Engineered *Akkermansia muciniphila*: A promising agent against diseases (Review)

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Abstract. Achieving a harmonious gut microbial ecosystem has been hypothesized to be a successful method for alleviating metabolic disorders. The administration of probiotics, such as *Lactobacillus* and *Bifidobacteria*, is a known traditional and safe pathway to regulate human commensal microbes. With advancements in genetic sequencing and genetic editing tools, more bacteria are able to function as engineered probiotics with multiple therapeutic properties. As one of the next-generation probiotic candidates, *Akkermansia muciniphila* (*A. muciniphila*) has been discovered to enhance the gut barrier function and moderate inflammatory responses, exhibit improved effects with pasteurization and display beneficial probiotic effects in individuals with obesity, type 2 diabetes, atherosclerosis and autism-related gastrointestinal disturbances. In view of this knowledge, the present review aimed to summarize the effects of *A. muciniphila* in the treatment of metabolic disorders and to discuss several mature recombination systems for the genetic modification of *A. muciniphila*. From gaining an enhanced understanding of its genetic background, ingested *A. muciniphila* is expected to be used in various applications, including as a diagnostic tool, and in the site-specific delivery of therapeutic drugs.

Contents

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1. Introduction

With increasing economic development, chronic non-communicable diseases have emerged as a substantial global concern due to common risk factors, such as unhealthy diets and environmental pollution (1,2). Although a range of pharmacological and surgical interventions are constantly being devised to address the increased numbers of cases of non-communicable diseases, the side effects and contraindications of certain medicines or radiotherapy have limited the number of patients that are able to receive such treatments (3,4). Furthermore, the unavoidable postoperative complications such as surgical site infection, abscess, active bleeding, hematoma and anastomotic leak, resulting from surgery may worsen a patient's state (5). Therefore, researchers have begun to consider other possibilities to cope with this global problem (6,7). The term dysbiosis refers to the major changes in the gut microbial ecosystems that contribute to a range of metabolic disorders, including obesity, type 1 and type 2 diabetes and inflammatory bowel disease (8-11). Numerous other types of disease like autism or allergies have also been associated with an imbalance in gut microflora composition (12,13). Several strategies to normalize human gut microbial ecosystems are available to treat different syndromes, including fecal microbiota transplantation, which has demonstrated promising results (14). At present, probiotics are commonly used to improve the intestinal environment (15-17).

To further determine the relationship between the gut microflora and a healthy human state, the use of metagenomics and metatranscriptomic sequencing techniques has been proven to provide a compositional snapshot of the microbial species in the gut, as well as to sequence their expressed genes (18,19). With the elucidated genetic background of different gut microflora, it also provides a wide range of possibilities to modulate the gut microflora composition genetically for greater therapy. Advances in synthetic biology have extended this therapeutic potential, as selected bacteria can be tailored to deliver drugs or molecules to act directly on the host (20). An increasing abundance of human-associated bacteria have been identified to provide health benefits, including *Lactococcus lactis* (*L. lactis*), *Escherichia coli* (*E. coli*) and *Bifidobacterium*, making them desirable engineering targets for therapeutic application (21-27). Similar efforts may also be applied to *Akkermansia muciniphila* (*A. muciniphila*), a microbial

species that has been proposed as a novel candidate for probiotic therapy (28). In the present review paper, the potential of *A. muciniphila* as an engineered bacterium was discussed by first reviewing other engineered bacteria. The review covered its colonization sites in human intestines, therapeutic effects and probiotic characteristics, prior to identifying potential avenues for modification.

2. Commonly engineered bacteria used for the treatment of diseases

The most commonly engineered bacteria provide a platform to develop probiotics as a novel direction in therapeutic studies. In the following sections, some of these cases are discussed in further detail. By noting similarities in their therapeutic effects, characterization status and the strategies used for their modification, the current review aimed to demonstrate why *A. muciniphila* is being considered for similar engineering approaches.

