ENVIRONMENTAL RISK ASSESSMENT OF GENETICALLY MODIFIED BACTERIA IN MEDICAL APPLICATIONS

ERABACMED

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Aline Baldo, Jeanne Vandermeeren, Amaya Leunda

Service Biosafety and Biotechnology, Sciensano

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Summary

Genetically modified bacteria (GMBs) are being developed as new treatments and diagnostic tools for several diseases, including cancer, gut disorders, and infections. Before these bacteria can be used in people, researchers must check whether they could pose any risk to others or to the environment. This process is called an environmental risk assessment.

So far, studies show that the GMBs used in clinical trials are generally safe for the environment and people who are not receiving the treatment. This is because the bacteria are designed so they cannot survive outside the body, cannot spread easily, and often require specific nutrients that are not found in nature.

Some GMBs, such as *Lactococcus lactis* and *Escherichia coli Nissle*, are originally harmless bacteria commonly found in food or the human gut. When used as treatments, they may be present for a short time in the patient's faeces or saliva but do not persist in the environment.

Other GMBs, such as modified *Listeria* or *Salmonella*, are derived from bacteria that can cause disease in their natural form. Even though these strains have been weakened to make them safer, special care is still needed to protect healthcare workers and vulnerable individuals.

Across all studies, the likelihood that these modified bacteria share their genes with natural bacteria in the environment appears to be very low. However, when the treatments do contain antibiotic-resistance genes, an in-depth assessment of their risk of transfer to other bacteria should be performed.

Looking ahead, new technologies are emerging. These include genetically modifying bacteria that are natural residents in tumours and harmless viruses that can edit bacteria already living in the gut, opening the door to more precise and personalised therapies. These innovations will also require new ways of evaluating safety to make sure they can be used safely.

Overall, genetically modified bacteria show strong promise for future medicine but need careful design and proper safety measures to be used without significant risk to the public or the environment.

List of abbreviations

AE	**Adverse event**
AMGR(s)	Antimicrobial resistance gene(s)
CFU	Colony forming unit
CR	Class of risk
CT	Clinical trial
DSS	Dextran sulphate sodium
EcN	*Escherichia coli* Nissle 1917
EFSA	European Food Safety Authority
ERA	Environmental risk assessment
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FDA	Food and Drug Administration
GI	Gastrointestinal
GM	Genetically modified
GMB	Genetically modified bacterium
GMBs	Genetically modified bacteria
GMOs	Genetically modified organisms
GMMs	Genetically modified microorganisms
GOI(s)	Gene(s) of interest
GRAS	Generally recognised as safe
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LAB	Lactic acid bacteria
Lm	*Listeria monocytogenes*
LPS	Lipopolysaccharide
MetaEdit	Metagenomic Editing
PPE	Personal protective equipment
QS	Quorum sensing
QSP	Qualified presumption of safety
TME	Tumour microenvironment

Glossary

Adjuvant: A component that enhances the immunogenicity of vaccines when administered in conjunction with vaccine antigens.

Attenuation: Use of genetic modification to suppress or inhibit the proliferation, survival, or other biological activity of an organism, thereby mitigating the risks posed by that organism to its hosts and the environment.

Auxotrophic/ auxotrophy: Inability of bacteria to proliferate or survive in the absence of some exogenously provided essential substance. Genetically modifying bacteria to be auxotrophic for an essential substance not found in humans is an attenuation strategy for therapeutic use of bacteria.

Bacteraemia: The presence of bacteria in the bloodstream.

Bacteriophage: A virus that infects and replicates within bacteria.

Biodistribution: Dispersion of a product within the patient's body from the site of administration.

Chassis: A chassis is a host micro-organism, often a bacterium, yeast or other cell, into which genetic elements, circuits or metabolic pathways designed by synthetic biology are introduced.

Facultative anaerobe: Bacteria that can survive in the presence or absence of normal atmospheric concentrations of oxygen.

Horizontal gene transfer: Transfer of genetic information between organisms by means other than parent-to-offspring vertical transfer. The below three terms are modes for horizontal gene transfer:

> **Conjugation**: Transfer of plasmids between bacteria by direct cell-to-cell contact;
>
> **Transformation**: Uptake and chromosomal integration of exogenous DNA;
>
> **Transduction**: Transfer of DNA between bacteria by a virus (a bacteriophage) or a viral vector.

Hypoxic: Sub-physiological levels of oxygen in a tissue.

Microbiota: A community of micro-organisms (e.g., fungi, bacteria, and viruses) that naturally resides in a particular habitat of the body (e.g., the gut and skin).

Oncolytic: The ability of a virus or bacteria to preferentially infect and kill cancer cells.

Payload: a payload is a functional molecule, such as a therapeutic protein, small molecule, enzyme, or siRNA, delivered directly to a disease site (e.g., tumor microenvironment, infection) to exert a targeted effect.

Probiotic: Live micro-organisms that, when administered in adequate amounts, confer a health benefit to the host.

Quorum sensing: cell-to-cell communication mechanism by which bacteria detect their population density through small signalling molecules and coordinately regulate gene expression once a threshold concentration is reached. **Tumour microenvironment**: The tissue local to the tumour comprised of solid tissue, immune effector cells, blood vessels, and signalling molecules. The tumour microenvironment is typically hypoxic, and its constituents support an immunosuppressive environment that allows the tumour to evade recognition and destruction by the immune system.

Virulence factor: Bacterial components that allow a bacterium to colonise its host and cause disease. Disruption of virulence factors by genetic modification can decrease risk the use of a bacteria for therapeutic means.

[main source : (Gulig et al., 2024)]

I. Introduction

Genetically Modified Bacteria (GMBs) are being investigated as potential treatments for a broad range of human medical applications including cancer immunotherapy, metabolic disorders, inflammatory diseases and infectious diseases. To date, two GMB based products have already been placed on the market in the European Union (EU) for the prophylaxis of typhoid fever (Vivotif®) and cholera (Vaxchora®).

Certain bacterial strains possess intrinsic properties that can be adapted for medical use through targeted genetic modifications. These strains may originate from the human microbiota, be used as probiotics, or be naturally pathogenic to humans and/or animals. Ongoing research, including preclinical studies not yet at the clinical stage, aims to optimise GMBs as therapeutic or diagnostic tools, integrating synthetic biology and artificial intelligence to support personalised bacterial engineering.

The term “genetically modified bacterium” used in this report refers to a bacterium whose genetic material has been altered in different ways in order to provide:

- a biological containment to the GMB, designed to prevent it from surviving, reproducing, or spreading outside the host,
- an attenuation of the GMB virulence necessary in the case of a pathogenic bacterium and/or
- a transgene or “gene of interest” (GOI) coding for a protein intended to provide a potential treatment for a given indication. Note that not all the current investigated GMBs as therapeutics carry a transgene.

As genetically modified organisms (GMOs), GMBs must comply with the EU legislation on biosafety particularly with regard to the contained use of genetically modified micro-organisms (GMMs) and the deliberate release of GMOs into the environment (Directive 2009/41/EC; Directive 2001/18/EC). This regulatory framework aims to protect the general population and the environment against potential risk arising from the use of GMOs. It provides for an environmental risk assessment (ERA) of the use of these GMOs in order to implement containment and other protection measures where needed.

Research and publications on GMBs as a therapeutic tool are focusing on optimising patient safety, efficacy and administration methods. Considerations relating to potential risks for the population in general and the environment are much less documented although there is an awareness that GMBs may be shed by the host exposing the environment and that further product development needs to deal with environmental and regulatory requirements.

The first part of the report provides an overview of past and current clinical trials (CTs) investigating GMBs as potential therapeutics for various human diseases and disorders. On the basis of this overview and on the information available for each GMB and its medical application, some GMBs and CTs have been selected and their analysis regarding biosafety has been performed in the second part of the report. For this purpose, the relevant GM strain bacteria are described with particular attention to their characteristics and their use in CTs, enabling an ERA. The objective is to improve the understanding of their potential risks for the general population and the environment, while contributing to the development of safer medical applications of GMBs. The third part of the report provides an overview of current preclinical research on GMBs. It highlights ongoing efforts not only to further refine and optimise strains that have already undergone evaluation in CTs, but also to develop new approaches that introduce innovative therapeutic or diagnostic strategies. This part of the report thus delineates both the incremental improvements being made to existing candidates and the development of innovative GMB platforms that may underpin future translational progress.

Potential benefits associated with applications with GMBs continue to drive research and development efforts aiming at creating safer and more effective therapeutic models. This report aims to identify and document potential risks that GMBs may pose to human health in general and the environment when used in medical applications, and to propose protective measures if needed to mitigate exposure to these organisms during medical use.

2. Clinical trials involving GMB

- Material and methods

To establish an inventory of CTs involving GMBs, 7 articles were reviewed discussing their principles, practice, recent advances and prospects (Ali et al., 2025; Becerra-Báez et al., 2022; Le et al., 2012b; Liu et al., 2022a; Mahdizade Ari et al., 2024), as well as their impact on human health (Dey and Sankaran, 2024) or risk assessment (Gulig et al., 2024). The review articles were selected using keywords such as “engineered bacteria”, “genetically modified bacteria”, “oncolytic bacteria”, “biocontainment”, “medical application”, “disease treatment”, “synthetic biology”, “artificial intelligence”, “microbiota”, “diagnosis” or “sensor”. Based on the current state of knowledge, a list of GMBs and their associated parental strains (the bacteria from which the GMBs derived) was compiled. This served as the foundation for consulting CTs websites, such as ClinicalTrials.gov, Clinical Trials Information System (CTIS) and EudraCT, to draw up a list of studies associated with each GMB. In total, 55 GMBs and 117 CTs were identified. Using the CT identification numbers, the “literature list” was supplemented with reports of CT data involving GMBs. All review articles, clinical reports, reports of preclinical data and retrospective studies contributed to further characterise the 55 GMBs and their corresponding trials.

Each GMB was evaluated for multiple variables including parental bacterial strain, genetic modifications made, involving the transgene and the mitigation strategies to ensure biosafety and containment, along with the purpose of genetic modification.

Each trial was described by the status, phase, year of launch, GMB dose administered, route of administration, type of treatment (monotherapy or combination), number of patients treated, type of disease targeted, and GMB shedding assessment (details, see supplementary excel file). Shedding corresponds to the dissemination of the GMB into the environment by excretions or secretions, the skin and the blood from the treated individual (Schenk-Braat et al., 2007). For the purpose of the current project, the shedding variable aims to verify whether a shedding analysis was performed during the CT and if so, what is the type of samples evaluated and the duration of the assessment.

- Results

Drawing on the identified list of 117 CTs, a systematic inventory was generated to document the bacterial species employed in these studies (Figure 1). Bacterial species under clinical investigation include human pathogens such as *Listeria monocytogenes, Mycobacterium tuberculosis, Bordetella pertussis, Shigella flexneri 2a, Salmonella enterica*, and pathogenic *Escherichia coli* strains, which are evaluated for their strong immunogenicity. Also under study are members of human microbiota and opportunistic species, including *Staphylococcus epidermidis* and *Phocaeicola vulgatus*, which are members of the skin or gut microbiota and generally possess low pathogenic potential. In addition, several probiotics, such as *Lactococcus lactis, Lacticaseibacillus casei, Limosilactobacillus reuteri*, and *Bifidobacterium longum*, are being developed as safe chassis for therapeutic delivery. Finally, *Clostridium novyi* and *Neisseria lactamica* are also explored for applications leveraging their properties, including selective tumour colonisation or replacement of pathogens in the nasopharynx respectively.

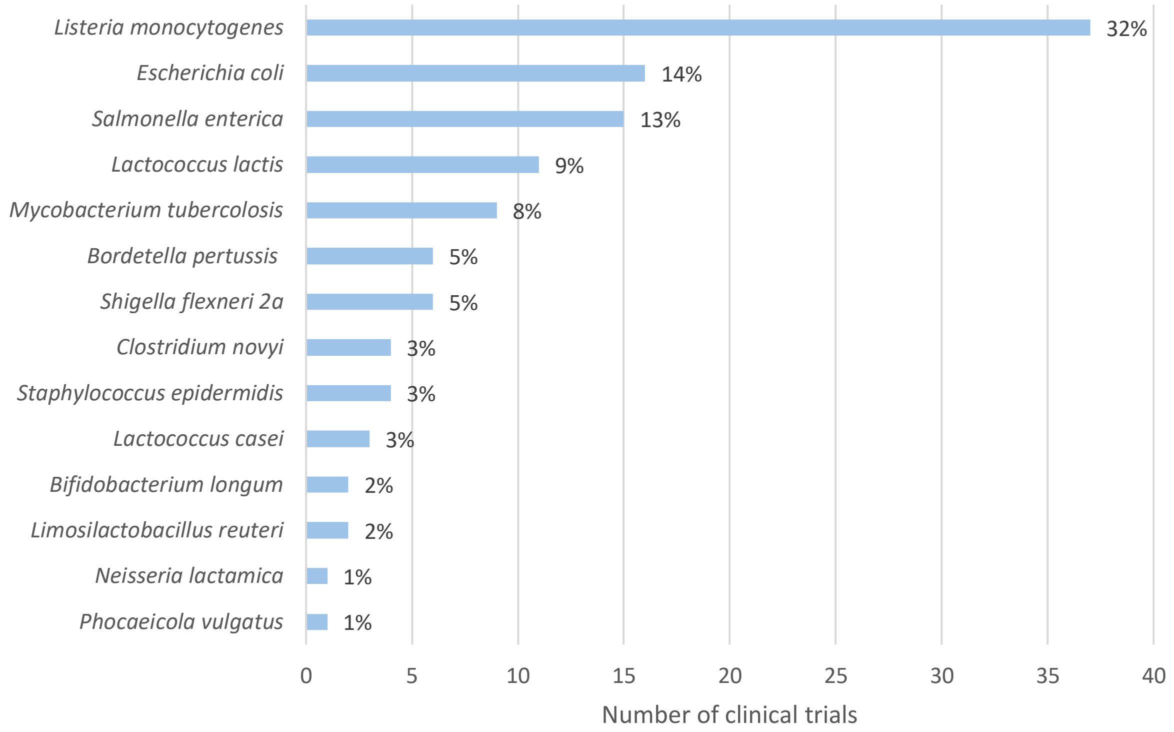

Figure 1. Horizontal bar graph showing the number of CTs categorized per type of parental bacterium. For each category, the fraction of CTs using a particular type is shown as percentage of the total number (117) of clinical trials inventoried in this review.

