Provisional Translation*

Points to Consider for Gut Bacterial Products Based on Microbiome Research

－Considerations for the Development and Evaluation of Live Biotherapeutic Products－

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Subcommittee on Microbiome of the Science Board

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Preface

Live biotherapeutic products (LBPs) originating from human intestinal microbiota are being developed for a growing range of diseases, such as immune disorders, gastrointestinal diseases, and cancer. The concept underlying the development of LBPs is as follows: intestinal bacteria, which form the intestinal microbiome, are thought to maintain homeostasis in the intestinal tract, and disrupting the homeostasis of the microbiome causes many diseases. Bacterial candidates from healthy human feces, which may play an important role in human health, have been explored for LBPs. The Science Board of the Pharmaceuticals and Medical Devices Agency in Japan compiled a report on the expected aspects of LBPs originating from human intestinal bacteria. This paper summarizes the discussion and the points to consider proposed by the expert group.

Introduction

The human intestinal tract is inhabited by hundreds of bacterial species, and approximately 100 trillion bacteria (1-4) form the intestinal microbiome. Through various metabolites produced by these bacteria along with their interactions with the human intestinal tract, these bacteria affect various physiological functions, such as immune function and metabolic activity in the human host. This indicates their involvement in the maintenance of human health.

Additionally, it has become clear that the microbiome of patients with some diseases is abnormal and that abnormalities in the intestinal microbiota may lead to the development of various diseases. Furthermore, several studies have reported that fecal microbial transplantation (FMT) from healthy individuals can alter the microbiome of these patients and may help treat various diseases.

The species of bacteria from fecal microbiota have not been clearly proven to be effective in treating disease; moreover, either these species or strains have not been precisely identified, despite some studies showing promising finding(s). Therefore, it is desirable to identify bacterial species of particular interest. These effects may indicate that treatment with single or multiple species of live biotherapeutic products (LBPs) can be used to treat diseases.

This document presents challenges specific to LBPs currently under development for treating various diseases. We summarize perspectives that should be considered in developing therapeutic agents using LBPs, including elucidating the mechanism of action, manufacturing process, quality characterization, and design of preclinical and clinical studies. The document also summarizes issues to be considered when conducting quality

and safety evaluations in clinical settings based on the characteristics of LBPs. The development of LBPs is advancing rapidly, and these issues need to be reviewed. Although bacteria, fungi, and phages can form the basis of LBPs, we here only consider bacterial agents. However, we believe that many fundamental concepts stated herein can be extended to other intestinal microbial species.

1. Current Status for the development of LBPs for infectious immunologic, and non-immunologic diseases

Recent advances in omics technology have revealed that the microbiome has a marked influence on the regulation of human biological functions. Based on these findings, FMT and LBPs consisting of fecal microbiota have been developed to treat infectious diseases, treat immune modulation therapy, and improve metabolic functions. LBPs may include naturally occurring and genetically modified bacteria and yeasts isolated as specific strains. LBPs are intended for the treatment of infections and diseases, but do not include biological products such as vaccines, which protect against infection through an immune response. Currently, many companies are developing LBPs for treating various diseases, including *Clostridioides difficile* infection (CDI), inflammatory bowel disease, food allergy, and cancer. Pharmaceutical companies are also interested in partnering with innovative companies to develop such products.

LBPs are currently developed as single- or multiple-bacterial products; LBPs may have a limited impact on the gut environment compared to FMTs because they contain only defined bacterial species; however, their safety may be more manageable.

Most LBPs are currently in the non-clinical/Phase I trials and in the early stages of development, except for several in phase II/III trials overseas. Therefore, there is great interest in exploring methods for the development of LBPs in a scientifically rational manner.

The current indication of the live bacterial products approved for clinical use in Japan is to improve various symptoms caused by abnormal gut microbial composition, and the products consist of freeze-dried viable organic acid-producing bacteria, such as those producing lactic acid and short-chain fatty acids. The effects of administering the live bacteria in the human body have not been fully elucidated, and the formulation has been approved for the treatment of bowel incontinence, soft stools, constipation, and abdominal distension.

So far, the clinical development of LBPs in Japan has barely progressed. FMT, in contrast, is being developed in Japan as an advanced medical treatment and for specific

clinical research. While the effect of FMT has been reported in several studies, it has not been tested in a clinical trial. In addition, the risk of contamination with harmful bacteria has been highlighted, and there are concerns regarding safety management.

1.1 Major disease areas for which LBPs are being developed are as follows(5-7);

- CDI and multi drug-resistant bacterial infections
- Inflammatory bowel disease
- Cancer immunotherapy (in combination with immune checkpoint inhibitor antibodies)
- Food Allergy
- Metabolic diseases

1.2 FMT

Several reports have suggested that FMT is effective in various diseases, such as inflammatory bowel disease (8). Additionally,

the clinical use of FMT could also be a platform for the development of LBPs; the responsible bacteria which confer the disease improvement can be identified based on the clinical results obtained from FMT and the analysis of the bacterial composition in the fecal sample. (9, 10).

1.3 Challenges in LBP Development

- In most cases, bacterial strains in LBPs are selected for the following reasons; The strains whose relative abundance is decreased in patients with the disease
- The strains related to the effects of FMT therapy
- The strains which regulate specific pathways or affect certain host pathways or phenotypes

However, in such ways, it is not always clear whether the correlation is causal and whether cellular level responsiveness is consistent with host-level responsiveness. Therefore, the effects of the candidate LBPs in vivo may be unclear until confirming the clinical outcomes or improvement of pharmacodynamic indices.