E. *coli*. *E. coli* is an inhabitant of the human gastrointestinal tract; its well-characterized genome, accessible and versatile plasmid vector, susceptibility to genetic manipulation and high recombinant protein synthesis rates renders it one of the most desirable hosts for the expression of recombinant proteins (29,30). Since recombinant human insulin was first produced in *E. coli* by Genentech in 1978, numerous genetic engineering strategies have been developed for *E. coli*, providing a genetic circuit model for the subsequent genetic manipulation of bacteria (31). For example, by deleting the arginine repressor gene of *E. coli Nissle* (EcN) and integrating a feedback-resistant arginine synthase into the intergenic region controlled by the fnrS promoter, Kurtz *et al* (24) generated the SYNB1020 clinical candidate for the treatment of hyperammonemia. In addition, Whelan *et al* (32) ligated a functional nematode gene into the pMu13 plasmid and transformed it into EcN; in the EcN, the expressed nematode cystatin, reported to have anti-inflammatory properties, decreased the inflammatory monocyte/macrophage migration and positively affected the epithelial barrier function in both mice and piglets. The introduced genetic material was able to overcome the defense barrier of the host cell and was stably maintained as a plasmid with the aid of selectable markers and a compatible origin of replication, or by integration into the genome (24).

Lactobacillus (LAB). LAB is a commensal intestinal microbiota species with widespread use in the production of fermented foods (33,34). By virtue of its numerous health-promoting effects in humans and its decoded genetic sequence, LAB has become one of the most convincing engineered probiotics (35-37). In 2015, Yang *et al* (27) constructed the recombinant strain *LAB plantarum* (*L. plantarum*) NC8, which expresses angiotensin-converting enzyme inhibitory peptides (ACEIPs) for prolonging antihypertensive effects; the recombinant expression vector pSIP409-ACEIP was built by replacing the gusA gene in the pSIP409 plasmid with genes encoding ACEIPs. Through incubating its DNA with available methyltransferases *in vitro* to match the host's DNA methylation patterns, the transformation efficiency of *L. plantarum* was raised to a level comparable with that of *E. coli*. This vector was subsequently transformed into *L. plantarum* NC8. An antihypertensive effect was noted following the oral administration of the engineered strain to spontaneously hypertensive rats, as evidenced by a reduction in abnormal systolic blood pressure and in triglyceride, endothelin and angiotensin II levels. For further consideration, the expression levels of the integrated genes should be monitored and regulated (38).

Different promoter-repressor systems have been constructed for the induction of recombinant protein expression in *L. plantarum* to evaluate their stability and efficiency (39). An increasing number of systems have emerged, such as the quorum-sensing system, chemical-based induction system and temperature-sensitive system, which enhanced the abilities of microbes to sense, respond to and record their local environment, as well as improving the ability to evaluate and control the expression levels of the desired genes, which are designed to produce the required product (40-42).

Bifidobacterium. Of all the commensal bacteria inhabitants in the mammalian gut, bacteria of the *Bifidobacterium* genus represent some of the most prevalent probiotic species, which have been used to prevent or treat colorectal cancer, diarrhea, necrotizing enterocolitis and IBD (43-48) In view of these prominent therapeutic characteristics, molecular genetic studies are of crucial importance. Among the *Bifidobacterium* genus, *Bifidobacterium longum* (*B. longum*) was identified to exert more significant positive effects on the gut environment compared with others (49). The complete genome sequence of this strain has been deciphered and it frequently used in genetic manipulation. As it was discovered to selectively grow in the hypoxic regions of solid tumours, genetic modifications to *B. longum* for cancer therapy have been proposed (45). In a previous study, the tumstatin gene was inserted into a plasmid and electrically transformed into the *B. longum* NCC2705 strain, which generated an anticancer effect in tumor-bearing mice by inhibiting the apoptotic vascular endothelial cells of the transplanted tumours (50). A similar strategy was employed in other *B. longum* strains, enabling them to express more anticancer drugs (51-53). These achievements demonstrate the strength and utility of engaging the immune system at the level of the intestinal mucosa using ingested microbes.