The four bacteria most investigated in CTs are *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli* and *Lactococcus lactis*, which together account for 68% of the CTs listed. This aligns with the traditional focus on model organisms such as *Escherichia coli* and lactic acid bacteria in genetic modification applications, a focus that is now expanding with the development of synthetic biology toolsets (Arnold et al., 2023). With a few genetic modifications, these specific bacterial strains have properties particularly suited to therapeutic uses.

Since the early 2010s, therapies involving GMBs have been continuously developed and clinically tested, showcasing their therapeutic potential (Dey and Sankaran, 2024) (Figure 2). This surge in GMB based therapies can be attributed to a combination of scientific advances, regulatory support and investment in bacterial therapy development. For instance, in 2018, companies like Synlogic launched CTs using GM *Escherichia coli* strains to treat metabolic diseases, while ActoBio Therapeutics explored *Lactococcus lactis* to prevent type 1 diabetes in patients. Additionally, European Union funded projects like WHILYAS (https://cordis.europa.eu/project/id/804438/factsheet/fr) were initiated in the same year to develop GMB therapies aimed at treating chronic wounds in diabetic patients. These initiatives reflect a clear intensification of interest and activity in microbiological therapies during this period.

The field of oncology, in particular, has seen significant advances in the use of GMB innovations. Since 2010, a substantial portion of CTs has focused on cancer treatments, with 56% of all CTs involving oncology, followed by 23% for infections, 12% for autoimmune diseases, and 9% for metabolic diseases (Figure 3).

The distribution of therapeutic indications across bacterial strains is further clarified in Figure 4, with strains like *Listeria monocytogenes*, *Clostridium novyi*, *Bifidobacterium longum*, and *Salmonella enterica* specifically linked to oncology applications. This further underscores the growing potential of GMBs in cancer treatment and beyond.

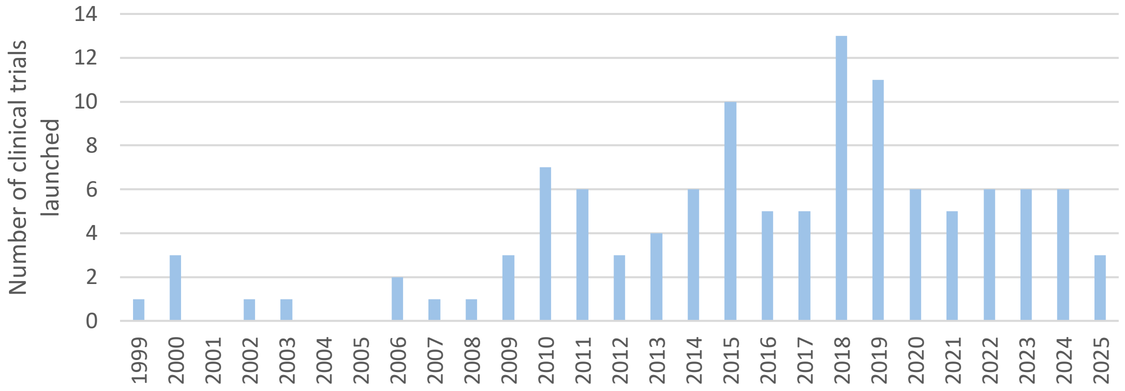

Figure 2. Bar graph showing the distribution of the 117 CTs launched each year between 1999 and June 2025.

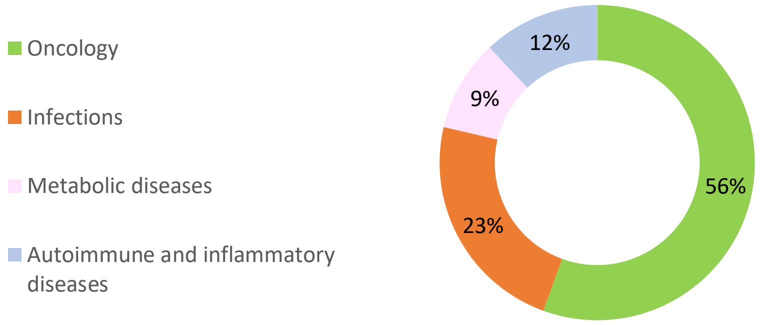

Figure 3. Donut plot showing the relative portion of analysed CTs (117), for each of the main disease categories: Oncology (n=65), infections (n=27), metabolic diseases (n=11) and autoimmune and inflammatory diseases (n=14).

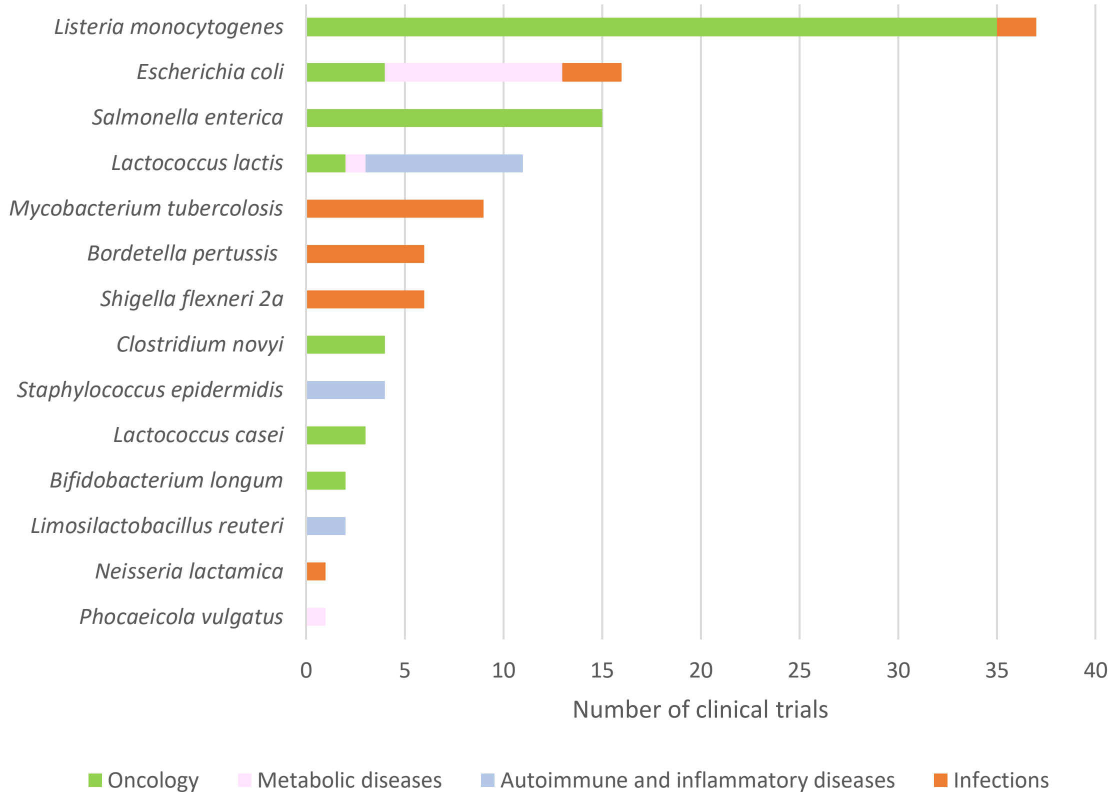

Figure 4. Stacked bar graph representing how many CTs were performed using a particular type of parental bacterium, subdivided per indication: Oncology (green), Metabolic disease (pink), Autoimmune and inflammatory disease (blue) and infections (orange).

Of the 117 identified CTs involving GMBs, about half have been completed since 1999. In contrast, 24% of trials have been stopped permanently or temporarily (Figure 5). Reported reasons include insufficient efficacy, unacceptable toxicity, or technical and operational issues. For instance, in the NCT03447730 trial, which studied *Escherichia coli* Nissle 1917 derivative SYNB1020 aimed at reducing intestinal ammonia absorption in patients with hyperammonemia, interim futility analyses showed that the GMB was not more effective than placebo, leading to study termination. Similarly, the trial NCT03234465 testing AG013, a live recombinant *Lactococcus lactis* administered as a mouthwash for chemoradiation-induced oral mucositis, was discontinued due to lack of efficacy. Moreover, the NCT02291055 study investigating a GM *Listeria monocytogenes* was placed on hold by FDA following the death of one trial participant.

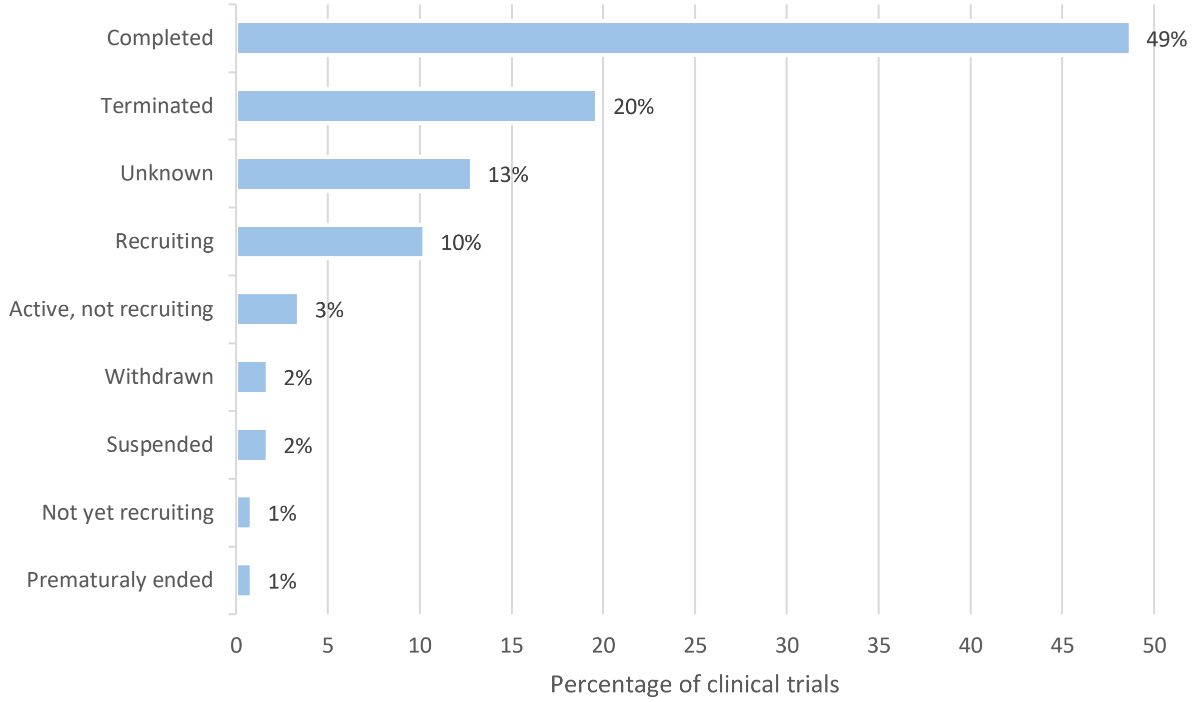

Figure 5. Horizontal bar graph representing the number of CTs categorized by status. The numbers beside the bars represent the relative fraction of CT (n = 117) per status category. ***Completed****: The study has ended normally, and participants are no longer being examined or treated;* ***Terminated:*** *The study has stopped early and will not start again. Participants are no longer being examined or treated;* ***Unknown:*** *A study whose last known status was recruiting; not yet recruiting; or active but that has passed its completion date, and the status has not been last verified within the past 2 years;* ***Active, not recruiting:*** *The study is ongoing, and participants are receiving an intervention or being examined, but potential participants are not currently being recruited or enrolled;* ***Not yet recruiting:*** *The study has not started recruiting participants;* ***Recruiting:*** *The study is currently recruiting participants.;* ***Suspended:*** *The study has stopped early but may start again;* ***Withdrawn:*** *The study stopped early, before enrolling its first participant.*

Over the last three decades, promising candidates have been tested in CTs but most failed to progress beyond phase I (Figure 6). 47% of the CTs, irrespective of their status, reached phase I, which assesses safety and identifies side effects. About a third reached phase II, involving a larger group of patients to determine the GMBs effectiveness and safety, and 20% are in phase I-II combined.

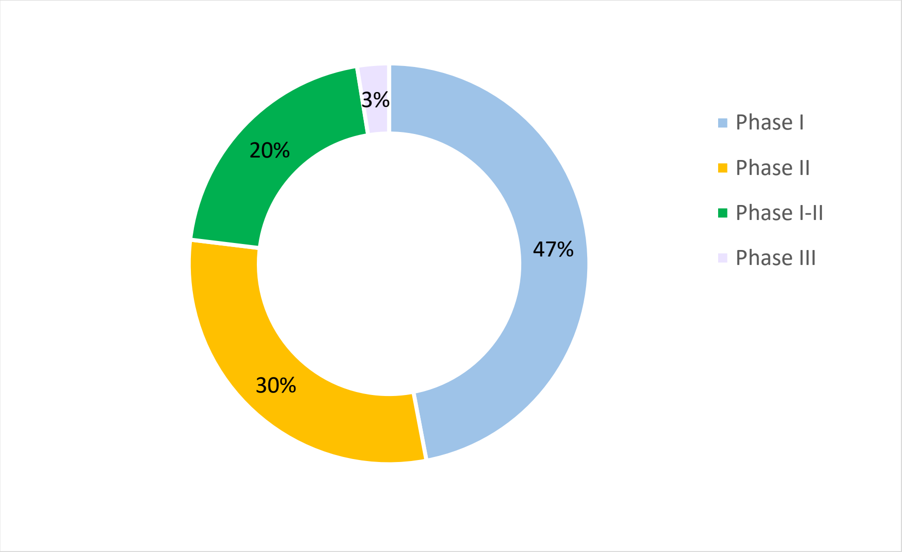

Figure 6. Circular plot of the relative fraction of the GMB-CT inventory (n = 117) displayed for clinical trial phases: ***Phase I:*** *Researchers test a drug or treatment in a small group of people for the first time. The purpose is to study the drug or treatment to learn about safety and identify side effects;* ***Phase II:*** *The new drug or treatment is given to a larger group of people to determine its effectiveness and to further study its safety;* ***Phase III:*** *The new drug or treatment is given to large groups of people to confirm its effectiveness, monitor side effects, compare it with standard or similar treatments, and collect information that will allow the new drug or treatment to be used safely.*

A limited number of launched clinical studies have progressed to phase III. These include :

- A trial launched in 2023 using an *Escherichia coli* for the treatment of phenylketonuria, a metabolic disorder. This CT is currently listed as "terminated".
- A trial conducted between 2015 and 2019 involving *Listeria monocytogenes* ADXS11-001, which was administered following chemoradiation as an adjuvant treatment for high risk locally advanced cervical cancer.
- A trial initiated in 2022 to evaluate the efficacy, safety and immunogenicity of *Mycobacterium tuberculosis* MTBVAC in healthy HIV unexposed and HIV exposed uninfected newborns in tuberculosis-endemic regions of Sub-Saharan Africa. This trial is currently recruiting and aims to be completed by 2029.

