a series of complex antagonistic mechanisms and microbial interactions (Ibrahem, 2015). Much attention has been given recently to develop conventional probiotics such as lactobacilli and bifidobacteria as diagnostic and therapeutic agents (Zuo and Marcotte, 2021). In fact, some probiotics have already been used for treatment or prevention of various diseases, such as, inflammatory bowel diseases, infections, and autoimmune disorders (Tavares et al., 2020). Previously, some studies also reported the successful gene editing in probiotic bacteria specially *Lactobacillus* strains. A RecT-assisted CRISPR-Cas9 approach has already been developed to perform codon saturation mutagenesis and gene deletions in the chromosome of *Lactobacillus reuteri* ATCC PTA 6475 (Oh and van Pijkeren, 2014). A similar approach was used in *Lactococcus lactis* NZ9000 and seamless genomic DNA insertion or deletion was efficiently performed (Guo et al., 2019). A recent study revealed that the genetic engineering feasibility of the method used can vary depending on the targeted gene and strain (Leenay et al., 2019). Therefore, while many advances have been made to harness the potential application of probiotics in different areas, there remain several challenges for future research. Molecular studies and mechanisms of action of specific probiotic strains of the gut microbiota need to be elucidated. Accurate genome editing will help in understanding the molecular mechanisms of probiotics and their interaction with host and ultimately will promote the development of next-generation probiotics with improved characteristics and tailored

functionalities. CRISPR-Cas-based genome editing approaches along with synthetic biology strategies will further enhance the knowledge and application of probiotics (Zuo and Marcotte, 2021). This project is designed to employing advanced gene editing tools to develop next-generation probiotic strains that can reduce fatigue via lactate metabolism. Detailed genome studies will also be performed to elucidate mechanism of actions of probiotic strains.

Key Scientific Issue to be Solved

The main scientific problem to be solved in this project is to develop next-generation probiotic strains that can be used to reduce fatigue along with its conventional health benefits. CRISPR gene editing might help us in revolutionising the methods of conventional probiotics which depend on using pre-determined mixture of good bacteria. CRISPR offers the possibility of manipulating bacterial strains and hence creating unlimited opportunities in the field of probiotics. Genetic manipulation within the genomes of probiotic strains will provide improved strategies for strain development and applications. Findings suggest that lactate generated during sustained bouts of exercise could be accessible to the microbiome and converted to the short chin fatty acids that can help to reduce fatigue and improve athletic performance. The development of lactate utilizing next-generation probiotic strains may solve the problem of fatigue and acidosis and improve athletic performance along with health benefits.

Main Research Content

Based on the scientific issue to be solved, the main research content of this project are as follows:

1. Whole-genome sequencing and genome annotation will be performed for the identification of bacterial strains and their probiotic potentials as next-generation probiotics.
2. Characterization of metabolite associated genes and pathways.
3. Use of endogenous gene editing system for modulating metabolite gene expression to develop next-generation probiotics.
4. Investigating the potential of next-generation probiotic strains in reducing fatigue.

2. EXPECTED GOALS

Overall Objective

Major objectives of this research include

1. Identification and characterization of genes related to biosynthesis of secondary metabolites using genome annotation, metabolomic profiling, bioinformatics, phylogenetic, and comparative genomic analysis.

2. Development of next-generation probiotics with improved metabolite gene functionality using endogenous CRISPR-Cas system.

3. Investigating the potential of next-generation probiotic strains in reducing fatigue.

4. Publish research papers in high impact academic journals.

5. Provide guidance for undergraduate and postgraduate students in gene editing, phylogenetic studies, bioinformatics, and omics-based analysis.