Commonly engineered pathogens. Foodborne pathogens, such as *Salmonella typhimurium* (*S. typhimurium*) and *Listeria monocytogenes* (*L. monocytogenes*), have also been engineered using an attenuation operation for therapeutic purposes (54). Examples of attenuation strategies include interrupting the transport of lipids, purines and/or metabolites (54). A previous study developed an attenuated *S. typhimurium* strain, VNP20009 DNase I, which contained defective adenine and lipopolysaccharide metabolism genes, and a plasmid with a humanized toxin DNase I sequence inserted; the results indicated that the combination of VNP20009 DNase I and triptolide significantly reduced tumor volume, prolonging the survival of mice (55). A similar strategy has been adopted for modifying the *L. monocytogenes* strain for use as a vaccine for different types of disease; for instance, the administration of the Lmdd-multiple peptide fusing genes (MPFG) strain, which was based on a vaccine against hepatocellular carcinoma (HCC) (56),

created an antitumor response towards the human leukocyte antigen (HLA) epitopes of MPFG (HLA-A0201), presenting a potentially feasible strategy for the prevention of HCC (57). Biocontainment and biosafety are crucial factors in the clinical application, to avoid the harm that engineered pathogens like *S. typhimurium* and *L. monocytogenes* cause, thus the attenuation of these strains to lower the expression levels of pernicious genes is a critical step. At present, to achieve greater control and safety, kill switches and genetic firewalls have been added into genetic circuits (58).

3. Next generation of engineered bacteria: *A. munciphila Akkermansia*

Overview of A. muciniphila. A. muciniphila was first isolated from a fecal sample in anaerobic medium containing gastric mucin (its sole energy source) in 2004 by Derrien *et al* (59). *A. muciniphila* was discovered to directly bind to enterocytes to enable colonization, while its degradation of mucin was identified to stimulate mucin production and increase mucin thickness, thereby strengthening epithelial integrity (60). In addition, metabolites, mainly short-chain fatty acids, produced by *A. muciniphila* were found to be absorbed in the colon and serve as an energy source for colonocytes, and they also exhibited potential therapeutic and anti-inflammatory effects in various types of metabolic disorder, such as obesity, IBD, and diabetes (61-63), as illustrated in Fig. 1. Moreover, the effects of some exposed active molecules of *A. muciniphila* have been demonstrated to remain after pasteurization; for instance, as Amuc_1100 is heat-stable, it is able to replicate almost all of the effects of live *A. muciniphila* or inactivate the inhibitory compounds for live *A. muciniphila* (64,65).

A. muciniphila in metabolic disorders and other types of disease.

Obesity. Globally, the prevalence of excess weight between the years 1980 and 2013 has increased to 27.5% in adults and 47% in children, with 2.1 billion people in the world classifying as overweight (BMI >25 kg/m^2) and over 500 million being classified as obese (BMI >30 kg/m^2) (66). Obesity has become a worldwide health concern, with current medical and lifestyle interventions largely failing to offer adequate solutions. Increasing evidence has indicated that probiotics are involved in gut barrier maintenance and inflammation normalization, suggesting that their adoption could eventually result in a long-term treatment for obesity (67,68).

In recent years, *A. muciniphila* has been proposed as a potential probiotic for the treatment of obesity, as significantly decreased levels of *A. muciniphila* were observed in obese or overweight individuals (69,70). Everard *et al* (71) demonstrated that administering a daily dose of live *A. muciniphila* to mice with diet-induced obesity significantly lowered their body weight and sanguineous lipopolysaccharide levels (71). However, this treatment was reported to increase fat mass development and alter adipose tissue metabolism. Similarly, a study of overweight and obese insulin-resistant volunteers indicated that oral supplements coated with pasteurized *A. muciniphila* normalized the mean adipocyte diameter and lowered plasma leptin concentrations (72).

Type 2 diabetes. The prevalence of diabetes has increased in parallel with the global rise in obesity, with type 2 diabetes accounting for >90% of all cases of diabetes (73-75). Both obesity and type 2 diabetes have been associated with changes in nutrition and more sedentary lifestyles, thus adopting *A. muciniphila* interventions for the treatment of diabetes has been hypothesized to exert similar therapeutic implications (10,76). Previous studies reported that prediabetic patients and patients with type 2 diabetes had lower amounts of *A. muciniphila* in the gut compared with healthy individuals (77,78). The relationship between *A. muciniphila* and type 2 diabetes was also insinuated following metformin treatment, which induced high levels of *A. muciniphila* in a previous study (79). Notably, Depommier *et al* (72) observed more significant improvements to insulin sensitivity and reductions in insulinemia following the use of pasteurized, instead of live, *A. muciniphila*.