The efficacy of GMBs in disease treatment can be assessed through their capacity for colonisation after entering the body (Liu et al., 2022). In fact, the efficiency of the treatment is partially linked to the route of administration, which determines the tissues and organs most directly exposed to the GMBs (Gulig et al., 2024). Several routes of administration have been used in CTs involving GMBs including oral, topical, transcutaneous, intradermal, intravenous, intratumoural, nasal, intravesical, and intravitreal. The selection of the route of administration depends on the disease, the target tissue, the parental bacteria species including their inherent pathogenic potential (Hosseinidoust et al., 2016; Mahdizade Ari et al., 2024). In addition, the administration route can facilitate colonisation at the intended site and thereby help reduce off-target effects of expressed bacterial products (Dosoky et al., 2020; Liu et al., 2022).

Among all administration routes tested, the most common are oral and intravenous (Figure 7). Intravenous delivery is restricted to cancer therapies, likely due to its high potential for systemic circulation (Mahdizade Ari et al., 2024). In contrast, the oral route represents a wide range of bacterial species and therapeutic indications. However, it faces the particular challenge of the gastrointestinal (GI) environment, characterised by harsh conditions (low pH) and a highly diverse microbiota. This complexity influences encapsulation strategies, dosing, and the release of the administered GMBs in the faeces (Dey and Sankaran, 2024).

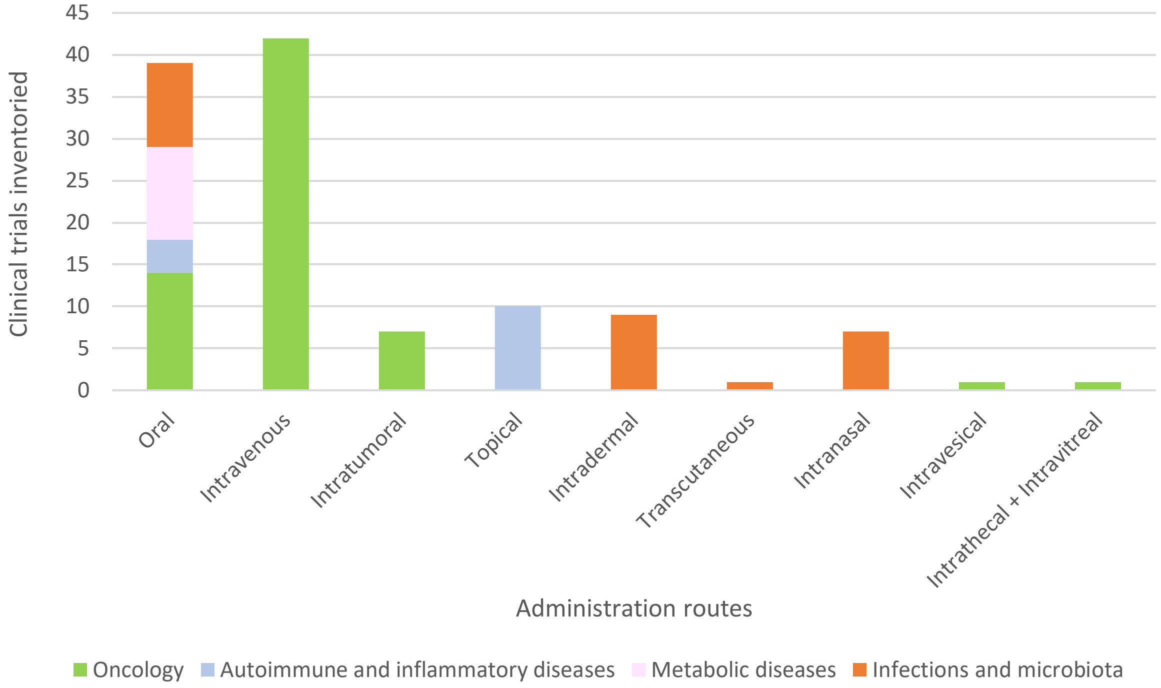

Figure 7. Stacked bar plot showing the number of CTs that use a particular route of administration. Each bar represents an administration route which is subdivided the four main disease categories: Oncology (green), Metabolic disease (pink), Autoimmune and inflammatory disease (blue) and infections (orange).

The intratumoural route has been investigated in CTs for the treatment of solid tumours as an alternative to intravenous or intravesical routes, which are specific to oncological diseases. This mode of administration significantly reduces the potential systemic toxicity associated with live GMBs (Mahdizade Ari et al., 2024; Taniguchi et al., 2010). Other routes have shown indication specific applications: topical delivery is mainly explored for autoimmune diseases, while transcutaneous, intradermal, and intranasal routes are under investigation primarily for infectious diseases. In particular, intranasal immunisation is more suitable for preventing systemic allergies and inflammation of the respiratory tract.

Intratumoural or intravenous administration of GMB represents routes of exposure that differ from the naturally occurring host entry route of the parental wild-type strain and may therefore influence biodistribution, persistence, and shedding, with potential implications for environmental exposure. To characterize potential environmental exposure to GMBs, shedding is typically assessed through regular sampling of patient excreta (e.g., urine, faeces, and saliva) during CTs, although the specific sampling strategy may be adapted according to the administration route. Detection of GMBs is generally performed using culture based methods and quantitative PCR for bacterial DNA. Culture based detection identifies GMBs that are intact and capable of survival, and therefore represent the most biologically relevant measure of potential environmental exposure. This method is less sensitive than qPCR, which may be used for initial screening but do not distinguish between viable and non-viable bacteria. This distinction is essential for interpreting shedding data.

3. Environmental risk assessment

The number of CTs involving different GMBs and being investigated for different medical indications demonstrates that research in this field is promising and will continue to develop. Besides the regulations linked to the clinical development of medicines concerning, in particular, quality, efficacy and safety aspects, CTs involving a GMB based medicinal candidate should comply with the requirements of the GMO regulatory framework in Europe, in particular directive 2001/18/EC on the deliberate release of GMOs (Directive 2001/18/EC). Following this Directive, an ERA must be carried out to evaluate the potential of the GMB to cause adverse effects on humans (beyond the treated subjects), animals, plants and other micro-organisms that might be exposed. The ERA considers the likelihood of such effects occurring. It follows a weight-of-evidence approach that incorporates both qualitative and quantitative considerations, and is described using qualitative terms ranging from high, moderate, low, or negligible risk. Once the overall risk has been characterised, the need for risk management measures is assessed to determine whether additional actions are required to minimise the likelihood of adverse effects. If no risks are identified, no risk management measures are necessary.

Key elements of the ERA include the assessment of the genetic stability of the GMB and its possible interaction with other organisms. It has to take into account the intrinsic characteristics of the parental bacterium, the characteristics of the inserted gene(s) of interest (GOIs), the biodistribution and shedding and the potential for horizontal gene transfer (HGT). A critical step in the ERA is the evaluation of possible exposure pathways through which the GMB may interact with other humans and the environment. Possible exposure pathways during a CT include leakage upon administration, accidental release during administration and shedding of GMBs by the patients. Blood and related products, like serum or plasma, could also be a source of exposure for personnel in clinical settings (e.g., during intravenous administration of the products) or for close contacts of the trial subjects (e.g., direct contact with open wounds).

The ERA does not cover purely medical aspects, such as the efficacy and safety of the GMB for the patient. Nevertheless, clinical safety data can provide valuable insights into the potential pathogenicity of the GMB and may inform the ERA. It is important to highlight that exposure of the general population and the environment is generally expected to be lower than that of the treated patient.

The following section presents an ERA of GMBs currently under clinical investigation, derived from four parental bacterial species: *Lactococcus lactis* (*L. lactis*), *Escherichia coli* (*E. coli*), *Listeria monocytogenes* (*L. monocytogenes*), and *Salmonella enterica* (*S. enterica*).

L. lactis is a member of the Lactic Acid Bacteria (LAB) group. It is considered safe for use in human and animal food due to its lack of acquired antibiotic resistance genes, significant pathogenicity or identified risk factors. The FDA in the USA has qualified the strain as GRAS (Generally Recognized As Safe) and EFSA in Europe has included the strain in the QPS (Qualified Presumption of Safety) list (Koutsoumanis et al., 2023).

E. coli species includes strains with very different pathogenicity. *E. coli* Nissle 1917 (EcN) is a beneficial commensal strain, non-pathogenic and sometimes used as a probiotic. In contrast, enterotoxigenic *E. coli* (ETEC) is a pathogenic strain classified as a class of risk (CR) 2 pathogen for humans and animals in several risk classification lists (e.g., Directive 2000/54/EC), Belgian, German and Dutch lists of risk classification).*L. monocytogenes* and *S. enterica* are both pathogenic bacteria assessed as a CR 2 micro-organism for humans and/or animals (Directive 2000/54/EC, Belgian, German and Dutch lists of risk classification).

The selected CTs are intended to reflect a diversity of GMBs, thereby illustrating the range of potential risks to human health and the environment associated with their use. To this end, CTs involving GMBs derived from both non-pathogenic and pathogenic bacterial species have been included. In addition, the number of trials conducted with a given GMB, as well as the corresponding number of patients enrolled in these trials, were also considered in the selection.

3.1. Mitigation strategies to ensure (bio)safety of the selected GMBs

For the four parental bacteria of interest, genetic modifications involve techniques that focus either on upregulating the gene encoding the key enzyme involved in the biosynthesis pathways of the target product; on lifting the inhibition and/or repression of the key enzyme; or on interrupting the synthesis pathways of by-products (Xu and Zhang, 2016). Like any other living bacteria, the resulting GMBs have the potential to evolve during treatment, which may impact their pathogenic potential or shedding profile (Mahdizade Ari et al., 2024). Their clinical application therefore requires a careful balance between biosafety and efficacy. This balance depends on effective biological containment strategies that ensure functionality within the host, while allowing selective elimination to prevent dissemination into the environment.

In GMBs currently tested in human CTs, biological containment strategies primarily rely on auxotrophy, which means that GMBs are not able to synthesise an essential compound required for their growth or survival. The essential gene is first deleted from the bacterial chromosome and conditional restoration of the bacterium’s capacity to synthesise the missing compound is generally achieved through a plasmid based system, an integrated gene under the control of an inducible promoter or a mechanism dependent on a non-natural cofactor. The reintroduced gene alleviating auxotrophy leads to the maintenance of the plasmid vector even in the absence of external selective pressure. Mitigation strategies for pathogenic strains used as therapeutic tools focus on virulence attenuation such as the use of an attenuated parental strain or the deletion of virulence genes.

3.2. Reported shedding analysis during CTs with the selected GMBs

Based on available data, shedding assessments were reported in approximatively 40% of CTs across all phases; however, results were not available or were not found for around 20% of the CTs in which shedding analyses were stated to have been conducted.

Regarding the bacterial strains from which the GMBs are derived, shedding assessments were conducted in CTs for each of the four selected bacterial strains (Figure 8). Shedding analysis was carried out in 24% of the trials involving *L. monocytogenes*. For the other three bacterial strains, shedding was assessed in approximately half of the trials. Overall, there appears to be a limited amount of data, or publicly available information related to shedding assessments in analysed CTs involving GMBs.

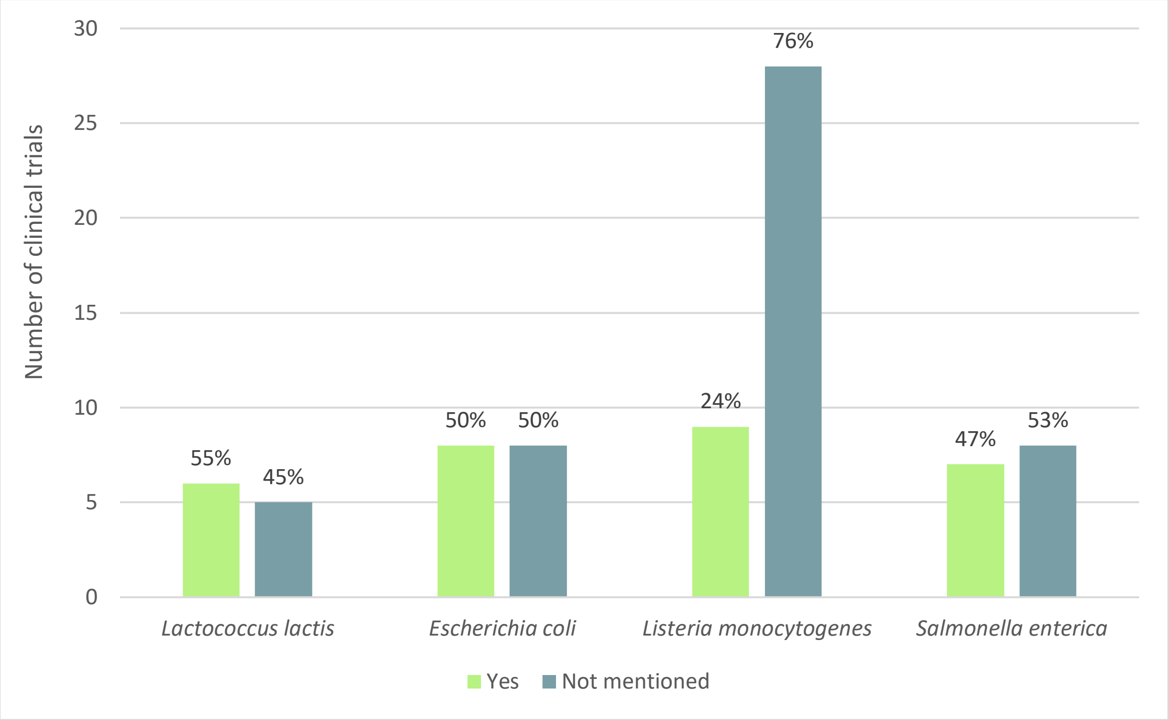

Figure 8: Number of CTs and shedding assessment percentage implying the four main genetically modified bacteria (L. lactis, E. coli, L. monocytogenes, S. enterica).