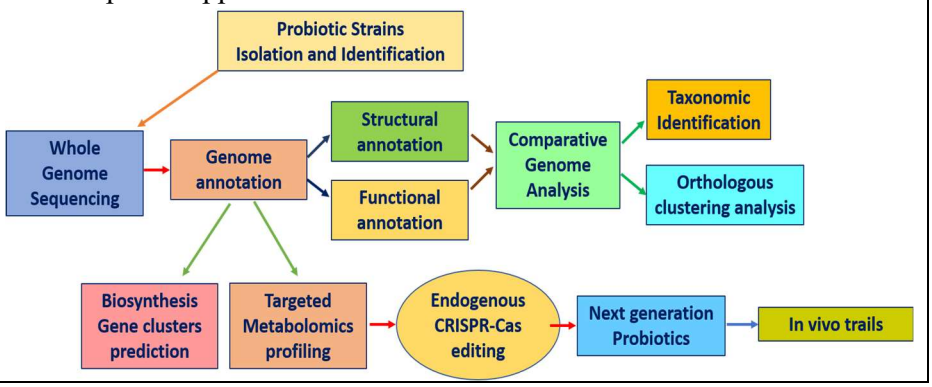
3. RESEARCH PLAN

Academic Thinking

This research involves whole genome sequencing, genomic annotation, comparative genome analysis, metabolomic profiling, and gene editing. Genomic annotation of whole-genome sequence data will be used for strain identification, functional probiotic gene prediction, and characterization. 16S rRNA gene and single-copy protein-coding gene of orthologous gene clusters will be used for taxonomic identification of strains. Comparative genome analysis including bioinformatics, orthologous, and phylogenetic analysis will be carried out for functional probiotic gene prediction and identification of gene clusters associated with biosynthetic pathways of metabolite production.. Repurposing of probiotic strains will be carried out using metabolomics-informed CRISPR-Cas gene editing. The effectiveness of probiotics to reduce fatigue will be determined by high quality randomised controlled trials.

Technical Approach

This research will adopt genomics, metabolomics, and metabolic genetic engineering for the development of next-generation probiotics for biotherapeutic application.



Innovation

CRISPR-Cas gene editing approach will be used for developing next-generation probiotic strains with improved probiotic efficiency and their performance as fatigue reducing agents will be determined by human trials. The development of lactate utilizing next-generation probiotic strains may solve the problem of fatigue and acidosis and improve athletic performance along with health benefits.

Feasibility Analysis

CRISPR-Cas gene editing has recently been employed in gene editing of probiotic bacteria specially lactic acid bacteria to develop next-generation probiotics for therapeutic applications. This study will be an effort to use the existing techniques and methods to design next-generation probiotics with enhanced probiotic efficiencies and biotherapeutic potential. Ethical permits from appropriate bodies will be obtained.

Topic Setting

Based on the scientific problem to be solved and the overall research goal, the research study is divided into 4 topics.

Topic 1: Whole-genome sequencing and genome annotation for the identification and screening of bacterial strains for probiotic potentials as next-generation probiotics

Expected goal: Taxonomic identification of bacterial strain from stool samples, putative probiotics gene prediction, and characterization

1. Isolation and identification of probiotic bacteria

Human stool samples will be grown on de Man Rogosa and Sharpe (MRS) media. Plates will be incubated overnight at 30◦C (Ayala et al., 2019). Pure isolates will be stored as glycerol stock cultures for further studies. All isolates obtained will be identified presumptively, using routine morphological and cultural characteristics, gram staining reaction, and analytical profile index (API).

2. Whole-genome sequencing of probiotic strains

Phenotypically identified isolates will be cultured overnight in Luria Bertani broth. Genomic DNA will be extracted and purified with a DNA extraction kit, according to the manufacturer's instruction and DNA quantification will be performed using nanodrop spectrophotometer. DNA library will be prepared with DNA library prep kit and sequenced using Miseq sequencing kit for Illumina sequencing (Balbin et al., 2020).

3. Genome annotation

Raw sequences from whole genome sequencing will be analyzed, edited, filtered and trimmed via Torrent, Geneious and Trimmomatic software suites. Processed sequence reads will be assembled using SPAdes software into contigs. Contigs will be annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotation using the Subsystem Technology (RAST) (Jabbar et al., 2020; Khan et al., 2020).