Additionally, it is necessary to determine each genome sequence to confirm the presence or absence of putative virulence factors such as transmissible drug resistance genes or toxins, and to design strain-specific primers to confirm the colonization of each strain in the gut.

2. New technologies for evaluation of LBPs

Several *Lactobacillus* products which have therapeutic properties to control intestinal conditions have been approved in Japan. However, since these products have been approved as drugs half a century ago, the test methods listed to evaluate these products are classical microbiological methods. Indeed, the methods for confirmatory testing and quantitative analysis of active ingredients listed in the Japanese Pharmacopoeia were published in the 1990s. On the other hand, during the past three decades, there has been remarkable progress in molecular biological methods due to the development of PCR-related technologies and molecular biological technologies such as NGS, which have significantly changed the ability to identify and quantify microorganisms.

2.1 Recent trends in classification and identification techniques

In classical microbiology, the classification and identification of prokaryotic microorganisms is based on cell morphology, including Gram stainability, and physiological characteristics. However, rapid progress in molecular genetics has provided a clear notion that the classification system deviates significantly from natural classification based on evolutionary phylogeny (11). Currently, the classification and identification of microorganisms are shifting to a state-of-the-art classification system based on molecular phylogenetic inference and identification based on genomic information.

16S rRNA is now widely being used as a molecular phylogenetic marker. The 16S rRNA gene is approximately 1,500 nucleotides long, and all prokaryotic microorganisms possess this gene. It is considered a gene that does not propagate horizontally, and the evolutionary path of each microorganism can be inferred by molecular phylogenetic analysis based on the similarity of its nucleotide sequence.

In the case of the 16S rRNA gene, two microorganisms are likely to be regarded as the same species if they show 98.7% homology on comparison of the full length of the gene.

However, the current definition of prokaryotic species is based on the criterion of obtaining a value of 70% or higher by DNA-DNA hybridization (12), and it may not necessarily be appropriate to declare that the two microorganisms are the same species based on 98.7% or higher homology of the 16S rRNA gene. The DNA-hybridization method often shows a value of more than 70%, which corresponds to more than 95% when using whole-genome sequencing data (average nucleotide identity (ANI)). Therefore, more than 95% homology in ANI can be used for species discrimination between the two microorganisms (13).

In addition to identifying bacterial species at the genetic level, microbial identification

techniques considering the similarity of the total protein profile of each bacterium may be used. In many cases, similarity of the protein mass spectrum determined using MALDI-TOF-MS has been used to identify bacterial species (14). This method can generally identify differences at the strain level and can be used to determine whether two strains are the same. However, it is necessary to consider that protein profile may change owing to culture conditions and other factors, and this effect should be accounted for when using MALDI-TOF-MS for identification.

With regard to structural identification under a microscope, it is possible to visually identify microorganisms and measure their presence ratio in a sample using fluorescent in situ hybridization method targeting 16S rRNA or a fluorescent staining method using antibodies against specific antigens of the target bacteria.

2.2 Trends in methodologies for characterization of microbial consortia

For LBPs containing multiple microbial strains, the composition of the consortia (that is, the identity and abundance of the different strains) is an important quality attribute. In non-clinical and clinical studies, microbiome community measurements are sometimes performed to evaluate the composition in either the model animal or the subject (e.g., the intestinal tract or feces) to assess the efficacy and safety of LBPs. In such cases, the abundance of the different strains should be carefully determined, in addition to the identity of the strains. Currently, various molecular biological analysis methods such as realtime PCR, amplicon sequencing, and other methods targeting the 16S rRNA gene, are commonly used. Shotgun metagenomic analysis of the entire genomes using NGS is also increasingly being used and offers several benefits compared to 16S rRNA gene sequencing.

There are two major NGS-based microbiome measurement methods; 1) amplicon sequencing analysis and 2) metagenomic analysis (Fig. 1). In amplicon sequencing analysis, phylogenetic marker genes, such as 16S rRNA or gyrB genes, are amplified by PCR using broad spectrum primer sets that cover a wide range of phylogenetic groups, and the PCR products (amplicons) analyzed by NGS. In case of 16S rRNA gene amplicon sequencing, identification of strains to the species- or strain level may not be possible and the resolution of the method should be considered. In comparison, metagenome sequencing can provide identification with higher resolution down to the strain level.

In addition to identity of the strains, sequencing of microbial consortia can also provide quantification of the strains. However, bias in the measurement should be considered, especially for amplicon sequencing due to the use of PCR for library construction. For metagenome sequencing, deeper sequencing is required than for

amplicon sequencing for quantitative analysis because it involves analyzing the entire genome sequence rather than specific marker genes. Metagenomics has the added benefit that this method not only provides taxonomic classification but also genome-scale information (sequences of various functional genes and their quantitative information) and enables reconstruction of the genomic information of individual microorganisms. Further, it is can also provide insights into the metabolic potential of the microbiome and interactions between individual microorganisms. Additionally, since metagenomics, unlike amplicon sequencing analysis, does not depend on specific PCR primers, bias due to primer selectivity is circumvented and more accurate microbiome analysis can be achieved.

In addition to the above analytical methods, direct microscopic detection and identification of microbial cells is also possible by fluorescence in situ hybridization with nucleic acid probes targeting 16S rRNA or by fluorescence staining using specific antibodies. Hybridization methods targeting 16S rRNA can be employed for identification using probes for various phylogenetic levels; however, strain-level identification is challenging with these methods. In contrast, strain-level identification may be possible using fluorescent staining methods with specific antibodies.