3.3. Environmental risk assessment of clinical trials involving the selected GMBs

Lactococcus lactis

L. lactis is a non-sporulating Gram-positive bacterial species recognized as a GRAS strain for administration in humans and does not possess endotoxic lipopolysaccharides (LPSs) or other toxic substances. It is commonly found in and added to food products and has a long history of safe use. *L. lactis* is sensitive to a wide array of antibiotics (De Fabrizio et al., 1994). While *L. lactis* is not a natural inhabitant of the human GI tract, it does survive gut passage but does not colonise it (Wegmann et al., 2007).

The strain *L. lactis* subsp. *cremoris* MG1363 is a derivative strain of the natural isolate *L. lactis* National Collection of Dairy Organisms (NCDO) 712 in which all of the five different plasmids were removed (Gasson, 1983; Wegmann et al., 2007). As all plasmids were removed during the isolation of recipient strain MG1363, the strain has essentially lost the capacity to access its main energy and amino acid sources. In consequence, the habitat of MG1363 is confined to artificially supplemented culture conditions.

L. lactis MG1363 strain has been used as vector for GOI expression and tested in several CTs. In the following group of MG1363 based GMBs (AG0xx), the *thyA* gene was replaced with expression constructs in the bacterial chromosome (Table 1). The resulting strains express the GOI while being strictly dependent on thymidine or thymine, which is an essential component for growth and survival (thymidine auxotrophy). No resistance marker is required to guarantee stable inheritance of the GOI and these GMBs are self-limiting because of their thymidine auxotrophy (Steidler et al., 2003). GM *L. lactis* MG1363 has been shown to have the GOI stably integrated for at least 75 generations (Biosafety advisory council, Advice 2008_SC_634). Genetic instability would result in the loss of the GOI expression. Owing to high diversity at *thyA* loci, successful acquisition would probably be possible only from the homologous *L. lactis* subsp. *Cremonis cremonis* (Steidler et al., 2003). In case of the removal of the inserted gene by homologous recombination, the strain would revert to non-modified state which is not pathogenic.

In the context of live bacterial therapy, HGT can occur between orally administered bacteria and native bacteria present in the host microbiota (Munck et al., 2020). Stable integration of the GOI in the bacterial chromosome and the fact that the GMBs do not harbour plasmids or conjugative transposon prevent the HGT of the GOI (Bringel et al., 1991). Phage replication, which facilitates exchange of genetic material among bacteria, is severely hindered as the GMBs are not able to produce thymidine.

The thymidine auxotrophic GM *L. lactis* **AG011** (also called ***LL-Thy12***) expresses human interleukin 10 (hIL-10) and was investigated to treat inflammatory bowel disease. Repeated systemic administration of recombinant hIL-10 has been extensively studied in both healthy volunteers and patients, for different indications including ulcerative colitis and Crohn's disease. Systemic IL-10 treatment is associated with considerable side effects (Fedorak et al., 2000). However, oral administration limits systemic exposure by confining IL-10 expression to the gut, thereby reducing its associated toxicity

AG011 was administered in two ways, capsule for oral intake and an enema formulation for rectal application. Shedding was evaluated in faeces during treatment and 28 days after first administration. The maximum amount of total, live and dead GMB was recovered on day 4 following administration with less than 6.25% of all GMB still alive. Two days after treatment cessation (day 9), qPCR detection was negative, indicating less than 10^4 cells/g stool (Braat et al., 2006). Recovered bacteria were unable to grow without thymidine, confirming effective biological containment.

AG017 is another *L. lactis* MG1363 based GMB expressing hIL-10 orally administered in combination with a gliadin peptide for the treatment of celiac disease. No published data are available concerning the shedding of AG017. Another genetically modified *L. lactis* derived from *L. Lactis* MG1363 (**AG014**) expressing an anti-Tumour necrosis factor (TNFα) antibody for the treatment of inflammatory bowel disease was tested in CT. No adverse effects were observed under the given conditions of administration indicating that this GOI does not have hazardous properties. But as for AG017, data were not found concerning the shedding of this GMB.

The MG1363-derived **AG019**, expresses human proinsulin (hPINS) and hIL-10. Human IL-10 and proinsulin are expressed locally and at low dose to reduce side effects. It is further genetically modified to accumulate intracellular trehalose during fermentation, increasing resistance to bile acids and improving survival in the GI tract. It was administered orally as monotherapy (Phase Ib) and in combination with teplizumab (Phase IIa) for type 1 diabetes. AG019 was detected in faeces in 82% of treated participants at one or more sampling timepoints; one week after last administration dose (d64 after the first dose), AG019 was undetectable in most participants and strongly decreased in others (Mathieu et al., 2024). No AG019 bacteria were detected in blood during treatment or one month afterwards. Teplizumab co-administration did not influence the shedding profile of AG019.

AG013 is a topical oral rinse formulation of a *L. lactis* MG1363 genetically modified to secrete mucosal protectant human trefoil factor (hTFF1) with the aim to reduce symptoms of oral mucositis induced by radiation therapy and/or chemotherapy. Upon topical administration of AG013 no bacteria were not detected in blood but live bacteria were detected in saliva and oral mucosa immediately after dosing and up to 24 hours (saliva) or 6 hours (buccal mucosa). Levels decreased after 90 minutes and were undetectable two weeks after the last dose (Limaye et al., 2013). No AG013 bacteria were detected in faeces.

Administration of GM *L. lactis* MG1363 orally and by rectal application is thus associated with shedding of GMBs in faeces; topical administration through mouth rinse is associated with shedding of GMBs in saliva. Using these administration routes, the GMBs were not detected in blood.

AUP1602-C is based on *L. lactis subsp. cremonis* AUC1000 (also derived from MG1363) with an *alr* gene deletion creating auxotrophy for D-alanine and encoding three human proteins (fibroblast growth factor-2, IL-4, colony-stimulating factor-1) aiming to treat chronic wounds (Kurkipuro et al., 2022). D-alanine is necessary to peptidoglycan and cell wall formation. The plasmid complementing *alr* and encoding the three human proteins is stably maintained for over 200 generations (Bron et al., 2002). Selection based on *alr* expression avoids the selection based on antibiotic resistance. In Phase I trials, AUP1602-C was administered as a topical product on diabetic foot ulcers. The GMB was detected at low levels near the assay limit (1 CFU) in 7% of urine and faeces samples, with no systemic presence (Schindler et al., 2024). Notably, protection of wounds and therefore physical containment of the GMB was foreseen during the treatment period (semipermeable transparent film and a secondary foam layer).

Studies have revealed that oral vaccination with recombinant *L. lactis* expressing whole antigens generated antigen-specific antibodies in mice, demonstrating that systemic humoral immune response has been successfully induced (Gu et al., 2009; Kim et al., 2006). Therefore, GM *L. lactis* has been tested in humans as vaccine against human papillomavirus (HPV). Two human oral vaccines, **NZ8123-HPV16-optiE6** and **NZ8123-HPV16-optiE7**, express codon-optimised E6/E7 HPV-16 oncogenes using the nisin-controlled expression (NICE) system to reduce the potential toxicity of E6 and E7 oncogenes of HPV (Mohseni et al., 2020; Taghinezhad-S et al., 2019). This system allows tight regulation and constant expression of a group of proteins of various functions and origins (Mierau et al., 2005). NZ8123-HPV16-optiE6 and NZ8123-HPV16-optiE7 were genetically modified by introduction of a plasmid containing the genes of interest and a chloramphenicol resistance gene. This marker gene confers resistance to an antimicrobial considered highly important for human health by WHO (https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf) raising concerns about the risk of HGT to exposed bacteria in the GI tract or the environment. There is a consistent lack of information on biological containment if any and on the shedding of these GMBs into the environment. Based on the CTs information available in the literature, the risks associated with exposure of the general population or with environmental dissemination of these vaccine candidates are considered to be low. However, as a precautionary measure and because these vaccines have been administered orally, steps must be taken to limit any direct contact (see the “management measures”).

L. Lactis MG1363					
GMB Name	**Gene(s) of interest**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
AG011	hIL-10	Deletion of *thyA* gene	Chromosome	- Oral - Rectal	in faeces
AG019	hPINS + hIL-10		Chromosome	oral	in faeces
AG013	hTFF1		Chromosome	Topical - oral rinse	in saliva
AG017	hIL-10 (+ gliadine)		Chromosome	oral	No data
AG014	Anti-TNFα antibody		Chromosome	oral	No data
AUP1602-C	hFGF-2 + hIL-4 + hCSF-1	Deletion of *alr* gene	Plasmid with *alr* gene complement	Topical – wound application	in urine and faeces
NZ8123-HPV16-optiE6 NZ8123-HPV16-optiE7	nisin-controlled expression of oncogenes HPV-16 E6 HPV-16 E7	No data	Plasmid with chloramphenicol resistance gene	oral	No data

Table 1 : summary of the characteristics of some L. lactis based GMBs used in CTs

Escherichia coli

Several *E. coli* strains have been genetically modified and evaluated in human CTs (Tables 2 and 3). EcN is a non-pathogenic commensal strain widely used as a probiotic (Mutaflor®) for ulcerative colitis and diarrhoeal disorders. EcN was originally isolated from a soldier who remained healthy during a severe Shigella outbreak in World War I (Sonnenborn and Schulze, 2009). It holds GRAS status, and its complete genome has been sequenced (Reister et al., 2014). EcN fails to achieve sustained colonisation of the human gastrointestinal tract and exhibits a mean residence time of approximately 48 h (Rutter et al., 2022).

EcN has been genetically modified for the treatment of metabolic disorders caused by the absence or underperformance of enzymes that maintain the metabolism of the complex human diet and the excretion of toxic by-products and for the treatment of cancers. Mechanisms of action of these EcN GMBs have been described by Dey and Sankaran (Dey and Sankaran, 2024).

EcN naturally carries the pks island, a cluster of genes that enables the bacterium to produce colibactin, a genotoxin implicated in colorectal cancer mutational signatures in animal models (Nougayrède et al., 2021). Whether EcN is pro-tumourigenic remains unclear (Lynch et al., 2022). In **SYNB1353**, a EcN genetically modified to metabolise the amino acid methionine in the GI tract, the pks island was deleted to prevent colibactin production (Perreault et al., 2024). Although this deletion improves safety, evidence suggests colibactin contributes to some probiotic functions (Massip et al., 2019).

Genetic modification strategies for EcN avoid introducing antimicrobial resistance markers by either integrating therapeutic genes into the chromosome or using plasmids maintained through antibiotic-free mechanisms such as auxotrophic complementation.

Current EcN based GMBs used in CTs exhibit reduced environmental survival due to deletions of *dapA* and/or *thyA* genes. *DapA* encodes an enzyme required for diaminopimelic acid (DAP) synthesis, essential for peptidoglycan formation. Lack of DAP causes cell lysis. Because wild-type EcN (Mutaflor®) is excreted in faeces after oral administration, EcN based GMBs are expected to be released into the environment (Kurtz et al., 2018). Shedding analyses have been performed in multiple CTs with EcN-based GMBs.

SYNB1020 and **SYNB8802** are both EcN based GMBs with deletion of *thyA* gene making them dependent on thymidine or thymine for growth and survival (thymidine auxotrophy). SYNB1020 is further genetically modified to express 2 enzymes (argininosuccinate synthetase, ASS1 and argininosuccinate lyase, ASL) to reduce blood ammonia from the intestine and mitigate disease symptoms in conditions of hyperammonemia. In a Phase II CT, SYNB1020 administered orally was excreted in faeces within 7 days after the final dose (d28) but was no longer detectable (below the qPCR limit of detection) two weeks after the final dose (Kurtz et al., 2019). SYNB8802 is genetically modified by insertion into the genome of genes encoding several enzymes involved in uptake and degradation of oxalate. Shedding of SYNB8802 has been detected in mice faeces, but no shedding data were found in human (Lubkowicz et al., 2022).

SYNB1020 and SYNB8802 do not efficiently colonise the intestine, necessitating repeated dosing because they are eliminated after treatment cessation. This limited colonisation is considered risk mitigating, as any unintended effects would be transient (Gulig et al., 2024). EcN strains are susceptible to antibiotics and are inactivated upon exposure to serum.

SYNB1891 represents a different use of EcN. Unlike other EcN strains, it is injected intratumourally. SYNB1891 is genetically modified through deletion of two genes (*ThyA* and *DapA*) limiting its ability to proliferate in the absence of specific supplementation. It is further modified to stimulate an immune response against solid tumours by activation of the STING (Stimulator of Interferon Genes) pathway, a central innate immune signalling cascade that drives interferon production within the tumour microenvironment (Leventhal et al., 2020). Under hypoxic conditions characteristic of solid tumours, SYNB1891 expresses deadenylate cyclase (DacA) from *L. monocytogenes*, enabling local production of a potent STING agonist and subsequent induction of type I interferons. In a phase I CT testing SYNB1891 alone or in combination with atezolizumab, the GMB was not detected in blood of any patients at any time but was found within the injected tumour with clearance within the first week (Luke et al., 2023). Given the administration route and the absence of GMB in blood, SYNB1891 is not expected to be shed.

Other GM *E. coli* strains derived from pathogenic ETEC strains have been used as oral vaccines.

Wild-type *E. coli* ETEC is a host-adapted pathogen transmitted via the faecal-oral route, causing diarrhea in human by toxin-mediated intestinal secretion. ETEC also infect animals, particularly young livestock (especially post weaning pigs). Human and animal ETEC strains are distinct, so cross-species infection risk is minimal. Person-to-person infection can occur, but the probability of transmission and subsequent infection is low because the infectious dose is high, estimated to be at least 10^8 organisms (Pathogen Safety Data Sheet ETEC).

ACE527 is a live, oral, multivalent vaccine comprising three genetically attenuated ETEC strains. Attenuation involves deletion of all known toxin genes and antibiotic resistance determinants. Mutations in aroC, ompC, and ompF limit the environmental persistence ability of the GMB. Antigen encoding genes (LTB and colonisation factors CFA/I, CS1, CS2, CS3, CS5, CS6) are stably integrated in the bacterial chromosome (Turner et al., 2011). CTs have shown ACE527 to be safe and well tolerated (Darsley et al., 2012).