Atherosclerosis. Atherosclerosis is a pathological condition underlying adverse vascular events (80). Previous studies have identified that the gut microbiota contributes to atherosclerosis by controlling the direct invasion of the host, the activation of the innate and acquired immune system and alterations in metabolism. Thus, *A. muciniphila* has also been suggested for the treatment of atherosclerosis (81-83). Li *et al* (84) fed germ-free atherogenic mice lacking apolipoprotein E with *A. muciniphila* and revealed that the oral gavage of *A. muciniphila* significantly impeded atherosclerotic lesion growth by decreasing the intestinal permeability and inhibiting the proliferation and migration of macrophages; these effects persisted in spite of *A. muciniphila* pasteurization.

Autism-related gastrointestinal disturbances. Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder in which gastrointestinal disturbances are commonly reported (85). Through the analysis of fecal samples, Wang *et al* (86) reported a decreased abundance of *A. muciniphila* in children with ASD and their siblings, as well as a thinner gastrointestinal mucus barrier compared with control subjects. Other previous studies have also indicated that intestinal barrier impairment was aggravated in children with ASD and their immediate relatives, suggesting that *A. muciniphila* may guide the implementation of dietary interventions to reduce gut permeability in individuals with ASD.

Other diseases. In the majority of the studies discussed, when supplied in a viable form, therapeutic effects of *A. muciniphila* were noted for metabolic disorders. However, such treatment could also extend to other diseases. For example, in cancer treatment, *A. muciniphila* employment was suggested to enhance the effects of immunotherapy (87,88). The fecal matter of patients with cancer with positive responses to immunotherapy has been studied for *A. muciniphila*, as an abundance of the bacteria can reflect the state of immunotherapy (87). In addition, *A. muciniphila* was also reported to exhibit protective effects in immune-mediated diseases, including atopic diseases, IBD and liver damage (65,89,90). The association between *A. muciniphila* and immune-mediated diseases was explained using whole transcriptome analysis of intestinal tissue samples, which indicated that *A. muciniphila* regulated