4. Structural annotation

Open reading frames (ORFs), protein-coding genes and their multiple mRNAs on contigs will be located and ORFs will be translated to protein sequences for gene prediction via Prodigal in PROKKA, which uses several open source bioinformatic tools and databases. Protein coding sequences will be predicted through HMMER and Protein Basic Local Alignment Search Tool (BLASTP) search for protein homologs in the Universal Protein Resource (UniProt), Protein Families (Pfam), Conserved Domain Database (CDD) and Clusters of Orthologous Groups of Proteins (COGs) databases. For non-coding RNAs genes, tRNA gene prediction will be carried out using tRNAscan-SE while RNAmmer and Infernal will be employed to predict rRNA genes. Predicted non-coding RNAs genes will be classified via search for multiple sequence alignments in the RNA families (Rfam) databases (Jabbar et al., 2020; Hamidian et al., 2020).

5. Functional genome annotation

Functional genes specific for functional probiotics attributes based on niche adaptation, genetic stability/diversity, and safety selection criteria, will be screened for follwing:

1. **Niche adaptation criteria:** Genes required for carbonydrate, lipid and protein metabolism, primary and secondary metabolites; adhesion and aggregation factors encoding genes including genes coding for proteins involved EPS biosynthesis, mucus-binding proteins, fibronectin-binding protein, sortase proteins required for anchoring surface proteins (e.g. mucus-binding proteins) to the bacterial cell wall and S-layer proteins; genes encoding resistance to environmental, osmotic and oxidative such as acids, gastric juices, heat and cold shock, bile salt, etc. All genes required for successful colonization and niche adaptation will be predicted using protein data bank (PDB), antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH), bacteriocin genomemining tool (BAGEL), DIAMOND BLAST and BLASTP search of the UniProt bacterial protein database (Hussein et al., 2020; Chen et al., 2020).

	2. **Genetic stability/diversity criteria:** Genes encoding mobile genetic elements that influences chromosomal rearrangements such as duplications, deletions, and inversions will be investigated. E.g. insertion sequences, prophages, integron, plasmids, etc. Genes coding for the above mobile genetic elements will be predicted via ISfinder, Prophage finder, Integron finder and Plasmidfinder databases, respectively and NCBI GenBank databases using tBLASTn (Hamidian et al., 2020; Khan et al., 2020; Yao et al., 2020). 3. **Safety selection criteria:** Genes coding for virulence factors such as degrading enzymes (e.g. gelatinase (*gelE*), hyaluronidase (*hyl*), lecithinase (*lec*), hemolysin (*hly*)) and toxins (exotoxins and endotoxins), pathogenicity, infectivity, and antimicrobial resistance. These genes will be identified via Virulencefinder and virulence factor database (VFDB), Rapid Automated Scan for Toxins in Bacteria (RASTA-Bacteria), PathogenFinder, Comprehensive Antibiotic Resistance Database (CARD) and Resfinder databases (Balbin et al., 2020). **6. Comparative genome analysis** Comparative genome analysis will be performed to predict protein gene function, compare strains for; genetic differences and shared genetic features, and taxonomic identification of bacterial probiotic strains, using orthologous clustering and phylogenetic analysis. 1. **Orthologous clustering analysis:** All predicted protein sequences from genomic annotation will be used for comparative genome analysis of functional probiotic features, to determine genomic difference between probiotic strains. Protein sequences will be compared with other protein sequences on NCBI database using BLASTP alignments algorithm in the OrthoMCL software and grouped into orthologous gene families by clustering tool Markvoc cluster (MCL). Only proteins sharing both 60% amino acid identity and 80% sequence coverage will be considered as orthologous proteins. A venn diagram showing distribution of gene families shared amongst orthologous gene clusters will be drawn (Hussein et al., 2020; Yao et al., 2020). 2. **Taxonomic identification:** Taxonomic classification will be carried out based on comparison of the 16S rRNA genes and single-copy protein-coding genes of the orthologus gene clusters and other highly conserved genes. The genomic sequence will be compared with similar sequences on GenBank, using the NCBI Basic Local alignment search tools BLASTN and BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST). Following BLAST search on GenBank, isolates will be designated as the same species with bacterial organisms they share ≥ 99% sequence similarity with, or