Data from a Phase IIb CT showed that 97% of vaccinees shed ACE527 in faeces on at least one occasion (Darsley et al., 2012). Vaccine colonisation was self-limiting, with a median time to negative faeces culture postimmunization of less than 1 week after each dose (Darsley et al., 2012). In the phase I CT, the level of shedding of the attenuated vaccine was about 1% of that observed for virulent ETEC strains (Harro et al., 2011). No evidence of reversion to virulence of ACE527 has been reported during CTs. Although the vaccine is attenuated and with limited environmental persistence, the use of live attenuated vaccines still warrants caution in vulnerable individuals.

***E. coli* Nissle**

GMB name	**Gene(s) of interest**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
SYNB1020	Enzymes involved in ammonia metabolism	Deletion of *thyA*	Chromosome	oral	in faeces
SYNB8802	Enzymes involved in oxalate metabolism	Deletion of *thyA*	Chromosome	oral	No data
SYNB1891	*Lm* enzyme DacA	Deletion of *dapA* and *thyA*	Chromosome	Intratumoural	No data

Table 2 : summary of the characteristics of some E. coli Nissle based GMBs used in CTs

***E. coli* ETEC**

Name GMB	**Gene(s) of interest**	**Attenuation**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
ACE527	• heat-labile toxin B subunit (LTB) • colonisation factors	deletion of all known toxin genes	mutations in *aroC, ompC*, and *ompF*	Chromosome	Oral	in faeces

Table 3 : summary of the characteristics of some E. coli ETEC based GMBs used in CTs

Listeria monocytogenes

L. monocytogenes (Lm) is one of the most extensively investigated parental species for GMBs in cancer therapy. Numerous CTs have been completed or are ongoing with Lm-based GMBs, all of which are live attenuated bacteria (Table 4).

Wild-type Lm is a Gram-positive, facultative anaerobic and intracellular pathogen primarily transmitted by ingestion via contaminated food or water. Human-to-human transmission is rare, except for mother-to-child transmission during pregnancy or birth. Lm persists in diverse environments, growing across a wide pH range and at temperatures between 1°C and 45°C (Pathogen Safety Data Sheet *Listeria monocytogenes*) .

Lm possesses strong immunostimulatory properties, can infect antigen-presenting cells, and exhibits tropism for hypoxic tumour microenvironments. It can also deliver genetic material via plasmid release during intracellular infection. These features make attenuated Lm an attractive vector for cancer immunotherapy (Flickinger et al., 2018; Zhou et al., 2018).

ADXS11-001 (also known as ADXS-HPV or Lm-LLO-E7) has been evaluated in twelve CTs including one Phase III study, alone or combined with other treatments (chemotherapeutics or radiation) for HPV-16 related cancers. ADXS11-001 is derived from the *prfA*-defective Lm strain XFL-7, itself derived from streptomycin-resistant wild-type Lm 10403S (Wallecha et al., 2012).

prfA encodes a master activator of virulence genes such as *hly* and *actA* (de las Heras et al., 2011). *prfA*-defective mutants cannot express Listeriolysin O (LLO), required for phagosome escape, and ActA, required for cell-to-cell spread, resulting in a 4–5 log reduction in virulence (Verch et al., 2004).

ADXS11-001 contains a multicopy plasmid encoding a fusion protein of the full-length HPV-16 E7 and a truncated LLO lacking the haemolytic domain while retaining adjuvant activity (Miles et al., 2017). The plasmid also includes a mutated copy of *prfA* gene for plasmid retention, the introduced mutation leading to a weakened activity of the protein. A chloramphenicol resistance gene in the plasmid allows *in vitro* selection. The plasmids were shown to be stable both in vitro and in mice, remaining retained or stably associated with the bacteria regardless of the presence or absence of selection pressure (Wallecha, 2015 - patent ; Wallecha et al., 2009).

ADXS11-001 is administered intravenously. In a Phase I CT blood cultures were transiently positive in one patient after the first dose; all subsequent cultures were negative (Maciag et al., 2009). No urine or faeces samples tested positive. All treated patients experienced treatment related adverse events (AEs), with two requiring immediate antibiotics. One case of systemic listeriosis following ADXS11-001 administration has been reported (Sacco et al., 2016). No other data on shedding and clearance were found.

Several cancer vaccine candidates use the double-deleted Lm chassis LADD (or ANZ-100), derived from wild-type Lm 10403S with deletions of *actA* and *inlB*, preventing cell-to-cell spread and infection of non-phagocytic cells respectively and resulting in a 10,000 fold attenuation compared to wild-type bacteria in mice (Brockstedt et al., 2004; Shahabi et al., 2008). LADD based GMBs include CRS-207, JNJ-809, JNJ-757, pLADD, and ADU-623 and are all administered by the intravenous route.

CRS-207 expresses mesothelin as an ActA fusion protein under the *actA* promoter (Le et al., 2012a). Mesothelin is a glycosylphosphatidylinositol-linked cell surface glycoprotein, highly expressed in several human tumours but with limited expression on the surface of normal tissues. The expression cassette was stably integrated in the Lm genome. The GMB was investigated in eleven CTs alone or combined to other treatments (chemotherapeutics).

In Phase I shedding studies, LADD chassis was not detected in blood, faeces, urine, or sputum, whereas CRS-207 was transiently detected in blood but cleared by day 4 after administration (the last study time point). No faecal or urinary shedding was detected in culture. Later trials did not report additional shedding data.

JNJ-757, expressing EGFRvIII and mesothelin heteroproteins, and **JNJ-809** expressing four relevant prostate cancer antigens, were studied in Phase I and II CTs (Brahmer et al., 2021; Drake et al., 2022). JNJ-757 was transiently detected in blood on day 1 as monotherapy but not when combined with nivolumab. No positive cultures were observed in faeces, urine and saliva samples at any time point after each administration (last on day 15 after the first administration). For JNJ-809, several high-dose patients had transiently positive blood cultures (on day 1 of administration), but all later blood cultures were negative. None of the faecal, urine, or saliva samples tested positive for the GMBs.

CTs are investigating GMBs based on a Lm that has been genetically modified through deletions of the *actA* and *hly* virulence genes and the *dal* (alanine racemase) and *dat* (D-amino acid aminotransferase) genes. These two genes are essential for peptidoglycan synthesis and bacterial cell wall formation. Complementation is achieved by use of a plasmid devoid of antibiotic resistance markers, harbouring a copy of the nonhomologous *Bacillus subtilis dal* gene and carrying the GOI fused to truncated *hly* gene (Wallecha et al., 2009). The GMBs include candidates ADXS31-164, ADXS-504, ADXS31-142, ADXS-503, and ADXS-Neo (ADXSxx). All were administered intravenously; no data on shedding or presence in blood were found.

Overall, available shedding data for Lm-based GMBs remain limited and often come from early phase CTs with a small number of patients. Shedding was not detected in studied samples, although intravenous administration does not exclude the possibility of low level excretion. Co-administration with chemotherapeutics or radiation could influence biodistribution of the Lm based GMB, warranting dedicated shedding assessments.

In CTs involving Lm based GMBs, most patients experienced treatment-related adverse events, including some serious events, indicating that these strains retain residual pathogenic potential. Consequently, clinical protocols incorporate antibiotic rescue therapy at the end of treatment.

Wild-type Lm has a high infectious dose (10^6–10^8 CFU) in healthy individuals but significantly lower in susceptible individuals such as immunocompromised persons. Pregnant women and their unborn children are also a high risk group for *L. monocytogenes* infection as the bacteria can cross the placental barrier. Although it is expected that, in case of excretion, the number of GMBs excreted by the patients will be far below administered doses, repeated dosing may extend the window of potential environmental exposure.

Moreover, wild-type *L. monocytogenes* can infect a wide range of animal species. In the event of accidental exposure to these vaccines (Dhama et al., 2015), livestock such as sheep, cattle, and goats could be susceptible to infection, potentially leading to economic losses. This risk must be assessed, particularly given the high environmental persistence of wild-type L. monocytogenes (Pathogen Safety Data Sheet *Listeria monocytogenes*).

***L. monocytogenes* 10403S**

Name GMB	**Gene(s) of interest**	**Attenuation**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
ADXS11-001	truncated *hly* + HPV-16 E7 cancer antigen	Deletion of *prfA (*including *actA* and *hly)*	/	Plasmid with mutated *prfA* gene and chloramphenicol resistance gene	intravenous	• No shedding • Blood cultures positive
CRS-207	ActA + cancer antigen	deletions of *actA* and *inlB*	/	Chromosome	intravenous	• No shedding • Blood cultures positive
JNJ-757	EGFRvIII + cancer antigen			Chromosome	intravenous	• No shedding • Blood cultures positive
JNJ-809	prostate cancer antigens			Chromosome	intravenous	• No shedding • Blood cultures positive
ADXS31-164 ADXS31-142 ADXS-503 ADXS-504	Truncated *hly* + cancer antigens	deletions of *actA* and *hly*	Deletion of *dal* and *dat*	Plasmid with *B. subtilis dal* gene	intravenous	No data

Table 4 : summary of the characteristics of some L. monocytogenes based GMBs used in CTs

Salmonella enterica

S. enterica comprises multiple subspecies, including enterica, which contains clinically relevant serotypes such as *typhi* and *typhimurium*. It is a Gram-negative, facultative anaerobic, motile bacterium capable of surviving for extended periods in the environment depending on serotype and conditions. Transmission occurs primarily through ingestion of contaminated food or water or through contact with contaminated human or animal faeces (Pathogen Safety Data Sheets *Salmonella enterica* spp.).

In Europe, the most common serotypes of *S. enterica* subsp. *enterica*, including *typhimurium* are pathogenic to humans and animals. *S. typhi* infects only humans. Diseases associated with *S. enterica* are generally curable with appropriate antibiotic treatment. Shedding may continue for prolonged periods; some human carriers can excrete bacteria for years. Animals may also act as intermittent or chronic carriers.

Due to its tumour-targeting abilities and capacity to induce strong humoral and cellular immune responses after dissemination through systemic organs, *S. enterica* is heavily investigated in oncology (Zhang et al., 2025a). GM *S. enterica*, particularly *S. typhimurium* and attenuated *S. typhi* Ty21a, has been explored for cancer therapy (Tables 5 and 6).

Attenuation and biocontainment strategies for *S. enterica*-based vaccine candidates include:

- auxotrophy to limit growth;
- LPS modification to reduce virulence;
- deletion of the *htrA* gene to impair stress responses;
- chemical attenuation;
- facultative anaerobiosis, enabling hypoxia-induced expression of transgenes within tumours.

VXM01 is a GMB derived from the attenuated *S. typhi* Ty21a strain and is used in several cancer CTs. The chassis Ty21a was generated by chemical mutagenesis of *S. typhi* Ty2 strain in the 1970s and is an approved oral typhoid vaccine (Vivotif®). It is characterised by loss of the capsular polysaccharide virulence factor Vi and a mutation of *galE*, which restricts replication through toxic metabolite accumulation. Additional mutations (e.g., *rpoS, ilvD*) contribute to attenuation. It is known that Vivotif® vaccine can be transiently shed in faeces but secondary transmission has not been reported.

VXM01 uses a plasmid carrying VEGFR-2 GOI, a receptor tyrosine kinase overexpressed on proliferating endothelial cells in the tumour vasculature as well as on certain tumour cells (VAXIMM AG, 2019). The plasmid includes a kanamycin resistance gene for selection (Niethammer et al., 2012; Schmitz-Winnenthal et al., 2015). Oral administration of VXM01 has been investigated in four CTs, alone or combined with chemotherapeutics.

Shedding analysis was explicitly included in the objectives of trials but results were only found for one Phase I CT. In this study, transient faecal shedding occurred only at the highest dose; all other blood, faeces, urine, saliva, and tear samples were negative. Reported adverse events were mostly mild. Investigators anticipated VXM01 environmental release and implemented strict containment measures: during the CT, all trial participants were confined on site for 10 days plus three additional days, and all faeces were collected and incinerated (Niethammer et al., 2012).

Ty21a (Vivotif®) has an extensive safety record in humans and the VEGFR-2 GOI in VXM01 is not expected to alter the risk profile of Ty21a. However, in this GMB, the plasmid carries a kanamycin resistance gene that is relevant in veterinary medicine. If shedding is confirmed, exposure and transmission to people and animals cannot be discarded.

VNP20009 is an attenuated *S. typhimurium* strain with *msbB* and *purI* deletions. The *msbB* mutation modifies lipid A, reducing LPS toxicity and TNFα induction (Clairmont et al., 2000). The *purI* deletion establishes purine auxotrophy, thereby promoting tumour-selective growth. Deletions were reported to be genetically stable *in vitro* and *in vivo* in mice. Attenuation is estimated at 50,000-fold in mouse models (Lee et al., 2000). No transgene, plasmids or antibiotic resistance genes were introduced.

VNP20009 was administered intravenously to patients with metastatic melanoma or renal carcinoma (Toso et al., 2002). In this CT, no faecal or urinary shedding was detected. However, prolonged bacteraemia (7–8 days) occurred in three patients, and two additional patients developed later positive blood cultures at 48–96 hours. Some serious adverse events occurred. No more shedding data from other CTs were found.

TAPET-CD is derived from VNP20009 and expresses *E. coli* cytosine deaminase, converting 5-fluorocytosine to 5-fluorouracil (5-FU). 5-FU is then converted into its toxic metabolites blocking thymidylate synthase activity and inducing apoptosis (Nemunaitis et al., 2003). 5-FU is a common chemotherapeutic administered to cancer patients. The GOI is inserted in the bacterial chromosome. In a pilot study of three patients, intratumoural administration was tested, but no shedding or blood presence data were available.

Overall, additional shedding data are needed for both VNP20009 and TAPET-CD. Although purine auxotrophy restricts growth of these *S. typhimurium* based GMBs, the possibility of transient exposure to humans or animals should be further assessed, particularly given that VNP20009 has demonstrated residual pathogenic potential in some patients. Furthermore, domestic animals such as pets are natural hosts of wild-type *S. typhimurium* and could therefore become infected and develop disease if directly exposed to VNP20009.

