

# Exploring a transformative model for preventing hypercholesterolaemia: directed evolution of E.coli Nissle 1917 to obtain gene combinations with superior cholesterol-regulating effects

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## ABSTRACT

Hypercholesterolemia is one of the major influencing factors of Cardiovascular disease(CVD). Traditional lipid-lowering drugs such as ezetimibe have unavoidable drug side effects. The method of preventing hypercholesterolemia by reducing cholesterol absorption through intestinal microbiota, and then preventing CVD, may become a transformative model. Based on this, the LZU-China iDEC team is trying to use the *IsmA* gene that can directly metabolize cholesterol, the *BCoAT* gene that affects cholesterol absorption, and the *BSH* gene that promotes bile acid excretion to construct engineering *E.coli* Nissle 1917(ECN). We selected and optimised our effector gene modules by means of directed evolution to obtain combinations of genes with better cholesterol-regulating effects, and attempted to screen the optimal set of strain designs by means of directed evolution. We validated the function of the oleic acid inducer under both aerobic and anaerobic conditions, and proposed a design for directed evolution of the oleic acid inducer.

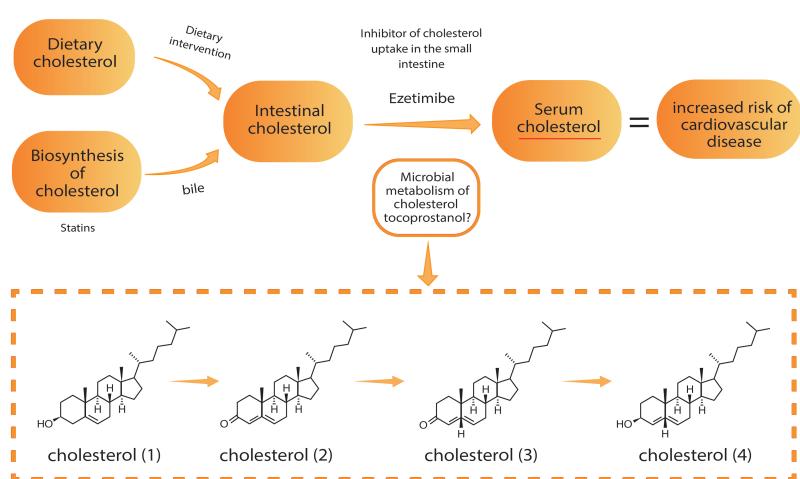
**Keywords:** Directed evolution, Hypercholesterolaemia, Cholesterol, *E.coli* Nissle 1917, Disease prevention

## Introduction

### Background and overall objectives

Since Sol Spiegelman's first in vitro evolutionary experiments in 1967, directed evolution has become one of the most powerful tools for achieving protein engineering and *function*<sup>1</sup>. Directed evolution is becoming an increasingly comprehensive field of study, and its concepts are becoming more widely understood and *accepted*<sup>2</sup>. The essence of directed evolution is to construct a molecular diversity library and screen mutants with improved traits from the library. Afterwards, the genes that can encode the improved traits are used as templates for the next round of evolution, and the required genes or enzymes and other requirements are finally obtained through multiple rounds of *repetition*<sup>3</sup>. At the same time, the concept and scope of directed evolution are also expanding, not only the research that fully conforms to the design of directed evolution is increasing. Due to the feasibility, scientific validity and universal applicability of the concept of directed evolution, the researches influenced by the concept of directed evolution and designed based on it are also increasing. Cardiovascular disease has been a serious threat to public health for a long time and is one of the common causes of death in the world<sup>4</sup>. Excessive cholesterol deposits in blood vessels, affecting blood flow and oxygen

transport, and increasing the risk of cardiovascular and cerebrovascular *diseases*<sup>5</sup>. High cholesterol is not only associated with the risk of diseases such as atherosclerosis, hypertension and coronary heart disease, but also adversely affects people with liver disease, Alzheimer's disease and *diabetes*<sup>6</sup>. Statins, also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, act on the liver and can not only effectively reduce total cholesterol (TC) and low-density lipoprotein (LDL), but also reduce triglyceride (TG) and increase high-density lipoprotein (HDL) to a certain extent<sup>7</sup>. Ezetimibe acts in the intestine and reduces intestinal absorption of cholesterol by selectively inhibiting intestinal cholesterol transfer *proteins*<sup>8</sup> ((Figure 1)). Although the existing drugs have good efficacy, they also bring inevitable side effects. In recent years, more and more studies have shown that probiotics are able to affect the synthesis, absorption and efflux of cholesterol<sup>9–11</sup>, and the use of probiotics to regulate cholesterol levels in humans is a practical strategy. The aim of this study is to make an attempt to construct an engineered ECN using the IsmA gene, which is capable of directly metabolising cholesterol, the BCoAT gene, which encodes the butyryl CoA-acetyl CoA transferase enzyme that affects cholesterol uptake, and the BSH gene, which promotes the excretion of bile acids. Afterwards, through the concepts and methods of directed evolution to select and optimize the effector gene modules to obtain the combination of genes that have a better cholesterol-regulating effect, and screened for the optimal strain design set. In addition, based on the concept of directed evolution, we proposed a directed evolution design scheme for the oleic acid inducer and made a preliminary attempt. This will make our product have the universality to be promoted to more people with the potential to produce corresponding effects in reality.