	same genus when sequence similarity of ≥ 97% is observed. Sequence reads of all identified probiotic strains will be submitted to GenBank, for ascension numbers. Sequences of identified probiotic strains will be aligned and trimmed by Clustal IW in the MEGA software. Phylogenetic trees highlighting the evolutionary relationship of probiotic strains will be constructed using the Neighbor-Joining algorithm (Adesina et al., 2019; Kweon et al., 2020). **Ratio of funds: 30%** **Topic 2:** Characterization of bacterial gene clusters for secondary metabolite production and metabolomic profiling **Expected goal:** To evaluate secondary metabolite diversity within probiotic strains **Research content:** To characterize known and novel metabolite biosynthesis gene clusters (BGC), elucidate metabolite biosynthetic pathways and evaluate diverse metabolite production within individual strains. **1. Identification of secondary metabolite pathways** Pathways of all secondary metabolites identified earlier by PROKKA and biosynthesis gene clusters (BGCs) will be predicted using Metacyc and Kyoto Encyclopedia of Genes and Genomes pathway analysis (KEGG) databases in MinPath, a stand alone antiSMASH and ClusterFinder, (Dong and Strous, 2019; Hannigan et al., 2019). **2. Metabolomics profiling** Secondary metabolites predicted by PROKKA and verified by the presence of their pathways will be confirmed via untargeted metabolomic profiling. Diverse metabolites will be profiled via GC-MS analysis. Result of GC-MS analysis will be processed by uploading raw data into Automated Mass Spectral Deconvolution and Identification System (AMDIS). Following deconvolution of chromatograms by AMIDS, Chromatogram will be aligned and metabolite peaks will be identified via the online portal SpectConnect and Golm databases, respectively. The significance of difference of the various metabolites will be analyzed statistically using the MetaboAnalyst (Lin et al., 2019). **Ratio of funds: 10%** **Topic 3:** Targeted metabolomics-integrated CRISPR-Cas gene editing **Expected goal:** Regulate secondary metabolite biosynthetic pathways to

enhance secondary metabolite production of interest via metabolomics-integrated CRISPR-Cas gene editing.

Research content: To characterize endogenous CRISPR-Cas system in probiotic strains, manipulate secondary metabolite biosynthesis regulatory genes with metabolomics directed endogenous CRISPR-Cas system and confirm successful modulation of secondary metabolite production by evaluating metabolite profile changes following CRISPR Cas gene editing.

Characterization of Endogenous CRISPR-Cas System

1. **Identification and characterization of endogenous CRISPR-Cas system**

The CRISPR-Cas system in each genome will be identified by CRISPRViz which uses Bash and Python pipelines that first identify CRISPR loci. In silico analyses will be performed to characterize the endogenous CRISPR-Cas System. Putative CRISPR arrays will be identified using the CRISPR recognition tool. The CRISPR-Cas System in the genome of each strain will be curated and annotated manually. Subtypes will be assigned based on the occurrence of signature Cas protein (Hidalgo-Cantabrana et al., 2019; Yang et al., 2020).

2. **Phylogenetic analysis of CRISPR-Cas Systems**

The amino acid sequences of CRISPR-Cas System subtypes will be aligned using the MUSCLE alignment algorithm. The neighbor-joining consensus tree will be assembled in the Geneious Prime software and depicted using the FigTree software (Pan et al., 2020).