Saltikva (or Salmonella-IL2) is based on *S. typhimurium* χ4550 with deletions of *cya* and *crp* genes coding for the adenylate cyclase and the receptor of cAMP respectively, resulting in reduced virulence (Leonard et al., 2013 - patent). The additional deletion of *asd* gene required for cell wall synthesis is complemented by a plasmid carrying an *asd* gene. This plasmid also carries the GOI coding for a less active, truncated interleukin-2 (IL-2) to locally stimulate the immune system. No antimicrobial resistance markers are used. In a Phase I CT in metastatic GI cancers, blood and faeces cultures were negative at all time points after oral administration. No vaccine-related adverse events were reported. A Phase II CT is ongoing combining Saltikva with chemotherapy. Limited sample sizes warrant additional data to fully characterise shedding and clearance of this oral vaccine candidate.

***S. Typhi* Ty21a**					
GMB name	**Gene of interest**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
VMX01	VEGFR-2	/	Plasmid with kanamycin resistance gene	oral	in faeces

Table 5 : Summary of the characteristics of some GMBs derived from S. typhi Ty21a and used in CTs

S. enterica						
Name GMB	**Gene(s) of interest**	**Attenuation**	**Biocontainment**	**Location**	**Administration route**	**Shedding**
VNP20009	/	deletion of *msbB*	*purI* gene deletion	Chromosome	intravenous	• No shedding • Blood cultures positive
TAPET-CD	Cytosine deaminase gene	deletion of *msbB*	*purI* gene deletion	Chromosome	intratumoural	No data
Saltikva	Truncated IL-2	deletions of *cya* and *crp*	/	Plasmid with *asd* gene	oral	No shedding

Table 6 : Summary of the characteristics of some GMBs derived from S. typhimurium and used in CTs

3.4. Discussion: ERA of clinical trials involving the selected GMBs

The development of GMBs for medical applications is driven by their unique therapeutic potential. While most scientific publications focus on optimising patient safety and therapeutic efficacy, this report has sought to focus on the potential risks that GMBs may pose to the general population and the environment. Under EU GMO legislation, their use in a CT requires an ERA prior to authorization by the regulatory authorities. A central component of the ERA involves identifying and characterizing possible risks and determining whether measures are needed to protect both the population and the environment.

A crucial step in evaluating risks associated with GMBs is assessing the likelihood of human and environmental exposure during CT. In practice, determining whether GMBs are excreted by CT participants is a major means of estimating this probability.

Among the used parental bacteria, *L. lactis* and EcN are non-pathogenic and hold a GRAS status. Moreover, the derivative strain *L. lactis* MG1363 can survive only under supplemented laboratory conditions. *S. typhi* Ty21a is a commercial vaccine strain (Vivotif®) with a long record of safe use. By contrast, *E. coli* ETEC, *L. monocytogenes* (Lm), and *S. typhimurium* are pathogenic for humans and animals. They transmit via ingestion, have high infectious doses and can persist in the environment for prolonged periods. All three are excreted in faeces, although Lm, being non-enteric, is shed to a lesser extent. Importantly, these parental strains are sensitive to commonly used antibiotics. All therapeutic GMBs derived from these pathogenic species have been genetically modified to attenuate their natural virulence. However, during CTs with Lm and *S. typhimurium* derived GMBs, patients experienced vaccine-related adverse events, indicating that these strains retain residual pathogenicity despite attenuation.

In the Lm based vaccine ADXS11-001, the plasmid carries a mutated copy of *prfA* virulence gene. Homologous recombination could occur between the plasmid encoded gene and gene sequences still on the chromosome, depending on the extent of the *pfrA* gene deletion. The outcome would be the reacquisition of virulence by GMBs, although to a lesser extent than the wild-type strain, since the restored gene is in a truncated form. Homologous recombination could, in principle, also occur in other GMBs complemented by a plasmid carrying an essential gene that has been deleted in the genome. This is the case for the *L. lactis* derived AUP1602-C, Saltikva and an *S. thyphimurium* GMB. In the ADXSxx vaccines, a *B. subtilis dal* complementing gene reduces the risk of recombination with the chromosome.

Approaches to expressing the GOIs vary across GMBs: some integrate the GOI directly into the bacterial chromosome, thereby enhancing genetic stability, while others rely on plasmids, which are more susceptible to HGT. In live bacterial therapies, HGT may occur between orally administered GMBs and native members of the host microbiota. Although all GMBs examined carry non-conjugative plasmids limiting conjugation, potential loss of plasmids from GMBs and subsequent acquisition by gut bacteria warrants further investigation, particularly for those carrying antimicrobial resistance markers.

Oral *L. lactis* based vaccines targeting HPV carry a chloramphenicol resistance gene, which warrants careful biosafety evaluation given that chloramphenicol is considered a highly important antimicrobial for human health by the WHO. Furthermore, recent EMA guidance stipulates that the presence of AMRGs in investigational gene therapy products should be avoided whenever possible, and if unavoidable, must be supported by a comprehensive risk assessment. Because *L. lactis* is not a resident of the human GI tract and the derived GMBs are unable to colonise it, the likelihood of HGT in gut is reduced. By contrast, the Lm–derived vaccine ADXS11-001 also contains a chloramphenicol resistance marker; however, its intravenous administration route minimises exposure of the gut microbiota to the GMB. In the event of release into the environment, the potential for HGT between the GMB and environmental bacteria must be carefully assessed, as such exchanges could facilitate the dissemination of antimicrobial resistance genes (AMRGs). The VXM1 vaccine carries a kanamycin resistance gene on a plasmid. Although kanamycin is used primarily in veterinary medicine, dissemination of this resistance gene to environmental bacteria through HGT represents a potential animal health concern that must be addressed.

Most GMBs express GOI that do not possess hazardous properties. Notable exceptions include the HPV oncogenes E6 and E7 expressed in *L. Lactis* based constructs and in Lm ADXS11-001. In these vaccines, the risks associated with these oncogenes are mitigated through tightly controlled expression systems. Likewise, GMBs expressing human cytokines or growth factors incorporate safety strategies such as the use of truncated, less active variants or restriction of expression to the local administration site.

Among the GMBs evaluated, *L. lactis* derived strains are the most thoroughly studied in CTs with regard to excretion. Following oral delivery, they can be detected in faeces, and after topical administration, in saliva. Although shedding data for EcN derived GMBs remain limited, these strains are expected to behave similarly to their parental organism. Orally administered ETEC based GMBs have been shown to be excreted. In CTs involving these organisms, shedding from treated individuals is therefore plausible. In contrast, shedding has not been observed for Lm or *S. typhimurium* derived GMBs, although available data remain insufficient to draw firm conclusions.

Nonetheless, the biocontainment strategies followed in most GMBs restrict their survival in the environment through deletions of essential genes involved in DNA synthesis or cell wall formation. As a result, these strains depend either on plasmid encoded functions or on microenvironmental factors to survive. Such biocontainment limits the capacity of GMBs to persist or spread beyond the intended site of action and therefore substantially reduce the risk of environmental exposure.

In summary, the overall risk associated with GMBs derived from non-pathogenic *L. lactis*, EcN, and from attenuated ETEC and *S. typhi* Ty21a is considered negligible for human health and the environment. Nevertheless, GMBs carrying AMRGs warrant additional assessment of the potential gene transfer. For GMBs derived from Lm and *S. typhimurium*, the overall risk is considered low owing to their residual pathogenic potential, although mitigated by their transmission characteristics and by the apparent absence of shedding. Accordingly, suitable management measures are required to prevent unintended human and environmental exposure.

3.5. Recommended risk management measures

The primary risk of exposure to GMBs concerns individuals in close contact with treated patients, particularly clinical personnel who handle, administer, or provide care to treated patients. Family members and other close contacts may likewise be at risk if they come into contact with contaminated biological materials such as faeces, urine, or blood. In addition, domestic animals such as pets which are natural hosts of wild-type *S. typhimurium* could become infected and develop disease if directly exposed.

At clinical sites, when direct handling of GMBs is required for preparation (e.g., dilutions, syringe filling) or administration (e.g., intravenous or intratumoural delivery), containment is necessary to prevent exposure of personnel and the environment. Measures include the use of biosafety cabinets to contain aerosols, appropriate personal protective equipment (PPE) such as laboratory coats, gloves, and masks to avoid oral exposure, particularly for bacteria transmitted via ingestion. Needle-stick injuries must be prevented by using hands-free techniques. Work surfaces and hands must be disinfected after each procedure. Close contacts of treated patients should avoid contact with potentially contaminated faeces, urine, or blood.

In CTs involving orally administered GMBs, direct contact with the product is not a concern. However, for strains carrying AMRGs, it may be advisable to chemically inactivate faeces during treatment and until negative cultures are confirmed, or until a more comprehensive assessment demonstrates that no risk to human health or the environment remains.

For treatments administered at home (e.g., *L. lactis* based therapies), household members, especially young children and immunocompromised individuals, must be protected from accidental exposure. GMB products should be stored securely, and any materials that have come into contact with the bacteria must be returned to the clinic for disposal as biohazardous waste. Strict hygiene, including sanitation of toilets and handwashing, is required.

Finally, as for all attenuated bacteria therapeutics, precautions should be taken to prevent exposure of vulnerable populations, especially immunocompromised individuals, young children, the elderly and pregnant women.

4. Preclinical research: ‘state of the art’ and improvements of current GMBs

4.1. Emerging strategies in preclinical development

The CTs involving GMBs described in the first part of this report primarily exploit native microbial traits that have been optimised through adaptive evolution. These include the intrinsic oncolytic activity of certain bacterial species, the probiotic properties of selected strains, and the attenuation of virulence in originally pathogenic bacteria. While available CTs indicate that GMB based therapies are generally well tolerated and show significant potential for the treatment of a wide range of human diseases, several limitations have been consistently identified. These include poor bacterial survival following administration, insufficient or unstable colonisation of target sites required to sustain long term therapeutic efficacy, variability in clinical outcomes, and the need for antibiotic mediated clearance in strains that retain residual pathogenicity, such as *L. monocytogenes* and *S. typhimurium*.

In response to these challenges, current research efforts have focused on refining bacterial strains that have already been clinically evaluated, notably through the optimisation of therapeutic GOIs, the implementation of tighter and more context dependent control of gene expression, and the introduction of additional safety features (Table 7). At the same time, intensive research and development activities are exploring innovative and increasingly complex strategies that integrate concepts from microbiology, synthetic biology, materials science, physics, and even artificial intelligence (AI). These multidisciplinary approaches aim to improve the precision, safety, and robustness of diagnostic and therapeutic applications based on GMBs. This section of the report outlines selected GMB development strategies currently under investigation for their potential use as therapeutic or diagnostic tools and discusses the associated ERA considerations.

Disease model	***Administration route***	***Mechanism***	***Inducer and induction system***	***Gene(s) of interest***	***References***
Constitutive system					
DSS induced IBD mice	Oral delivery	Expression of Il-35	/	Il-35	Zhang et al., 2018 For the description of the mouse model: see Kihara et al., 2003
DSS-induced IBD mice	Oral delivery	Expression of catalase and superoxide dismutase (SOD) to reduce ROS for the treatment of intestinal inflammation; coating with effective biofilms (chitosan and sodium alginate) to improve bioavailability.	/	Catalase and SOD	Zhou et al., 2022
DSS-induced IBD mice	Oral delivery	Expression of (R)-3-hydroxybutyrate for the treatment of colitis	/	3-hydroxybutyrate	Yan et al., 2021
Inducible gene expression system					
In vivo tumor model: subcutaneous MC38 tumor	Intratumoural injection	EcN colonises tumor and converts intratumoural ammonia into L-arginine (L-arginine-producing strain)	Ammonium chloride	*ArgA*^{fbr} for the production of arginine ΔargR deletion of arginine repressor gene (*ArgR*)	Canale et al., 2021
DSS-induced IBD mice	Rectal administration	Expression of a modified curli nanofiber matrix with anti-inflammatory domains (TFFs) to promote *in situ* intestinal epithelial restitution	Arabinose: AraC-P_{araBAD}	trefoil factor (TFF)	Praveschotinunt et al., 2019
Adenoma bearing mice (*Apc*$^{Min/+}$ model of CRC)	Oral administration	EcN colonises adenoma and synthesizes salicylate (non-invasively detected in urine for diagnosis); release immunotherapeutic proteins (anti-PD-L1/CTLA-4, GM-CSF); lysis circuit.	SLIC lysis circuit couples quorum sensing to the expression of a phage lysis gene	production of luciferase; Salicylate, anti-PD-L1/CTLA-4, GM-CSF	Gurbiatri et al., 2024 For the description of the mouse model: Zeineldin and Neufeld, 2013.

Colorectal and mammary carcinoma cells engrafted in mice; Transgenic mice with mammary tumours	Intravenous administration or intratumoural administration	EcN colonises tumors, protected and helped by iCAP in presence of inducer.	IPTG	Theta toxin	Harimoto et al 2022

Table 7 : Examples illustrating current strategies investigated in pre-clinical studies with the genetically modified Escherichia coli Nissle 1917 bacterium for disease treatment and diagnosis. EcN: Escherichia coli Nissle 1917. DSS: dextran sulphate sodium. IBD: inflammatory bowel disease. SOD: superoxide dismutase. ROS: reactive oxygen species. CRC: colorectal cancer. SLIC: Synthetic lysing circuit. TFFs: trefoil factors. iCAP: Inducible capsular polysaccharide. IPTG: isopropyl-β-D-1-thiogalactopyranoside.

4.1.1. Improvements to bacterial strains already evaluated in clinical trials

Investigators are currently evaluating newly identified GOIs in animal models using bacterial chassis that have already been tested in CTs and are therefore well characterised. For instance, to further enhance the therapeutic efficacy of GMBs for the treatment of IBD, additional immunosuppressive cytokines have been expressed, including IL-27 in *L. lactis* and IL-35 in EcN (Hanson et al., 2014; Zhang et al., 2018). As an alternative strategy, direct inhibition of key inflammatory pathways has also been explored through the use of GMBs expressing anti-TNF-α antibodies or nanobodies as novel GOIs (Papamichael et al., 2022; Agus et al., 2021; Lavelle and Sokol, 2020).

In oncology, GMBs that have already been evaluated in CTs but showed limited efficacy due to dose toxicity are also being further refined with the aim of improving their safety profile. For example, the *S. typhimurium* strain VNP20009, which was initially attenuated by deletion of the virulence associated *msbB* gene, has been further attenuated through the downregulation of endotoxin related genes (Mi et al., 2019).