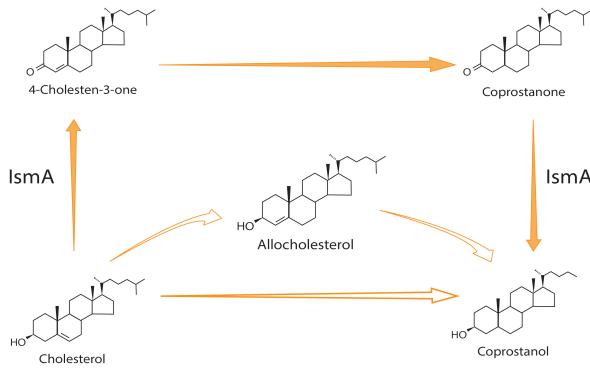


**Figure 1.** Serum cholesterol levels are important for human health and can be regulated by a number of factors, including the potential metabolism of cholesterol by gut microbes. Gut cholesterol levels are influenced by dietary and host-derived cholesterol.

Interventions with dietary changes or the use of statins can affect gut cholesterol levels, whereas the use of ezetimibe can block intestinal cholesterol uptake. Metabolism of cholesterol by gut microbes may also reduce intestinal absorption of cholesterol, leading to lower serum cholesterol levels. In the microbiota, the microbial pathway for conversion of cholesterol (1) to faecal prostaglandinol (4) involves the intermediates cholestenone (2) and faecal prostaglandinone (3).

### IsmA

Direct degradation of cholesterol is a potential mechanism for microbial regulation of cholesterol levels, which can be converted to coprosterols by bacteria in the gut, resulting in lower concentrations in the cholesterol pool and lower serum cholesterol levels. Coprosterols are cis-derivatives of cholesterol that are barely absorbed by the gut. Based on activity assays and multi-omics analyses, the intestinal sterol metabolism A gene (IsmA), which is homologous to *E. coprostanoligenes*, encodes a microbial cholesterol dehydrogenase. This enzyme catalyzes the conversion of cholesterol to 4-cholest-3-one and coprostanone to coprostanol. It is the only enzyme known to convert cholesterol to coprostanol (Figure 2).

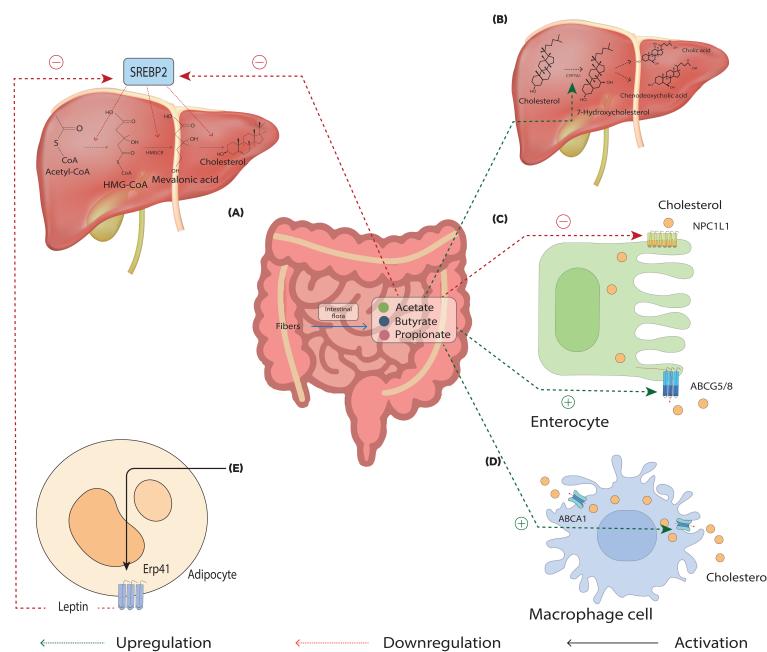


**Figure 2.** The gut microbiota-catalyzed transformation of cholesterol to coprostanol

Three proposed pathways for the transformation of cholesterol to coprostanol are shown, including indirect catalysis via 4-cholest-3-en-3-one and coprostanone, indirect via allocholesterol and direct reduction of cholesterol. The confirmed reactions are shown as a unbroken line. The hypothesized reactions are shown as a broken line. The intestinal sterol metabolism A gene (IsmA) has been identified from *Eubacterium coprostanoligenes* and unculturable bacteria found in the human gut, while other enzymes and mechanisms for these pathways remain unknown.