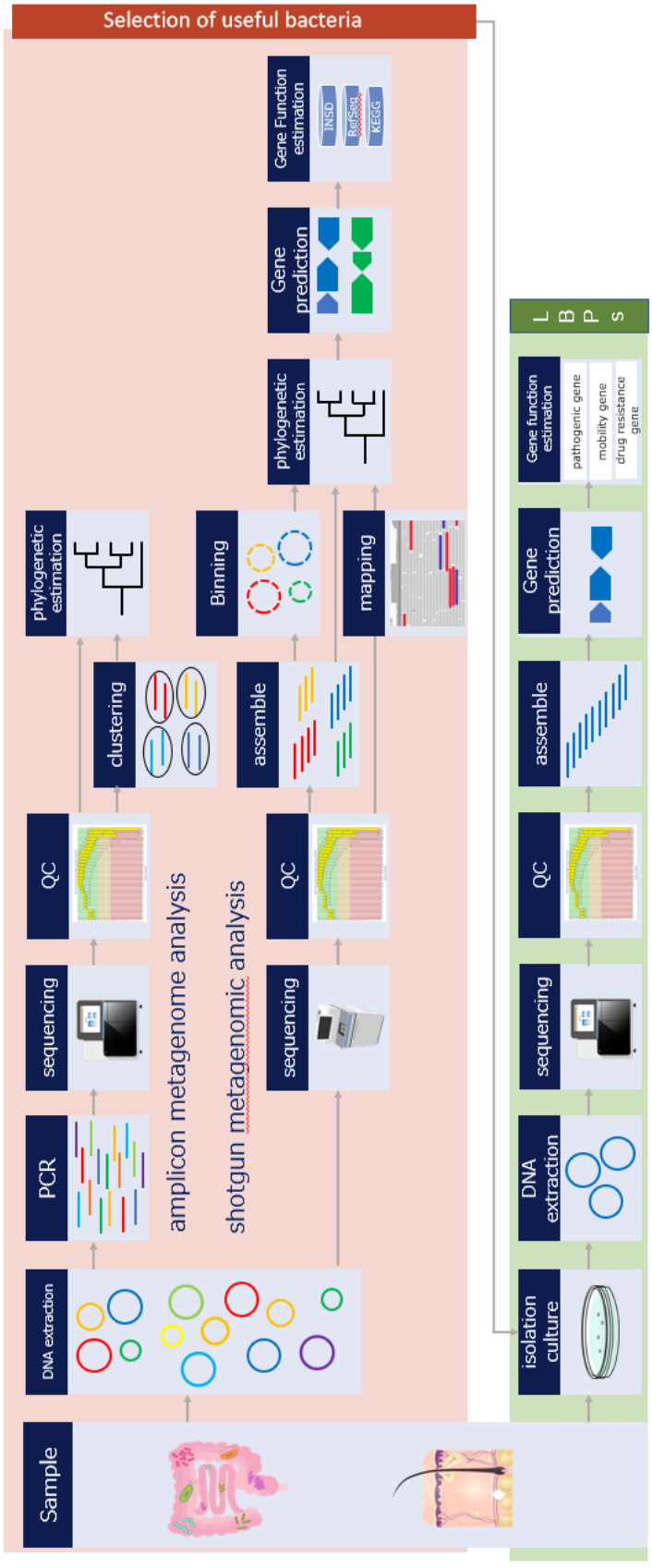
Selection of useful bacteria
Sample
DNA extraction
PCR
sequencing
QC
clustering
phylogenetic estimation
amplicon metagenome analysis
shotgun metagenomic analysis
sequencing
QC
assemble
Binning
mapping
phylogenetic estimation
Gene prediction
Gene Function estimation
INSD
RefSeq
KEGG
isolation culture
DNA extraction
sequencing
QC
assemble
Gene prediction
Gene function estimation
pathogenic gene
mobility gene
drug resistance gene
L
B
P
S

Fig. 1 Flowchart of genome and metagenome analysis.

Bacterial candidates for LBPs are identified using metagenomic analysis targeting bacterial communities in the human intestine, skin, and oral cavity as well as in the natural environment. The identified LBP candidates are genomically analyzed, and promising LBPs candidates are selected through in silico safety assessment, including the prediction of virulence genes and drug resistance genes.

2.3 In silico safety evaluation

It is crucial to evaluate the safety or effect of each bacterial strain that constitutes the LBPs and to comprehensively understand how a particular bacterium affects the human body at the molecular level. Therefore, complete genome sequencing of individual bacterial strains (genome assembly, gene annotation, presence of plasmids and phages, identification of mobile genes such as transposons (Tn), identification of toxin genes), transcriptome analysis, and metabolome analysis under various environmental conditions should be performed.

The physiological functions of bacteria should be clarified based on multi-omics analyses. It should be noted that functions of 30—50% of all genes in bacterial genomes are unknown. Although it seems to be challenging to determine the function of these genes, it is helpful to estimate whether these genes have an effect on pharmacological action and safety. It is also vital to refer to the information on the origin of the strains to be used. For LBPs, it is assumed that strains isolated from healthy donors should be used; however, obtaining information on the infectious agents present in the donor and their medical history is desirable.

(1) Estimation of gene functions

By reading the genome sequence information of an isolated bacterial strain, it is possible to estimate all the genes possessed by the bacterium from databases. Information on these genes includes estimating transcriptional and translational regions of genes in the genome sequence and the functions of these genes. Recent marked progress in genome sequencing technology provides many practical methods to estimate target genes, and estimation methods based on probabilistic models (such as GeneMarkS (15) and Prodigal (16)) can be used to estimate gene regions with high accuracy of 90–95%.