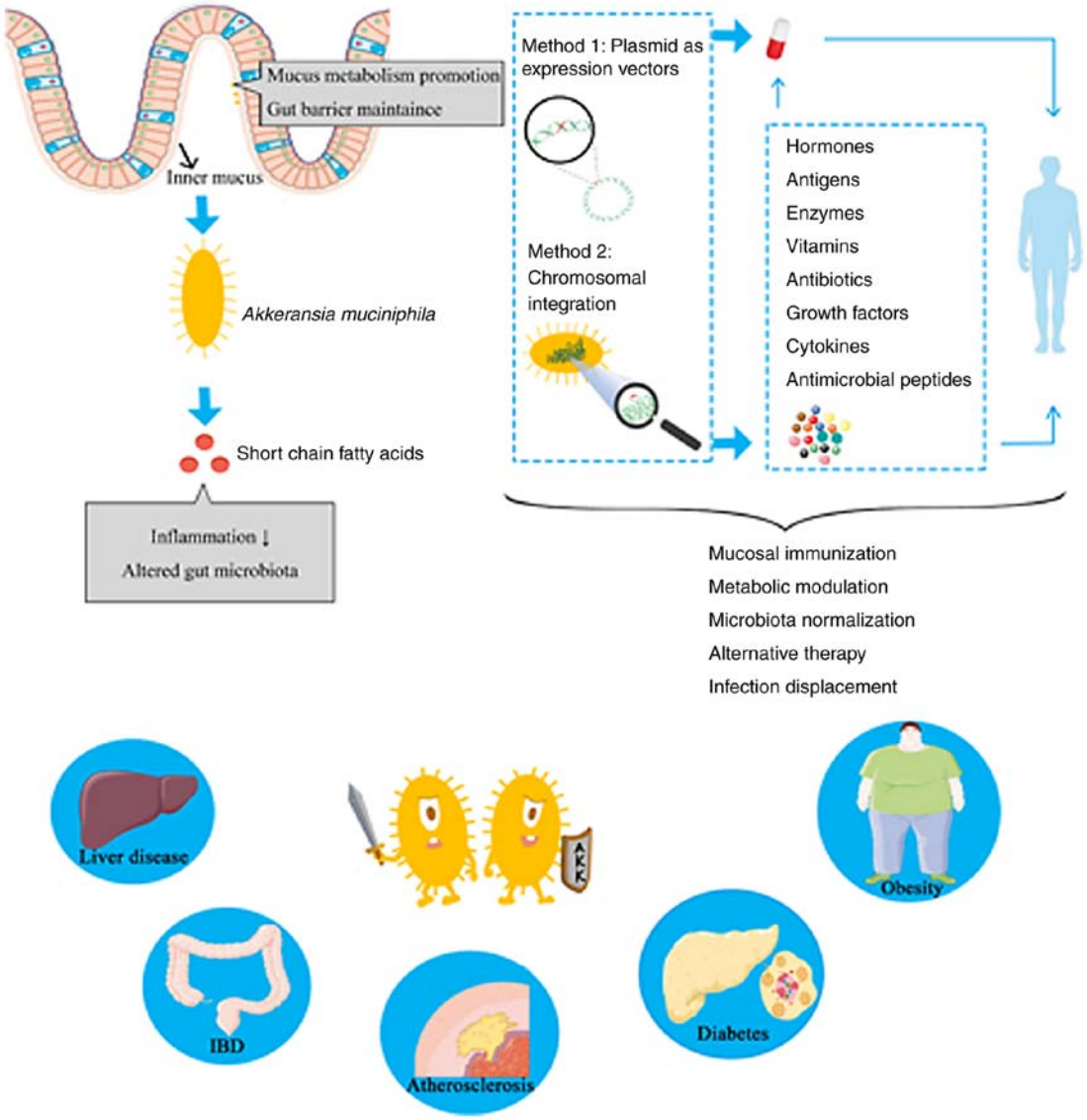

Figure 1. Probiotic effects and applications of *A. muciniphila* therapeutics. *A. muciniphila* makes use of gastrointestinal mucin to produce anti-inflammatory short chain fatty acids, which can serve as energy sources for colonocytes and other commensals. In addition, the mucin metabolism can stimulate mucin production and increase mucin thickness, thereby strengthening the epithelial barrier. Hence, *A. muciniphila* has been demonstrated as a promising therapeutic agent against obesity, diabetes, atherosclerosis, liver diseases, IBD and other diseases. *A. muciniphila* is expected to be converted into an engineered bacterium, either by chromosomal integration or designed plasmids, to further exploit its probiotic effects. Different benefits relating to mucosal immunization, metabolic modulation, microbiota normalization, precision therapy and infection displacement can subsequently be conferred depending on the oral supplement. *A. muciniphila*, *Akkermansia muciniphila*; IBD, inflammatory bowel disease.

the expression of the majority of the genes associated with immune responses (90-94).

Evidence for the viability of engineered A. muciniphila. The advent of next-generation sequencing and whole-genome sequencing has provided additional scope for more bacteria to be genetically modified. Based on this, the prospects for engineering *A. muciniphila* are promising.

The genome of *A. muciniphila* BAA-835 was first sequenced in 2011, from which *A. muciniphila* was predicted to synthesize all 20 canonical amino acids, as well as important cofactors and vitamins (95). In 2015, genes from the *A. muciniphila* strain Urmite were assigned to strain ATCC BAA-835, suggesting that the majority of these genes were involved in metabolic reactions (96). Recently, 39 new *A. muciniphila* strains were sequenced and analyzed, with several gene flow and recombination events being noted, indicating the development of a feasible background for future genetic engineering studies (97).

Moreover, an efficient and scalable workflow for the cultivation and preservation of *A. muciniphila* cells has been developed, resulting in viable *Akkermansia* colonies with high yields and very high stability, as well as up to 97.9±4.5% survival of >1 year when stored in glycerol-amended medium at -80˚C (98). The growth of *A. muciniphila* can be monitored and controlled by various quality assessment and control procedures to ensure that viable cells of *A. muciniphila* are available. In addition, although *A. muciniphila* is an anaerobic bacterium, it has demonstrated an ability tolerate and even benefit from nanomolar concentrations of oxygen in liquid medium (99). These properties extend the possibility of *A. muciniphila* to be manipulated for engineering (Fig. 1).