For these GMBs, the same ERA considerations as those identified in the previous CTs apply. In particular, their risk profile for the general population and the environment will mainly depend on the intrinsic pathogenicity of the parental strain, the presence of AMRGs, the nature and function of the GOI, and the potential for shedding of the GMB by the host.

4.1.2. Inducible gene expression systems

The majority of GMBs evaluated in CTs rely on constitutive expression systems, in which the promoter remains continuously active, resulting in sustained expression of the GOI. However, prolonged and uncontrolled production of therapeutic molecules may impose a substantial metabolic burden on the bacterial chassis and can also lead to adverse effects in the host (Zou et al., 2024).

Recent advances in synthetic biology have enabled the development of inducible gene circuits that rely on promoters activated in response to exogenous stimuli, endogenous bacterial signals, or host pathophysiological cues (Niu et al., 2025). Such systems allow for tighter spatial and temporal control of GOI expression. In particular, GMBs can be designed to rapidly and precisely switch between “ON” and “OFF” expression states in response to predefined conditions, thereby improving both the safety and efficacy of their use for diagnostic and/or therapeutic applications. The following sections present different sensing systems currently tested in GMBs in animal models and discussed potential risks arising from their use.

4.1.2.1. Exogenous signals

Diverse exogenous signals have been employed to trigger the expression of GOIs, including both chemical and physical stimuli (see Niu et al., 2025 for a comprehensive review in the context of cancer therapy). Among chemical inducers, small molecule responsive genetic circuits are commonly used. These systems are typically based on operon model in which a repressor protein binds to a specific DNA sequence to regulate bacterial gene expression in response to externally supplied molecules, such as isopropyl-β-D-1-thiogalactopyranoside (IPTG) or L-arabinose.

Despite their versatility, chemically inducible systems present several limitations that may constrain their clinical applicability. These include potential cytotoxicity, off target effects, the emergence of resistance, metabolic perturbations, complex pharmacokinetics and pharmacodynamics, and limited bioavailability. In addition, the difficulty of eliminating residual chemical inducers and the often poor tissue penetration of small molecules complicate the achievement of precise spatial and temporal control over GOI expression.

In addition to strategies based on the detection of chemical compounds, alternative approaches rely on physical stimuli to control gene expression. These include light inducible systems, which combine photosensitive proteins with regulatory genetic elements (Zhang et al., 2022), temperature responsive systems that typically exploit localized increases in temperature through thermosensitive genetic components (Li et al., 2022), and radiation inducible systems that rely on promoters activated in response to DNA damage (Gao et al., 2020).

From an ERA perspective, these approaches are not expected to introduce additional risks to human health or the environment compared with GMBs already evaluated in CTs. The use of external chemical or physical signals allows GOI expression to be controlled in a more localised and transient manner. Consequently, in the event of accidental exposure, GOI expression would be strongly limited and strictly dependent on the presence of the specific inducer. Other potential risks associated with exposure to GMB, whether direct or via shedding, remain comparable to those previously identified and are primarily related to the intrinsic pathogenicity of the parental strain, the presence of AMRGs, and the potential for HGT.

The primary risk associated with these approaches concerns the host and is mainly related to the potential toxicity of the external inducing signal, whether chemical or physical, as well as to the nature of the inserted gene of interest when it encodes a toxic effector molecule. In particular, insufficiently controlled activation of gene expression outside the intended target environment may lead to unintended exposure and adverse effects, thereby raising safety concerns.

4.1.2.2. Endogenous host signals

Endogenous signals derived from the host or the local microenvironment have also been harnessed to drive the expression of GOIs in GMBs. In this context, researchers have genetically modified EcN strain that preferentially colonises tumour tissue and responds to tumour specific metabolic cues by converting intratumoural ammonia into L-arginine. This metabolic rewiring leads to a local increase in L-arginine concentrations, a key determinant of T-cell activation and effector function, thereby promoting enhanced antitumour immune responses *in vivo* (Canale et al., 2021; Chen and Han, 2025).

Bacterial innate sensing mechanisms have been coupled with synthetic gene circuits to transduce specific microenvironmental cues (such as hypoxia, acidic pH, and elevated lactate concentrations) into localised therapeutic responses. These strategies exploit physiological conditions that are characteristic of the diseased tissue but largely absent from healthy organs.

A representative example is provided by hypoxia responsive gene expression systems based on a hypoxia sensing promoter (pPepT for example) and the FNR-like (fumarate and nitrate reduction) transcriptional regulators (Niu et al., 2025). These regulators are naturally present in facultative anaerobic bacteria, including *E. coli* and *Salmonella* spp., and control gene expression in response to anoxic conditions. As tumour tissues are frequently characterised by hypoxia, FNR dependent circuits enable preferential activation of therapeutic gene expression within the tumour microenvironment.

This strategy provides a biological containment, as GOI expression is tightly coupled to tumour associated conditions. As a result, gene expression and functional activity are expected to be minimal or absent outside the tumour microenvironment, including following accidental exposure or shedding in the environment. Consequently, risks remain primarily associated with the intrinsic properties of the bacterial chassis, such as pathogenicity, the presence of AMRGs, and the potential for HGT.

4.1.2.3. Endogenous bacterial signals

Other inducible systems exploit endogenous bacterial signals that are selectively amplified within the disease microenvironment. One of the most widely used mechanisms is quorum sensing (QS), a bacterial cell-cell communication process that regulates population density dependent behaviours through the accumulation of diffusible signalling molecules. When these molecules reach a critical threshold concentration, they interact with specific regulatory proteins to activate target gene expression via QS responsive promoters. In GMBs, QS circuits are frequently coupled to bacterial lysis circuits, enabling the controlled release of the expressed GOI and their clearance.

For example, to achieve precision antimicrobial targeting, EcN has been genetically modified to detect *Pseudomonas aeruginosa* in mouse gut via acyl-homoserine lactone (AHL)–mediated QS signals (Hwang et al., 2017). Upon sensing AHLs, the GM EcN undergoes programmed self-lysis, releasing the bacteriocin pyocin S5 together with a biofilm dispersing enzyme. This design enables highly localised elimination of pathogenic bacteria and disruption of biofilms within the intestinal environment, while limiting off-target effects.

QS based programmable lysis circuits have also been applied in cancer therapy. In these systems, the accumulation of AHL molecules reflects high intratumoural bacterial density and triggers synchronised bacterial lysis, resulting in the localised release of therapeutic payloads such as hemolysin E (HlyE), CTLA-4–blocking nanobodies, and other antitumour effectors. This coordinated lytic response enhances intratumoural drug delivery while simultaneously constraining bacterial proliferation, thereby contributing to effective tumour destruction (Din et al., 2016; Gurbatri et al., 2024). However, achieving the required bacterial density may be challenging in heterogeneous tumour regions, particularly in micrometastases or invasive tumour fronts, potentially leading to incomplete activation of the system.

From an ERA perspective, QS based systems provide a biological containment as GOI expression and payload release by bacterial lysis are conditional upon the attainment of a sufficient bacterial population density within the target site, tumour or infection site. As a consequence, expression is expected to be minimal in non-target tissues or following accidental exposure of the general population and the environment. Residual ERA concerns therefore remain linked to strain pathogenicity, persistence and dissemination, as well as the potential for HGT.

4.1.3. GMBs encapsulation strategy

A novel approach currently under investigation in preclinical studies involving GMBs consists of coating or encapsulating bacterial cells. The primary objectives of this strategy are to enhance GMB viability under complex GI conditions or within specific pathological environments, and to achieve precise spatial and temporal control over bacterial persistence and payload release at lesioned sites. Research on GMB coating is largely inspired by advances in the field of probiotic formulation and delivery (Brittain and Finbloom, 2025; Dey and Sankaran, 2024; Teng et al., 2025; Zhang et al., 2025b). In this context, a wide variety of coating materials have been explored, including natural and synthetic polymers, applied either externally or internally, and in some cases biosynthesised directly by the GMB itself. Brittain and Finbloom distinguish two main coating strategies inspired from the bacterial biology: single-cell coating and biofilm inspired bacterial encapsulation (Brittain and Finbloom, 2025).

Single-cell coating involves covering the bacterial outer membrane with an additional lipid based membrane or capsular polysaccharides. In the context of cancer therapy using GMBs, a representative example is the development of carcinoma cell mimetic bacteria (CCMB), in which the attenuated *S.*

typhimurium strain VNP20009 is coated with tumour cell derived nanoshells (Liu et al., 2022b). In this system, GMBs are first internalised into tumour cells, after which ultraviolet irradiation induces cancer cell apoptosis and the formation of apoptotic bodies that spontaneously envelop the bacteria. The resulting coating masks bacterial immunogenic determinants, thereby improving host safety, while simultaneously enhancing tumour specific accumulation.

In a complementary strategy, Harimoto et al. have genetically modified EcN to be capable of synthesising a natural biopolymer in the form of a capsular polysaccharide (Harimoto et al., 2022). A synthetic gene circuit was introduced to induce capsule biosynthesis upon activation by the exogenous inducer IPTG, resulting in inducible capsular formation (iCAP). The strain was further modified to express the pore-forming theta toxin to kill cancer cells. Following administration *in vivo*, dilution of IPTG leads to cessation of capsule production and gradual loss of the polysaccharide layer. This transient encapsulation enables temporary evasion of host immune surveillance, while subsequent capsule loss promotes bacterial clearance and increases the maximum tolerated dose of the GMB.

From an ERA standpoint, both examples of single-cell coating strategies do not fundamentally alter the nature of the hazards associated with GMB exposure but introduce additional layers of control over bacterial persistence, localisation, and clearance in the host. These approaches may therefore reduce the likelihood and duration of unintended exposure following shedding. While immune evasion strategies may theoretically increase the persistence and dissemination potential of GMBs, the approaches described here rely on transient and context dependent mechanisms, limiting risks for people and the environment in case of exposure. Residual risks remain primarily linked to the pathogenicity of the bacterial chassis, the presence of AMRGs, and the potential for HGT.

A second encapsulation strategy for GMBs currently under investigation is inspired by bacterial biofilms. Biofilms are composed of extracellular polymeric substances, primarily polysaccharides and proteins, which assemble into a nanostructured extracellular matrix that can be regarded as a biological hydrogel. This biofilm encapsulates and protects bacterial communities from adverse environmental conditions, while simultaneously promoting adhesion to abiotic and biological surfaces and permitting the diffusion of nutrients and small molecules. From a therapeutic perspective, biofilm inspired encapsulation therefore represents an approach to enhance GMB viability, functional stability, and colonisation capacity. As with single-cell coating strategies, the encapsulating material may be applied exogenously or biosynthesised by the GMB itself.

In this context, Zhang et al. developed a programmable oral *E. coli* in a hydrogel based on chitosan and sodium alginate, two polysaccharides commonly associated with bacterial biofilms, with the objective to protect GMBs from harsh GI environment and to achieve spatiotemporal control over the production and release of nanovaccines (Zhang et al., 2023). This system incorporates a QS based synchronous lysis genetic circuit that translates bacterial population growth into coordinated cycles of partial *in situ* lysis, thereby enabling periodic and controlled release of the therapeutic payload (BMCH-AH1- A5 nanovaccines).

Another example of GMB self-encapsulation has been described in the context of intestinal barrier protection (Praveschotinunt et al., 2019). In this system, EcN is genetically modified to secrete a specialised fibrous extracellular matrix composed of curli nanofibres covalently fused to trefoil factors (TFFs), which promote epithelial repair and reinforce mucosal barrier integrity. Importantly, expression of this matrix is placed under the control of an arabinose inducible promoter. Consequently, bacterial growth, survival, and therapeutic protein production are strictly dependent on the presence of arabinose.

From an ERA standpoint, biofilm inspired encapsulation strategies may increase GMBs persistence and adhesion, which could theoretically enhance exposure following shedding. However, the incorporation of inducible expression systems and, in some cases, programmable lysis circuits provides contextual and temporal constraints on bacterial activity. As a result, functional activity is expected to be limited outside the target site or in case of shedding. Risks for human health and the environment remain primarily associated with the intrinsic properties of the bacterial chassis, including pathogenicity, AMRGs, and the potential for HGT.

It is important to mention that only a limited number of GMB coating development strategies could be addressed in this report, given the breadth expansion of research in this field. For example, emerging approaches such as the chemical modification of bacterial outer membranes, which were not discussed here, may significantly influence interactions with the host and the environment and therefore warrant dedicated consideration in future ERA.

4.1.4. Emerging strategies for diagnosis developments

In the field of diagnostics, advances in synthetic biology have enabled the development of biosensor molecules that couple therapy with real-time imaging and adaptive responses. These living devices integrate sensing, computation, and reporting functions into whole-cell platforms, enabling real-time, non-invasive detection of disease associated biomarkers such as nitrate, thiosulfate, NO, and calprotectin (Tanniche and Behkam, 2023). For example, to circumvent the need for invasive diagnostic procedures for colorectal cancer, a GM EcN strain has been developed that can both detect and treat colorectal neoplasia (Gurbatri et al., 2024). For lesion detection, EcN selectively colonises adenomas and is modified to produce salicylate, chosen for its role in colorectal cancer chemoprevention and for the feasibility of its non-invasive detection in host urine (adenoma bearing mice) following oral administration of the GMB. This enables a simple, urine based readout of tumour presence. Therapeutically, the same EcN strain is genetically modified to locally express a suite of immunomodulatory agents at the neoplastic site, including the cytokine GM-CSF and nanobodies targeting the immune checkpoint proteins PD-L1 and CTLA-4 (Gurbatri et al., 2024). These effectors collectively enhance antitumour immune responses within the tumour microenvironment. Importantly, this platform integrates its therapeutic GOI with a genome encoded lysis circuit designed to trigger controlled bacterial self-destruction. This circuit based on quorum sensing and a reporter molecule not only maximizes the release of the immunotherapeutic molecules but also provides a biocontainment mechanism by limiting bacterial persistence and proliferation *in vivo*.

Some other advanced designs for diagnostic integrate electronic interfaces with GMBs. For instance, microcapsules containing genetically modified probiotic strains have been coupled to photodetectors and electronic circuitry capable of wirelessly transmitting metabolic signals to external devices (Inda-Webb et al., 2023). Such hybrid bioelectronic systems enable remote sensing and monitoring of bacterial activity *in situ*. The capsule is designed to transit the GI tract intact and is recovered in the faeces after use. As a result, the GMB and genetic material remain physically contained within the capsule, which is easily recovered and thus prevents their release into the external environment.