However, it should be noted that prediction accuracy tends to decrease in regions where the genome sequence features differ from those of other regions acquired by horizontal gene transfer. Additionally, depending on the settings of the

analysis software, it is necessary to consider that short genes cannot be detected. Sequence homology searches against gene sequence databases (such as GenBank/EMBL/DDBJ (17) and KEGG (18)) and motif databases (such as InterPro (19) and Pfam (20)) have been used to estimate gene functions. When the amino acid sequences are homologous, with 30% as the lowest limit of homology, it can be presumed that the functions of the target genes are similar.

(2) Estimation of pathogenicity (e.g., toxin-related genes)

The virulence factor database (VFDB) (21), a genome database of pathogenic bacteria and virulence factors, is commonly used to estimate pathogenicity factors by homology search. Software and analysis tools for estimating virulence factors by referring to VFDB from metagenomic sequence data are available (e.g., PathoFact (22)). PATRIC (23), and these can be referred to as integrated databases of genomic and omics data of pathogenic bacteria, which can help estimate virulence.

(3) Drug resistance

The presence or absence of antibiotic resistance genes (ARGs) in target bacterial strains constituting LBPs is also essential. For the estimation of ARGs, sequence homology searches against ARGs databases such as CARD (24), ARDB (25), and MEGARes (26) have become mainstream methods. With recent progress in AI technology, DeepARG (with DeepARG-DB) (27), which predicts ARGs through deep learning, and ARGMiner (28), an integrated analysis platform, have been developed.

(4) Plasmids and other transposable genetic factors

Identifying known mobile genetic elements (MGEs), such as phage genomes, plasmids, and transposons (Tn), is also crucial for evaluating the efficacy and safety of LBPs. ACLAME (29) is the most commonly used database for collecting and classifying MGEs. However, it should be noted that, as of 2021, the data have not been updated since more than 10 years and do not reflect the latest information.

Many software has also been used to predict mobile genes, such as plasmids, insertion elements (IS), Tn, and other transposable elements (30). There is also a software that can predict phages based on sequence homology and software that predicts phages based on sequence patterns using deep learning methods based on recurrent neural networks (PHAST/PHASTER/). PHASTEST (16), VirFinder (31),

VirSorter2 (32), Seeker (33)).

It is crucial to consider the advantages and disadvantages of each of these databases and software and combine them for analysis to improve the prediction accuracy. Analysis pipelines that combine various tools to predict MGEs from metagenomic data have also been published (e.g., eMGE (34)). It is important to clarify what type of analysis method (software) was used to obtain the results and to also consider the reliability of these results.

(5) Characterization of growth ability from genome sequencing information

When a bacterial genome is sequenced by the random shotgun method using short-read NGS, the coverage of short reads continuously decreases from the replication start point (ori) to the termination point (ter) of the circular chromosome. This phenomenon is attributed to the theta-type replication of bacteria, and techniques have been developed to estimate the growth rate of individual bacteria in a population (such as PTR (35), iRep (36), and GRiD-MG (37)). Using these methods, it is possible to estimate whether the target bacteria are growing in the population and also their relative growth rate.

2.4 In vitro evaluation

(1) Human intestinal model

In vitro culture systems that mimic the human intestinal tract and the intestinal microbiota have been developed to evaluate the functional effects of both foods and drugs on the intestinal tract. For example, to analyze the uptake of zinc through the human intestinal epithelium and its transport from the lumen to the epithelium, Caco-2 cells, a continuous cell line (CCL) derived from human intestinal cells, mucin-secreting cells, are utilized to evaluate in vitro digestion(38, 39) . Although these models can reproduce specific functions and features of the intestine, due to the complex microanatomy of the small intestine, contractile peristalsis of the intestinal gut wall, heterogeneous cell population oxygen gradient and diversity, and analysis of vast bacterial flora are often not included.

Recently, new in vitro models have been developed using micro-flow path technology to mimic the dynamic structural environment, signal transduction, and other vital features of the small intestine; however, all of these models should be considered for reproducibility, which is likely to be limited.

In contrast, reproducible systems have been developed for the large intestine that mimic intestinal microbiota and metabolites, and Polyfermentor has been developed to mimic the stomach, small intestine, and large intestine (for example,

PolyFermS (40), TIM-2 (41), SHIME (42), EnteroMix (43), Lacroix (44)).

However, even if the intestinal microflora of the large intestine can be reproduced, it is difficult to reproduce the bacterial flora as mimics of the upper gastrointestinal tract, including the small intestine. The recently developed single-vessel culture system (KUHIMM(45)) has advantages such as the ability to simultaneously perform multiple intervention tests and reproduce disease-specific bacterial flora using feces from patients with a certain disease, and the ability to simulate therapeutic intervention.

However, the effect of the mucin layer, which is expressed on intestinal epithelial cells, should be considered. The long-term stability of these colonic models has not been reproduced, and caution should be exercised regarding the extrapolation of the data obtained.

(2) Human Intestinal Organoids

An in vitro evaluation method using organoids has been developed to assess cell-bacterial interactions, especially those in the intestinal epithelium, constituting LBPs.

Organoids are mini-organs derived from tissue stem cells in vitro and are formed as three-dimensional tissue-like structures by self-organization using the self-renewal and differentiation capabilities of stem cells. In vivo, all intestinal epithelial cells differentiate from intestinal stem cells at the bottom of the crypt to maintain homeostasis in the intestinal epithelial tissues.