Potential genome editing tools for engineering A. munciphila. In general, plasmids are the first tool considered when genome editing is required. Plasmids contain appropriate DNA as the bacterial origin of replication, an antibiotic resistance cassette and the gene of interest, which is transcribed from a prokaryotic promoter (100,101). Adequate expression of the therapeutic gene or genes is ensured by using appropriate promoters and other regulatory elements (100,101). In previous years, the genetic toolbox of plasmids has been greatly expanded by adding sensors, regulators, memory circuits, delivery devices and kill switches (102). Once the recombinant plasmid carrying the desired gene tracks down signal molecules secreted by target cells or tissues, it releases therapeutics locally, and is subsequently self-digested as programmed to avoid any infection (103,104). After construction, plasmids are converted to the hosts by chemical, mechanical or physical techniques, with mammalian cell 'poration' systems (electroporation and sonoporation) being the most important and common techniques used (105-107).

In addition, extra genome integration in a chromosome of the host cell has been discovered to support the development of engineered bacteria (108). Normally, a designed homologous single-stranded DNA donor is provided based on the introduction of a site-specific double-strand DNA break (DSB) into the locus of interest (109). Information encoded on this template can be used to repair the DSB, resulting in the addition of the desired gene at the site of the break (109). Recombination systems carried by helper plasmids are crucial during this process (109-112). In the following sections, several mature recombination systems developed in *LAB* or *E. coli* are described, which could be applied to *A. muciniphila* once limitations relating to species differences have been eliminated.

Nisin-controlled gene expression (NICE) system. The NICE system is one of the most widely used tools for chromosomal integration exploited for engineering *Lactobacillus*. It is constructed for gene expression based on nisA and nisF promoters via a two-component regulatory system consisting of the histidine protein kinase, nisK, and the response regulator, nisR (113-116). When a gene of interest is placed behind the inducible promoter, PnisA, on a plasmid and transformed into a nisRK strain, the expression of the cloned gene can be activated by the addition of nisin (Fig. 2). Using the dual plasmid system, the classic NICE system can be successfully introduced into the majority of bacteria. For example, Mohseni *et al* (117) genetically engineered *L. lactis* using a NICE system with pNZ8148 to express the native and codon-optimized recombinant E7 [E7 is a good candidate protein for vaccine development against human papillomavirus (HPV)-related cervical cancer] oncogenes isolated from HPV; the results for the overall production of E7 by *L. lactis* NZ9000 containing codon-optimized E7 was >2.7-fold higher compared with NZ9000 containing the native E7 strain. The findings also indicated that the amount of recombinant E7 oncoprotein accumulation depended on the concentration of nisin added, with the highest concentration achieved in the presence of 10 ng/ml nisin for both recombinant *L. lactis* strains. However, the exposed drawback of the system was that its basal expression was leaky; therefore, it may not applicable for production of the desired proteins or for the expression of toxic proteins (118).

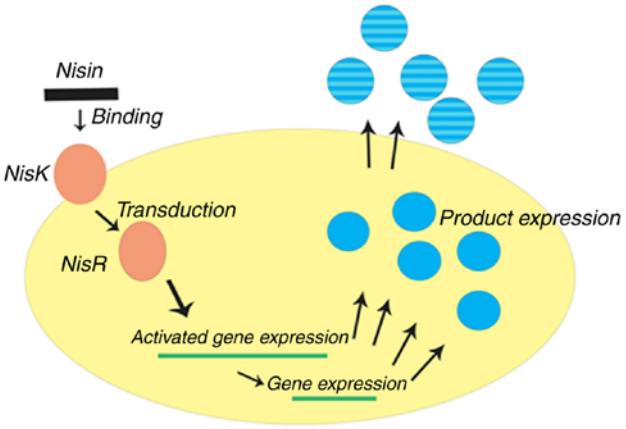

Figure 2. NICE system regulates gene expression. NICE, Nisin-controlled gene expression.