3. **Targeted metabolomics-directed endogenous CRISPR-Cas gene editing**

Prior to gene editing by the endogenous CRISPR-Cas gene editing, probiotic strains will be cultured and targeted metabolomic profiling will be used to quantify metabolites by measuring the concentration of specific metabolites. Cloning hosts will be cultured overnight in Luria-Bertani (LB) medium supplemented with antibiotics (ampicillin) and glucose. Plasmids will be extracted subsequently, using a plasmid extraction kit, following the manufacturer's instruction. Plasmids will be amplified, purified, and digested with high fidelity DNA restriction enzymes. Ligation will be carried out with a ligase master mix, according to the manufacturer's instruction. A CRISPR-Cas plasmid system for gene editing will be constructed using selection cassette, sgRNA expression cassette, and Cas9 (CRISPR-associated protein 9) cassette. The constructed plasmid will be transformed into competent cells of probiotic bacterial strains of interest via electroporation. Transformed

	probiotic strains will be screened on Luria-Bertani media with ampicillin and glucose. Transformant will be selected, amplified in a polymerase chain reaction, and sequenced to confirm successful gene edits (Ding et al. 2020; Huang et al. 2020). Following successful transformation, transformed probiotic strains will be cultured overnight. Targeted metabolomic profiling will be used to measure the concentration of specific metabolites, to validate metabolite profile changes (Yue et al., 2020; Zhou et al., 2019). **Ratio of funds: 30%** **Topic 4** Investigation the potential of next-generation probiotic strains to reduce fatigue **Expected goals:** To test the feasibility of next-generation probiotics in vivo **Research content:** Investigating the effect of supplement with lactic-acid producing probiotics on fatigue and physical activity **Human trial** The study will designed for human trails with a total duration of six weeks per participant. During the first two weeks baseline observations without treatment will be assessed (to provide baseline mean values of faecal microflora and calprotecin levels) followed by 2 weeks of treatment and a 2 weeks follow-up period. The probiotic product will be administered twice daily at a concentration of 10^8 cfu/ml of the probiotic strains. Fatigue and physical activity at baseline, at the end of treatment and at the end of the study will be assessed by the participant's self-reported physical activity level. Stool samples will be collected before administration of the probiotic, on the last day of administration and two weeks after the administration. Faecal concentrations of calprotectin will be determined by ELISA. Microbiological analysis of stool sample will also be carried out to isolate and enumerate bacteria (Sullivan et al., 2009).

SPANISH PARTNERS **(When you know a potential Spanish company, write its name and contact details in this section) Please, make a description of the desire type of Spanish Technology Partner.**
Biomedicine and general health

YOUR ENTITY DESCRIPTION

(Entity Website, Research and development guidelines, strategic alliances, competitive position, etc)

(The minimum information to show the potential of your company)

The company has joined the East China Industrial Research Institute of life sciences of Peking University and maintained good cooperative relations with many companies. The core team of the company has many years of experience in agricultural biotechnology R & D and development. The core members of the team have graduated from famous universities at home and abroad, such as the second University of Rome in Italy and the University of Punjab in Pakistan. Dr. Wang Yan, the project leader, has studied microbiology for many years. The team members have strong strength in the field of agricultural and livestock product research and development. The team members complement each other's advantages. The continuous innovation and R & D ability provides sufficient impetus for the development of the company. The company has many core patented technologies

Have the conditions of software and hardware facilities, and be able to carry out the R & D project smoothly. At present, probiotic products are still in the stage of technical development, and probiotic process platform and animal experiment platform have been built. The company has eight departments: strain agent factory, research center, marketing center and information department. There are several employees, including professionals in microbiology, soil chemistry, environmental protection and health care, with strong technical force and complete disciplines. The company has modern microbiology laboratory and advanced complete set of strain fermentation equipment. It is one of the production enterprises with the highest production technology, the best product quality and the lowest production cost in China. The company pays attention to technology development and research. From product quality to after-sales service, it is at the leading level in the same industry. At the same time, the company also hired experts from agriculture, environmental protection and medical and health care to form an advisory group to provide advice for the company's technological innovation and future development.

Dr. Huang Chao, the project leader, has been engaged in microbial research for many years. The team members have strong strength in the field of agricultural product research and biological research and development. The complementary advantages of the team members and the continuous innovative R & D ability provide sufficient impetus for the development of the company. The company has a number of core patented technologies and has the conditions of software and hardware facilities, so it can carry out the R & D project smoothly.