The mechanism of niche signaling that regulates the ability of intestinal stem cells to self-renew and differentiate has been elucidated previously, indicating that it is possible to culture intestinal epithelial cells in three dimensions for a long period using Matrigel, an extracellular substrate. Compared with conventionally cultured cells and spheroids, organoids exhibit anatomical and functional characteristics similar to those of in vivo organs, and it is possible to construct organoids derived from normal tissues (46).

Caco-2 cells have been widely used to evaluate the absorption of drugs and food ingredients in the intestinal tract; however, Caco-2 cells, which are one of the most commonly used CCLs, have a disadvantage in that they are derived from cancer cells and have only a part of the original functions of the intestinal tract. Use of colonic organoids prepared from colon tissues of healthy individuals makes it possible to evaluate the interaction between intestinal bacteria and the normal intestinal epithelium (47, 48).

To evaluate the reproducibility of the interaction between individual enterobacterial strains and the intestinal epithelium in vivo in an in vitro system using organoids, it is necessary to inject bacteria after confirming the apical position of the 3D organoid, which is technically challenging. To overcome this difficulty, a new culture technique using a two-dimensional organoid, a monolayer of a three-dimensional organoid, has been developed (47). In this study, a mucin layer could be detected; the results suggested that it is present on the apical surface of 2D organoids, which may reproduce the colonic epithelial structure in vivo.

Importantly, the ability of strains to induce pore formation within in vitro culture systems correlated positively with the ability of bacteria to translocate to the mesenteric lymph nodes in gnotobiotic mice, to which each strain was administered(49), suggesting that this method may help evaluate the effects of individual strains on the host in vitro.

3. Non-clinical studies

3.1 Pharmacological Studies (including Efficacy Support Studies)

Supporting studies on the efficacy of LBPs should be conducted to confirm that the expected pharmacological effects proof-of-concept (POC) can be observed at the same dosage as that planed in clinical trials, or should verify the efficacy observed in clinical trials. Pharmacological studies should be conducted to qualitatively and quantitatively clarify the pharmacological properties of a product for which an application for approval has been planned.

In such studies, animal models of diseases may be used to demonstrate the ability to achieve POC, while pharmacodynamic indices (e.g., the amount of IL-17 in the blood or the number of specific immunoregulatory cells) can be used instead of testing clinical efficacy. If pharmacodynamic indices are used for evaluation, it is necessary to explain the rationale underlying their selection.

The choice of model animals and the necessity of pre-treatment before administration (e.g., antimicrobial treatment) should also be considered according to clinical applications. To demonstrate the efficacy of LBP, it may be necessary to clarify theduration of action after administration and the appropriateness of pre-treatment and dosing interval settings in clinical trials with non-clinical study results.

3.2 Pharmacokinetic Studies

Unlike development of chemically synthesized drugs or biotechnology drugs,

conducting pharmacokinetic studies or evaluating toxicokinetics may not be necessary when developing LBPs. However, the distribution and persistence of LBPs in the intestinal tract and their biological properties are considered to be affected by the microenvironment in the intestinal tract. Therefore, it would be useful to obtain such information through non-clinical intestinal distribution studies following the route of administration.

To analyze the biodistribution of the administered strains (s), it is crucial to establish a strain-level identification method (e.g., genomic information) that can distinguish them from bacteria already existing in the intestinal tract However, although characteristic drug resistance patterns and strain-specific nucleotide sequences may be available, it is often difficult to analyze the intestinal distribution of all strains in the case of complex preparation of many strains.

It is believed that Polyfermentor culture devices can also be used to estimate the optimal dose before entering clinical trials by examining the survival kinetics and distribution of LBPs under conditions mimicking those in the patient's intestine and with different compositions, although more data on the extrapolation of such *in vitro* studies are needed.

3.3 Non-clinical safety studies

Generally, during drug development, safety assessments using model animals and other in vitro models are conducted to predict and control risks before first-in-human studies and subsequent clinical trials based on an appropriate understanding of the clinical quality and pharmacological characteristics of the drug candidate.

However, when developing LBPs, the first key consideration is to understand the quality and pharmacological characteristics of the product to be developed to secure safety of the product in the administration to human by scientifically rational and practically possible way, with understanding of the quality and pharmacological characteristics of the product to be developed

(1) Evaluation strategy

When developing LBPs, it is necessary to evaluate the possibility of adverse effects related to the expected pharmacological effects when administered to humans (hereinafter referred to as "on-target toxicity") and other adverse effects (hereinafter referred to as "off-target toxicity") through non-clinical safety studies.

Evaluation of on-target toxicity

Since LBPs are expected to elicit therapeutic effects by transplanting intestinal

bacteria obtained from the human intestinal tract into patients, concerns about on-target toxicity as an extension of pharmacological effects are considered to be relatively low.

In addition, in non-clinical safety evaluation of pharmaceuticals, toxicity tests are generally conducted using healthy animals. It has been reported that the animals used in these toxicity tests have their native intestinal microbiota and composition and metabolic activity are markedly different from human intestinal microflora (50). Furthermore, it is possible that the administered bacteria of human origin may fail to colonize in animals, and therefore, the significance of evaluating the safety of LBPs in conventional toxicity tests with animals is considered to be limited.

For LBPs, while non-clinical safety should not be evaluated in toxicity studies using healthy animals, instead pharmacological studies using animal models (one animal species, including rodents) could be performed to demonstrate their effects in humans. The effects on major physiological functions (e.g., the cardiovascular system, respiratory system, and central nervous system) may be assessed by checking the general condition in such studies.

Because LBPs are of healthy human origin and the evaluating ability of them using toxicity studies in model animals is limited, it is not necessary to conduct other non-clinical safety studies on on-target toxicity (such as single-dose toxicity, repeated-dose toxicity, genotoxicity, reproductive toxicity, and carcinogenicity).