λ recombination system. The bacteriophage λ Red homologous recombination system has been studied over the past 50 years as a model system for the transfer of chromosomal DNA from species (119). The λ recombination system, designated 'Red,' consists of two proteins; α, an exonuclease that acts on double-stranded (ds)DNA, and β, a single-stranded (ss)DNA binding protein capable of annealing complementary ssDNA strands (120). Red-mediated recombination is assisted by the γ protein, which increases α and β activity on linear dsDNA by inhibiting *E. coli* RecBCD exonuclease (121,122). In the past, NICE restricted the integration of molecular weight DNA into the host strain; however, the new lambda Red recombinase-mediated integration strategy was found to transform higher molecular weight DNA of variable lengths into any non-essential locus in the host chromosome (123). Juhas and Ajioka (124) successfully integrated 15 kB DNA encoding sucrose catabolism and lactose metabolism and transport operons into the flsu locus of the flagellar region 3b in the *E. coli* K12 MG1655 chromosome; this approach preferred the use of overlapping DNA fragments for integrating the high molecular weight DNA. Elongation of the integrated DNA sequence is facilitated by the alternative use of kan and cat-yfp cassettes tagged in different DNA fragments, which is less time-consuming compared with the standard lambda Red recombinase-mediated integration (124). Under monitoring, this new strategy did not reveal any negative effects on the host strain. However, compared with *E. coli*, to the best of our knowledge, there are fewer reports regarding the use of this technique on other strains.

CRISPR-Cas system. In the CRISPR-Cas system, the small CRISPR RNAs encoded by CRISPR spacer sequences form a duplex with a trans-activating CRISPR RNA. The duplex with the Cas9 protein subsequently searches the presented DNA for a Cas-specific sequence (Fig. 3) (125-127). Upon recognition of the specific sequence, Cas9 induces the targeted DSB, enabling the modification of a target gene sequence through host bacterial DNA repairing systems (128,129). The presence of a homologous template ensures the insertion of the addition in the region of the DSB. CRISPR-based technologies have been implemented for *E. coli*, *Streptococcus pneumoniae*,