Additional diagnostic innovations include EcN strains genetically modified to overexpress ferritin within colonised tumours, thereby enhancing magnetic resonance imaging (MRI) contrast for early cancer detection. More broadly, genetic modification allows bacteria to generate a range of alternative signal modalities, including contrast agents for ultrasound imaging and positron emission tomography (PET). These approaches enable localised *in situ* detection as well as real-time monitoring of therapeutic responses.

From an ERA perspective, such technologies primarily affect the functional complexity of GMB based systems rather than introducing fundamentally new biological hazards. The main safety considerations remain linked to the bacterial chassis, the genetic modifications introduced, and the control of gene expression. However, the integration of electronic or imaging functionalities may increase system complexity and should therefore be assessed on a case-by-case basis. Such assessment should verify the robustness and predictability of signal generation and transmission, and ensure that these signals remain confined to the intended clinical context without resulting in unintended exposure or persistence.

4.2. Perspectives

4.2.1. Development of new bacterial chassis

Future directions of research with GMBs not yet tested in animal models point toward increasingly personalised, adaptive, and integrated GMB therapeutics. This is exemplified by recent efforts to genetically modify micro-organisms directly isolated from tumour tissues (Nejman et al., 2020). Because distinct tumour types harbour characteristic microbial communities, tumour resident bacteria are anticipated to exhibit superior biocompatibility and intrinsic tumour targeting capacity compared with conventional bacteria (Goto et al., 2023). For example, *Cutibacterium acnes* isolated from tumour environments has been explored as a potential tumour suppressive agent, leveraging its natural ability to selectively colonise and inhibit tumour growth in a mouse model (Chintalapati et al., 2024).

However, while such personalised cancer therapies develop from customised intratumoural microbiota may enhance the precision, efficacy, and personalisation of microbial cancer treatments. However, these strains may exhibit limited growth robustness, thereby restricting their translational potential or display uncharacterised interactions with the host, including potential toxicity or altered immunogenicity. Moreover, their environmental behaviour, persistence, and capacity for horizontal gene transfer remain largely unknown and would require thorough case-by-case evaluation.

The exploration of new strains will certainly develop, assisted by meta technologies (metagenomics, proteomics) and artificial intelligence (AI). Current GMB platforms predominantly rely on aerotolerant and easily culturable microorganisms, which may not be optimal for treating certain diseases or targeting specific anatomical niches. The exploration of dominant members of the gut microbiome as alternative chassis is particularly relevant, as these species may offer improved adaptation to the human intestine, enhanced colonisation capacity, and more balanced interactions with the immune system. The development of novel fabrication techniques compatible with complex nutritional and environmental requirements, as well as new genetic modification tools enabling the modification of previously intractable microorganisms, will unlock new therapeutic applications.

4.2.2. *In situ* genetic modifications

Another strategy that explores the modification of members of the gut microbiota consists in the *in situ* gene editing by bacteriophages. In the highly complex GI environment, phage based delivery circumvents the need for conjugative bacterial donor strains or *ex vivo* manipulation of bacteria, including electroporation. Genetically modified bacteriophages carrying CRISPR based editors can deliver genetic material directly to resident gut bacteria *in situ*. For example, Brödel et al. (2024) have developed an M13-derived phage capsid encapsulating a base editor, which successfully edited a β-lactamase gene in *E. coli* residing in the mouse intestine (Brödel et al., 2024). A single oral administration resulted in the desired point mutation in approximately 93% of the targeted bacterial population, with the edited strain persisting in the gut for more than six weeks.

Another approach enabling precise and durable *in situ* genome editing of gut bacteria is the recently developed Metagenomic Editing (MetaEdit) platform. This system exploits microbiome mediated delivery of CRISPR associated transposases (CASTs) to stably integrate large DNA payloads into the chromosomes of targeted bacterial strains *in vivo*. In this platform, a non-engrafting *E. coli* donor strain delivers mobile CAST plasmids carrying kilobase payloads into recipient gut bacteria via conjugation. In recipient cells, programmable guide RNAs (gRNAs) mediate strain specific chromosomal integration of the payload, resulting in precise and heritable genomic modifications. Using this approach, *Bacteroides thetaiotaomicron* was genetically modified directly *in situ* by integration of a 7.5 kb metabolic pathway conferring the ability to degrade inulin fibres (Gelsinger et al., 2025). Subsequent dietary supplementation with inulin selectively increased the growth rate of the genetically modified strain by 30 to 40 fold over several weeks. Conversely, withdrawal of dietary inulin led to a rapid decline of the modified population due to competitive exclusion by native microbiota, demonstrating diet mediated control and functional reversibility of microbiome editing. In contrast to traditional phage based editors, which are generally limited to small genetic modifications, MetaEdit enables pathway scale genomic changes in native microbiota that are already well adapted to their ecological niche. This offers an alternative to the use of non-native chassis such as *L. lactis* or EcN, which often face challenges related to long term engraftment and ecological stability. Moreover, the ability to modulate the persistence of genetic traits through orthogonal dietary inputs provides an additional layer of contextual control and reversibility.

While, future development will be necessary to extend the use of MetaEdit to diverse and challenging bacterial communities and environments, including improvements in vector delivery, DNA integration efficiency, and payload persistence.

These developments are accompanied by increased ERA complexity. Stable chromosomal integration of large genetic payloads in resident microbiota raises concerns regarding long-term persistence, genetic stability, and unintended horizontal dissemination of editing components or inserted functions. As such, robust, reliable, and well-validated risk mitigation and monitoring measures will be essential in future applications. These may include the incorporation of genetic kill switches, metabolic auxotrophies, or other stringent biocontainment strategies designed to restrict survival, dissemination, or horizontal transfer of MetaEdit components beyond the intended host and environment.

4.2.3. Artificial intelligence tool

AI and synthetic biology work together to improve the understanding and modification of the human gut microbiome (Kumar et al., 2022). AI plays a key role in analysing the massive metagenomic datasets generated from gut microbes, helping identify microbial signatures, key genes, and metabolic pathways involved in health and disease. In the design of GM bacteria, AI can contribute in identifying genetic targets to modify or introduce, in designing and optimising genetic circuits, predicting their behaviour, and simulating their function in different gut environments. AI also supports experimental automation in synthetic biology and enables the development of personalised probiotics capable of diagnosing pathological states or delivering therapeutic molecules directly in the gut.

While, the use of AI in the design of GMBs does not in itself constitute a direct environmental risk, it may indirectly influence the environmental risk profile of GMBs by enabling the development of more complex, persistent and potentially adaptive biological systems. This increased complexity amplifies uncertainties related to dissemination, environmental persistence, horizontal gene transfer and post-use control, thereby requiring an enhanced ERA supported by robust experimental data.

4.3. Discussion

In many cases, extensive preclinical and clinical data characterise the risk profiles of the parental strains commonly used as chassis for GMBs (see section 3.4.). EcN and *L. lactis* have long histories of safe human use and hold GRAS status, indicating minimal pathogenicity and low environmental concern. In contrast, attenuated *S. typhimurium* and *L. monocytogenes* derive from pathogenic species and therefore retain a small but residual risk, particularly for immunocompromised individuals and in uncontrolled environments. Nonetheless, decades of research and multiple clinical evaluations provide an understanding of their attenuation, safety measures, and biocontainment needs.

Certain preclinical studies rely on chassis previously evaluated in CTs, with a well-established safety profile, to achieve persistent expression of the GOI independent of external conditions. The expressed GOI encode therapeutic proteins which do not confer toxic effects. Therefore, from an ERA perspective, these GMBs remain broadly comparable to their parental strains.

However, current preclinical studies employ genetic circuits for targeted delivery of GOIs into the host and introduce novel therapeutic transgenes with potential toxic effects. The control of gene expression by signal responsive gene circuits or by encapsulation strategies limit the toxicity. Moreover, lysis circuit may enable the selective destruction of GMBs under predefined conditions, thereby enhancing their biosafety. The GOI typically encode therapeutic proteins, sensing modules, or regulatory elements, none of which confer increased virulence, environmental persistence, or transmissibility. Accordingly, from an ERA perspective, these GMBs remain broadly comparable to their parental strains, provided biocontainment measures and genetic safeguards remain genetically stable.

In contrast, emerging strategies based on *in situ* genome editing of resident gut bacteria using bacteriophage derived delivery vehicles or MetaEdit introduce a distinct set of biosafety considerations. In these systems, phage particles or GMBs introduce genetic editing machinery directly into endogenous bacterial populations without deploying exogenous GMBs. This approach raises important questions regarding the potential shedding of phage transduced bacteria or conjugated bacteria into the environment. If *in situ* GM bacteria are excreted, their survival, persistence, and interaction with environmental organisms must be assessed, especially since, unlike classical GMBs, these edited bacteria do not inherently possess biocontainment systems such as auxotrophies, kill switches, or lysis circuits.

Moreover, the risk profile of *in situ* genome editing is strongly dependent on the nature of the edits. Edits that alter metabolic pathways, surface antigens, antimicrobial resistance, or ecological competitiveness could, in principle, modify the behaviour of edited bacteria in ways that differ from both the native microbiota and conventional GMB chassis. Consequently, when considered from an environmental risk perspective, the following aspects warrant careful consideration and thorough assessment:

(i) the likelihood of edited bacteria being shed. The potential release of these edited bacteria in the environment may occur given that humans excrete substantial quantities of bacteria daily in feces. Although most bacteria are unlikely to survive for extended periods outside the GI environment, certain taxa may persist transiently (e.g., *E. coli*, *Enterococcus*);

(ii) the persistence of edited bacteria into the environment, and

(iii) the possibility of horizontal transfer of edited traits within microbial communities. Notably, bacteriophages developed by Brödel are reported not to contain AMRG and are designed to minimize dissemination, thereby limiting the potential spread of CRISPR associated sequences beyond the target bacterial populations (Brödel et al., 2025).

4.4. Conclusion

The here reported preclinical research and development studies show that the increasing combination of multiple strategies, such as environmental or host sensing systems, genetic kill switches, encapsulation technologies, and the convergence of diverse disciplines including synthetic biology, microbiology, materials science and artificial intelligence, significantly increase the system complexity. While these approaches may enhance functionality and safety, they can also complicate risk characterization and reduce the predictability of biological behaviour. Consequently, ERA for these advanced GMBs are unlikely to be generalisable and will need to be conducted on a case-by-case basis, supported by robust experimental data.

A further important observation is that many of the strategies described remain at the preclinical stage of development. Several systems are not yet fully optimized and continue to rely on classical genetic tools, including selection markers based on antibiotic resistance genes. The presence of such elements raises concerns regarding horizontal gene transfer and environmental impact, and highlights the need for further technological refinement before clinical deployment.

This report focus on the ERA aspects associated to GMB for therapeutic use. Consequently, the scientific literature primarily discuss advancements in clinical development and optimised preclinical testing with a primary emphasis on demonstrating improvements in therapeutic efficacy and patient safety. Scientific literature in this domain rarely focus on potential environmental risks or impact on human health directly. While advancements in the patient safety of the GMB are expected to benefit the safety for human health at large, no general conclusion can be drawn regarding the impact that advancements in patient safety or therapeutic efficacy may have on ecosystems. This underscores that the environmental release of GMB, if any, warrants rigorous, multidisciplinary evaluation to assess any environmental risk.

In parallel, the use of AI in the design and optimization of GMBs introduces new dimensions of uncertainty and governance challenges. While AI does not constitute a direct environmental risk, its role in shaping biological complexity, it is suggested that its use should at least be explicitly considered within existing regulatory frameworks. In the longer term, dedicated guidance or regulatory provisions addressing AI assisted biological design may be necessary to ensure transparency, traceability and adequate risk management.

Taken together, these observations emphasise that the protection of human health and the environment should be embedded throughout the entire lifecycle of therapeutic GMB development. Safety considerations should be integrated from the earliest design stages, through preclinical and clinical development, and into eventual deployment. Early identification of potential hazards and proactive risk mitigation represent a more effective strategy than reliance on downstream containment measures or post hoc evaluation alone. Integrating ERA considerations during the innovation process is essential to reduce uncertainties related to environmental dissemination, persistence, genetic stability, and unintended interactions with host associated or environmental microbiota.

From a legal perspective, the *in situ* genetic modification of bacteria belonging to the human microbiota may raise questions regarding its classification under European legislation. Although microbiota associated bacteria possess genomes distinct from that of the human host, their permanent residence within the human body as well as their crucial role in host health challenges the traditional distinction between host and non-host genetic material. In the absence of explicit legal definitions, such interventions could be interpreted as indirect forms of genetic intervention in humans, thereby triggering regulatory frameworks applicable to gene therapy. Clarification at the European level may therefore be required to position or distinguish microbiota targeted genetic interventions from gene therapy.

Overall, a precautionary, lifecycle based and integrative approach to safety, combining early risk identification and multidisciplinary assessment will be critical to ensuring that the development of next generation GMBs delivers therapeutic benefits while maintaining a high level of protection for human health and the environment.

5. General conclusion

Overall, the evaluation of GMBs currently under clinical development indicates that these organisms present a favourable environmental and human health risk profile when appropriate biosafety measures are applied. Most GMBs rely on non-pathogenic or attenuated parental strains with well-characterised safety records and incorporate genetic safeguards that restrict survival, replication, and dissemination outside the host. When shedding occurs, it is transient and detected at low levels, and the likelihood of environmental persistence or transmission remains low. Nevertheless, data gaps persist, particularly regarding systematic shedding assessments, the behaviour of GMBs derived from pathogenic species, and the potential for HGT involving constructs that contain antimicrobial resistance markers.

Emerging technologies such as tumour resident microbial chassis and *in situ* genome editing of host bacteria introduce new ERA considerations that will require refinement of existing risk assessment frameworks. Embedding these considerations early in the developmental process enables proactive risk mitigation and reduces uncertainties related to dissemination, persistence, genetic stability, and unintended biological interactions.

Continued monitoring of shedding, genetic stability, biocontainment performance, and potential exposure pathways remains essential as GMB applications expand. With rigorous containment strategies and harmonised ERA methodologies, GMB based therapeutics and diagnostics can be deployed safely while enabling significant advancements in precision medicine.

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