Evaluation of off-target toxicity

Because LBPs are generally composed of multiple bacterial strains that exist in the human intestinal tract, it is necessary to evaluate concerns regarding off-target toxicity (e.g., unintended biological reactions or undesirable inflammatory reactions) in humans based on relevant published data and pharmacological tests. If safety concerns are identified from such assessments, appropriate risk reduction measures in clinical trials should be taken based on such information rather than pursuing further risk assessment in toxicity studies.

Additionally, since LBPs often have a complex composition consisted of multiple strains, there may be concerns about the potentially toxin-producing or invasive properties and/or drug-resistant genes of the microorganisms constituting the product, and chemical or biological contaminants during the LBP manufacturing process.

In the process of developing LBPs, it is critical to check whether constituent strains possess plasmids, and if any, the nature of the plasmid. The presence of known

pathogenic factors, such as toxin-producing ability, on the genome should also be checked. Simultaneously, the risk of unintentional microbial growth or impurities in the manufacturing process should be reduced to the maximum possible extent. Safety of the products should be ensured based on the test results obtained in quality control tests (e.g., limit values for impurities).

When it is difficult to control the risks during the manufacturing process, and there are concerns regarding safety in humans, it is necessary to conduct non-clinical studies focusing on possible off-target toxicity.

4. Manufacturing (bank establishment) and quality control of LBPs

For manufacturing LBPs, the microorganism itself is the active ingredient of the drug, and the starting material (that is, bacteria) is cultured in large scale to manufacture the final product. Ideally, the characteristics (such as phenotype and genotype) of the starting cells must be the same as those of the final product. To ensure such quality consistency of LBPs, a robust manufacturing process, an in-process control system during manufacturing, and appropriate quality characterization methods should be established. These methods are critical to enable the constant quality characteristics and help establish a management system for LBP manufacturing. Consistency between product lots (batch-to-batch variation) should also be carefully determined.

When strains are selected for development into a LBP, information on pathogenicity, toxin production potential, and bacteriophage production should be clarified, based on genome sequencing and *in vitro* testing. Furthermore, genetic stability (or genetic drift) of master cell banks should be evaluated, for example, whether the genes that contribute to the pharmacological effects are stably maintained in the cultures.

Since manufacturing of LBPs involves cultivating bacteria, there is a risk of contamination with impurities that may remain in the drug substance, such as the components of the medium used for manufacturing, metabolites, microorganisms other than the intended ones, and phages. The impurities and contaminants may vary depending on various factors during the manufacturing process.

4.1 Approaches to drug substance manufacturing and cell banking

(1) Approach to the construction and characterization of cell bank systems (MCB and WCB)

Single or multiple strains should be selected from historically defined candidate microbial strains (sufficient information regarding factors such as isolation history and laboratory breeding history) for LBPs that were anticipated to be effective

through various basic research. When selecting seed cell lines, they should be purified and evaluated to determine whether the seeds have specific functions.

MCBs established from these seed(s) are used as starting materials for the production of LBPs and are stored under stable conditions.

MCBs are the starting materials for manufacturing LBPs; hence, it is necessary to completely analyze MCBs from various aspects. For example, the morphology of cells should be confirmed by microscopy (if the cells form colonies on agar plates, their morphology should be confirmed), and the target strains should be identified by 16S rRNA gene and/or whole-genome sequencing. Genotypic and phenotypic characterizations, including purity and identity, should be performed (Table 1).

The phenotype and genotype of LBPs manufactured under optimized culture conditions should be identical to those of the original MCB and WCB cells. It should be confirmed that the phenotype and genotype of end of production cells (EPC) or cells produced beyond the production conditions (definend as Cells at Limit of in vitro age (CAL) in ICH Q5D guideline) are identical to those of original cell banks. Furthermore, it should be ensured that not only the growth ability but also the quality attributes of the characteristics of EPC/CAL remain stable throughout the manufacturing process.

It is necessary to consider that the growth and characteristics of the strains may change depending on the culture scale. However, if the involvement of a relevant factor is known and is considered to not affect the quality characteristics of LBPs, changes in the growth and characteristics of the strain are acceptable.

In the case of strains that produce specific substances (e.g., pigments or extracellular polysaccharides), their production capacity is also considered a characteristic of LBPs.

When manufacturing LBPs, the expansion of cultures should be conducted using established and reproducible manufacturing methods. After a defined culturing period, cultured cells should be collected, washed, and suspended in an appropriate buffer solution to manufacture the active pharmaceutical ingredients (APIs).

At the end of the culture process, the bulk harvested bacteria are washed, and the tests required for API are conducted based on the results of characterization of MCB. It is also necessary to set up appropriate standardized tests to show equivalence with MCB and to reconfirm the characteristics and purity of each lot.