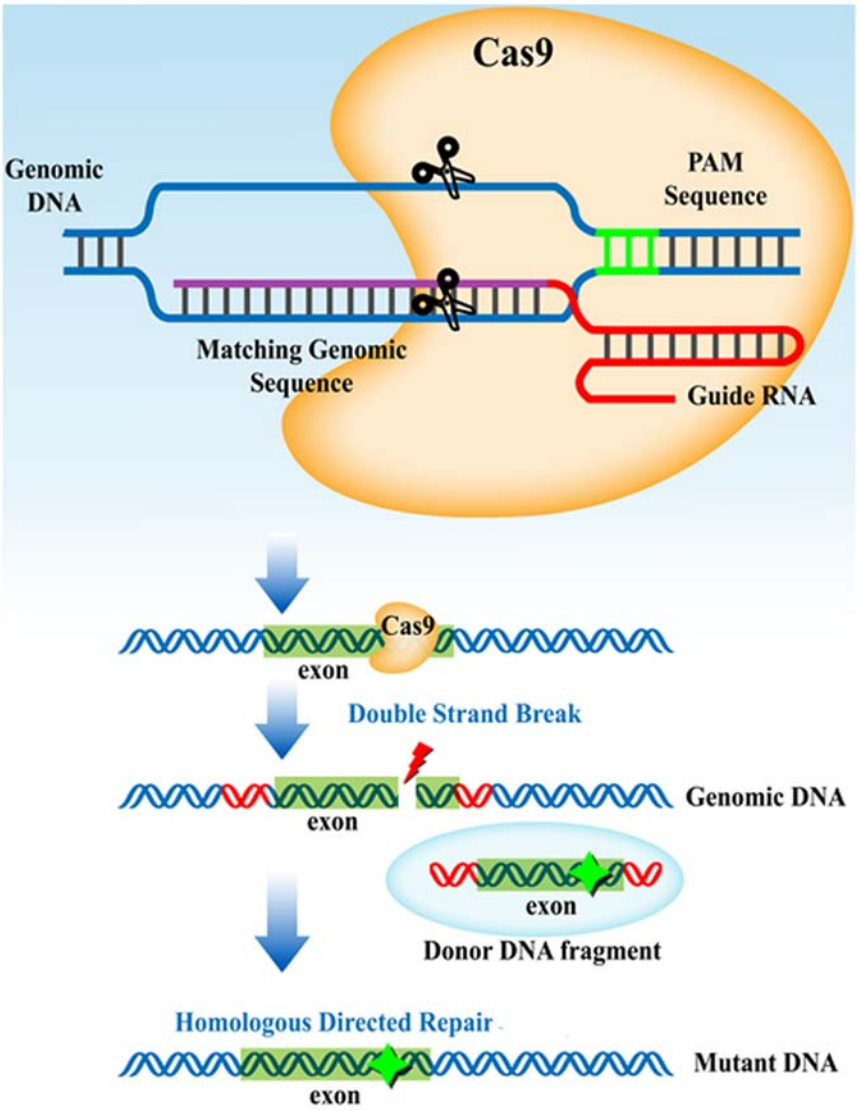

Figure 3. CRISPR-Cas9 regulates gene expression. The designed small CRISPR RNAs with the Cas9 protein subsequently searches for the Cas-specific sequence. Upon recognition of the specific sequence, Cas9 induces the targeted double strand break, initiating the homologous directed repair. The CRISPR targeting specificity is determined both by CRISPR RNAs and the binding between the Cas9 protein; and a short DNA motif commonly found at the 3' end of the target DNA, called the PAM. PAM, protospacer adjacent motif.

L. lactis and probiotic *LAB* species for the production of pharmaceutical products and precursors of high industrial significance (130-132). Various types of CRISPR are currently used for producing desired strains with therapeutic potentials. Δ-integration CRISPR enables strains to have multiple loci chromosomal integration, whereas CRISPR-based homology-directed repair allows site-specific integration (133). A catalytically inactive form of Cas9 (dCas9), has been developed to direct the promoter or coding regions to prevent transcription rather than cleaving the DNA, known as CRISPR interference (78). This technique has been used to control gene expression in *Corynebacterium glutamicum*, in which it was employed to downregulate multiple genes by concatenating single guide RNA sequences encoded on one plasmid (134). Genomic sequencing of the *A. muciniphila* strain determined the CRISPR loci, suggesting that the *A. muciniphila* system initiates the CRISPR defensive mechanism frequently and can be modified using CRISPR-Cas9 (95). An automated pipeline named CRISPR discovery has since been developed for the identification of CRISPR repeats and Cas genes in genome assemblies, to determine the type and subtype and to describe system completeness (135). With this knowledge, it is hypothesized that an endogenous CRISPR-Cas9 system can be developed for *A. muciniphila*, allowing it to avoid the host's immune system.

4. Conclusion

As illustrated in Fig. 1, *A. muciniphila* is a potential probiotic that binds to enterocytes for colonization, which can regulate the host's metabolism and immune response. It has been revealed to be a promising therapeutic for the treatment of obesity, type 2 diabetes, atherosclerosis, autism-related gastrointestinal disturbances and other types of disease (Fig. 1). Due to an increased understanding of how its genetics relate to its pathogenicity, as well as the

techniques required for effective culturing and preservation, *A. muciniphila* is expected to find use as one of numerous engineered bacteria. The present review described the potential of *A. muciniphila* as an engineered bacterium for the modulation of metabolic pathways and the production of desired proteins of therapeutic value, with high yields (using promoters, enhancers and terminators), and introduced several mature recombination systems that could be used for its genetic modification. Based on the deployment of other strains used in the aforementioned procedures, it was suggested that ingested *A. muciniphila* may be programmed to interact with signals secreted within its environment and respond to information. Thus, it could be applied to treat metabolic imbalances, pathological conditions in tissues and to assist postoperative recovery. Apart from its use as a diagnostic tool, it is feasible that *A. muciniphila* may also be designed for site-specific delivery of therapeutic compounds based on genetic circuit modulation. In addition, given that *A. muciniphila* has the capability to inhibit regulatory pathways that control immune responses, it may also be remodeled for application in vaccinations. Takei *et al* (136) successfully modified *B. longum* to express full-length antibodies against chronic hepatitis C virus infections in a murine model, demonstrating the ability to engaging the immune system using engineered commensal microbes.

However, despite its encouraging prospects, further studies of engineered *A. muciniphila* are still required. Currently, engineered bacteria are more frequently applied in animal or preclinical models, thus further clinical trials are required to check their efficacy and risk ratio. These clinical trials should include the following: i) Several control groups using the same dosage of normal bacteria for comparison; ii) randomization and double blinding for causality; and iii) statistical analyses for significance (137). However, Lawenius *et al* (138) discovered that pasteurized *A. muciniphila* did not protect against ovariectomy-induced bone loss. Thus, *A. muciniphila* treatment may not be as good as initially expected.

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Authors' contributions

TC conceived the idea for the review and designed its framework. YZ conducted the research and wrote the manuscript. Both authors edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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