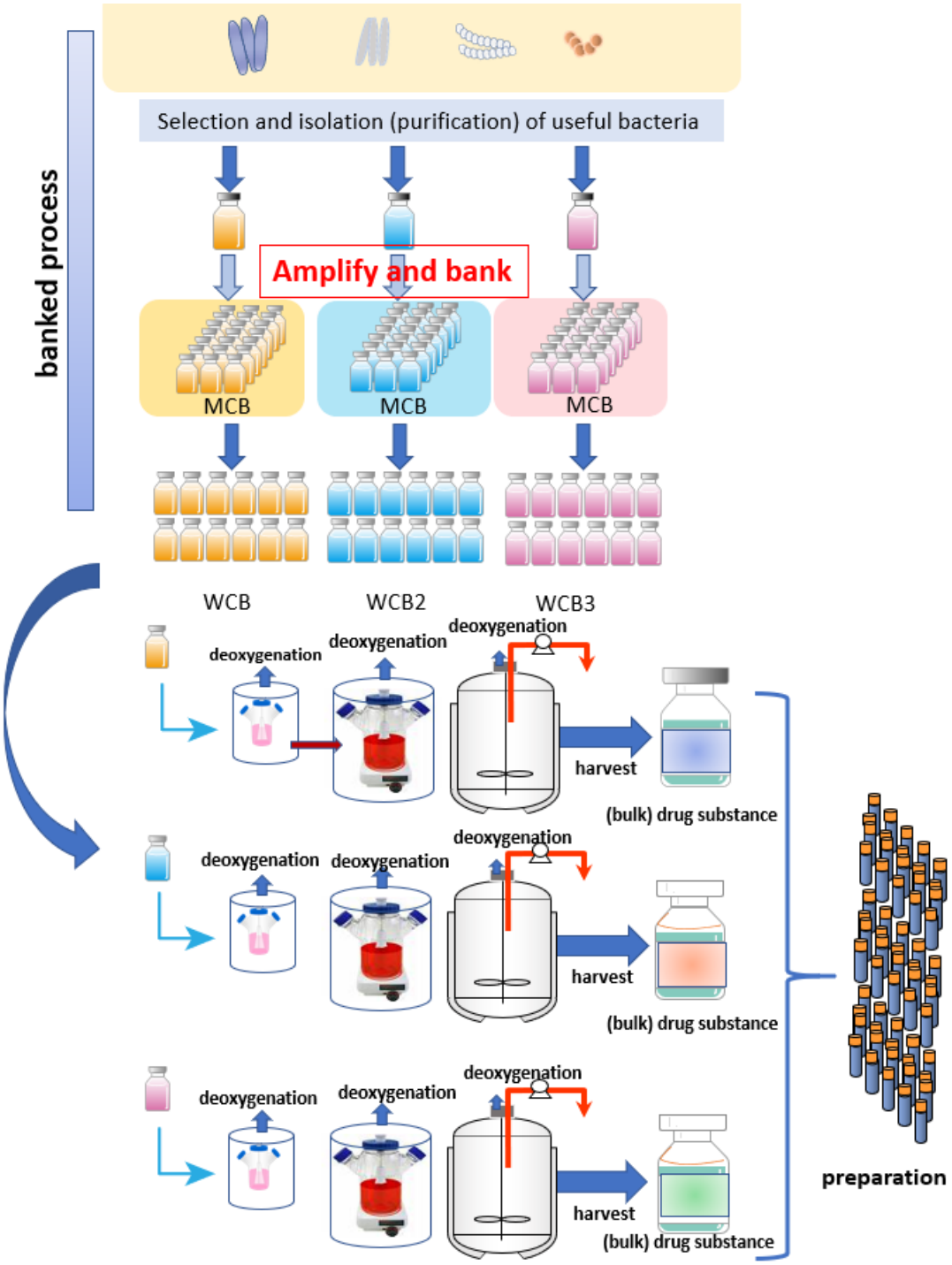

Fig. 2. Schematic flow-diagram for the manufacturing of LBPs

After the discovery of useful bacteria species, the bacteria are isolated and banked by species. During the manufacturing process, each useful bacteria should be cultured and expanded under optimal conditions, and each harvested bacteria should be purified and tested for quality as a drug substance. For LBP products consisting of multiple bacteria, these bacteria are mixed in a predetermined mixing ratio and formulated.

(2) Evaluation of production process to analyze EPC or CAL

- MCBs are the starting material for manufacturing LBPs, and it is necessary to comprehensively analyze the phenotypic and genetic characteristics of the established MCBs. In addition, the quality characteristics of the established MCBs, such as the absence of microbial contaminants and the ability to produce specific factors (such as proteins relevant to the expected pharmacological effects of the LBP), should be clarified and evaluated as a point of eligibility for starting materials of LBPs. Furthermore, generated data and analysis methods can help devise suitable tests that should be conducted when renewing MCBs and creating WCBs from MCBs.
- Bacteria in the cell bank of LBPs have essentially the same characteristics as the final products administered to humans. Therefore, the characterization methods used for the banks can be applied for the testing of APIs and formulated LBPs, as described below.
- Genetic stability: The genetic stability of banks should be evaluated under long-term storage and from the production process until the end of culture. The genetic stability of banks is expected to be evaluated in real-time by storing cells under stable conditions.
- A long-term culture may be less effective against gene instability in bacteria than in mammalian cells; however, the genetic sequences may be affected by culture conditions. For this reason, the cells can be cultured longer than expected at the time of product manufacturing, and mutations in the genetic sequence could be analyzed.

  The genetic stability test helps confirm any impact on the efficacy and safety of the LBP. If a genetic mutation is found in comparison with MCB/WCB, it is crucial to evaluate how the identified mutation may affect the efficacy and safety of LBPs.

4.2 Characterization of LBPs

Detailed characterization of LBPs is vital for identifying critical quality attributes (CQAs) and developing quality control strategies. For this purpose, multiple analytical methods should be applied to determine the characteristics of LBPs. A range of suitable test methods and acceptance criteria for the defined quality attributes should be established. The following characteristics may need to be assessed.

(1) Physicochemical property, including strain identification

- Morphological characteristics (morphology under the microscope and Gram staining)
- Identification using molecular phylogenetic marker genes such as 16S rRNA gene sequence (51).
- Physicochemical properties, including metabolites and cellular proteins
- Antimicrobial resistance
- Biogenic amine formation and drug metabolism profile
- Chemotaxonomic properties

In the case of LBPs composed of multiple bacterial strains, the quality attributes of each strain should be evaluated separately. Metabolic capacity, such as the drug resistance profile, biogenic amine production, and drug metabolism profile, may interact with each other and then they may need to be assessed using both pure and mixed cultures.

(2) Biological activity

For LBPs, it is also necessary to properly evaluate the bioactivity associated with the drug effect from a pharmacodynamic perspective. The assessment of biological activity of bacterial cells depends on the mode of action, and appropriate measurement strategy should be established. If the mode of action of a drug, such as a specific protein or metabolic capacity, is known, the metabolite or function of the target protein can be assessed directly. In contrast, if the mode of action is unknown, the number of microbial cells (or the number of viable cells) is considered as a proxy of biological activity, which serves as a basis for setting the dosage and other factors. Number of viable bacteria can be measured directly in terms of colony-forming units (CFUs) or cell counts in liquid culture (such as most probable number; MPN), or indirectly using culture-independent techniques such as flow cytometry for viable cells.

4.3 Specification test and acceptance criteria of LBPs

Based on the CQAs of LBPs, specification test of APIs is necessary to confirm that the quality is within the appropriate range as per the acceptance criteria. Appropriate specification tests should be established for the following aspects of LBP characteristics.

(1) Strain identity

- Morphological description: morphology under the microscope
- Confirmation of the 16S rRNA gene sequence
- MALDI-TOF MS analysis

  MALDI-TOF MS spectral analysis of the proteins produced by the target strain may be helpful in confirming its identity with reference material.

- Sequencing target genes related to pharmacological activity

  If the mechanism of the pharmacological activity of LBPs and the target gene group responsible for this activity is clear, sequencing of these genes may help in identity testing.

(2) Bioactivity

(3) Purity; i.e., contamination by microbes other than the target microorganism(s).

(4) Impurities

Process-related impurities in LBPs, such as medium components used in the cultivation process, must be evaluated.

(5) Potency

A potency titer is a standard on which the dose is based; it is usually based on the biological activity associated with the drug effect; however, it can also be based on the physical quantity, such as the number of viable bacteria in the LBPs. It may also be determined by the number of active ingredients produced by the microbiome.

(6) Amount of substance

It is assumed that the number of cells could be measured as the potency of the drug product. In many cases, the expected pharmacological effects may depend on the viable microbial cells.

4.3.7 Shelf life based on stability studies

As for other drug products, stability studies should be conducted to determine the shelf life of LBPs. For microbial consortia, such studies should evaluate the stability of each constituent strain in the LBP. For example, accelerated and caustic tests can provide valuable information by evaluating which bacterial species are affected; however, such tests cannot be used to predict the shelf life. The potency of a cocktail composed of multiple viable bacteria should depend on the component ratio of multiple viable bacteria; therefore, evaluating the number of viable bacteria in the composition is crucial.

4.4 Formulation Process Development

Most bacteria that reside in the intestine are anaerobic bacteria that are sensitive to oxygen. Therefore, to maintain sufficient viability during storage and delivery into the patient’s intestine, LBPs should be formulated using special capsules (e.g., seamless capsules) together with buffer solution and stabilizers. Additionally, the formulation development process for LBPs takes into account not only the stability of the target bacteria but also their efficient delivery to the intestine.

5. Considerations for clinical trials

In the clinical development of LBPs, the initial dose for clinical trials should be set based on the results of non-clinical studies. In addition, pre-treatment with antimicrobial agents is often used to ensure efficient colonization of administered microbes in the body. When antimicrobial agents are used, it is crucial to ensure the patient's safety; in some cases, preliminary testing of patient sensitivity to antimicrobial agents is necessary. It is also highly likely that dietary therapy will be required to promote engraftment and ensure the persistence of the administered LBPs. Therefore, it is necessary to explain the rationale for such a treatment. In the early stage of development (Phase I), the first human dose can be set based on non-clinical studies to evaluate safety in humans. Although pharmacokinetic studies are not usually required for LBPs, stool samples can be examined to assess the viability of LBPs indirectly.

For the clinical development of recombinant LBPs, it is necessary to evaluate their environmental effects.

Consideration should also be given to determining the optimal dosage for administration. The results of non-clinical studies and clinical data obtained using other bacterial preparations can be used as a reference to determine which methods are appropriate for dose escalation. The dose for clinical trials should be set based on biodistribution data in model animals. If persistence within the intestinal tract is insufficient, repeated administration may be considered.

Pharmacodynamic indicators such as immunosuppression and anti-inflammatory response can be used to appropriately assess the biodistribution and persistence of the drug in the subject's intestine to determine the optimal dose and the need for repeated administration. Trials to assess efficacy should also consider evaluating LBP using the true endpoint of the target disease. Therefore, it is vital to set up appropriately designed, double-blind comparative studies.

Characteristic Items		Considerations for conducting the test
Genotype	16S rRNA gene sequence	16S rRNA gene sequence unique to the strain: purity and identity of the target strain
	Whole Nucleotide Sequence Analysis	
Phenotype	Protein Expression Profiles	MALDI-TOF MS analysis: purity and identity of target strains (may vary depending on culture conditions)
	Morphology	Observation under the microscope, colony morphology
	Gram stainability	
	Ability to produce useful substances	Indicators related to drug efficacy and biological activity
	Proliferative properties	Depends on culture conditions
	Drug resistance	
Purity test		Heterologous microorganism denial test

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