### BCoAT

Butyric Acid (BA) is one of the Short-Chain Fatty Acids (SCFAs) produced by intestinal flora, which mainly exists in free form. A large number of butyric acid-producing microorganisms inhabit the gastrointestinal tract of both humans and animals. It has been confirmed that butyrate has many important physiological functions, such as substance metabolism, promoting intestinal tissue development, and enhancing body *immunity*<sup>12</sup>. The BCoAT gene, namely butyryl-coa:acetateco-transferase (BCoAT) gene, is closely related to BA *synthesis*<sup>13</sup>. SCFAs are important metabolites produced by probiotics that regulate cholesterol *levels*<sup>14</sup>. It has been shown that supplementation of SCFAs to hamsters fed a high-cholesterol diet significantly reduced the total cholesterol content in their serum, suggesting that SCFAs are effective components in lowering cholesterol *levels*<sup>15</sup>. There are five main mechanisms by which SCFAs lower cholesterol levels (**Figure 3**) .

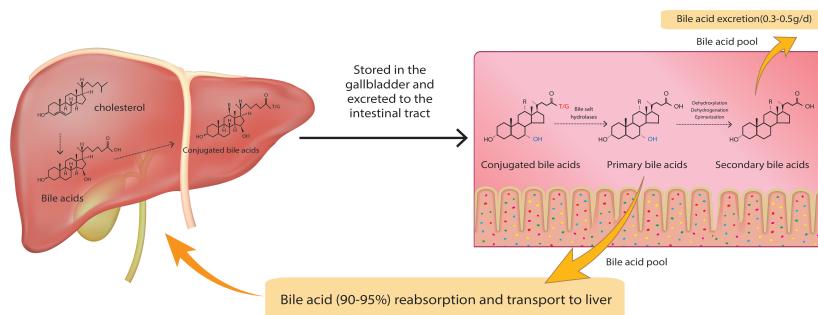


**Figure 3.** Proposed mechanisms by which short-chain fatty acids (SCFAs) reduce cholesterol levels

(A) SCFAs produced by fermentation of fiber by gut microbes decrease the expression of genes in the cholesterol synthesis pathway via SREBP2 regulation. (B) SCFAs also increase the expression of CYP7A1, which accelerates the transformation of cholesterol to BAs. (C) SCFAs also mediate cholesterol absorption in intestinal cells through upregulation of ABCG5/8 expression and downregulation of NPC1L1 expression.(D) In macrophage cells, SCFAs increase the expression of ABCA1 to enhance cholesterol efflux. (E) SCFAs also activate GPR41 in adipocytes to produce leptin, which further suppresses the expression of SREBP2. Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG5/8, ATP-binding cassette transporters G5 and G8; BA, bile acid; CYP7A1, cholesterol  $7\alpha$ -hydroxylase; GPR41, G protein-coupled receptor 41. NPC1L1, Niemann–Pick C1-like 1; SREBP2, sterol regulatory element-binding protein 2.

## BSH

The conversion of cholesterol to BA accounts for about 90% of the daily production of cholesterol and is the most important process in cholesterol catabolism. The two major BAs, Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA), bind to taurine or glycine in the liver and are released into the duodenum to promote lipid metabolism<sup>16–18</sup>. The bound BA is further hydrolyzed by microbial Bile Salt Hydrolases (BSH) in the small and large intestine to produce amino acids and primary BA, which initiates BA conversion in the intestine. BSH can reduce cholesterol levels in the host through two mechanisms (**Figure 4**) : (1) micelle formation in the small intestine; (2) increase BA excretion by feces. In the small intestine, BA forms micelles with cholesterol, fatty acids, and glycerol monoesters. The unconjugated BA produced by BSH is more hydrophobic and soluble than conjugated BA, which reduces their passive influx into enterocytes. Thus, increased BSH activity may have a cholesterol-lowering effect by reducing dietary cholesterol absorption and increasing BA excretion<sup>19</sup>. The enhanced BA excretion can further shrink the BA circulating pool in the gut and promote the conversion of cholesterol to BA, which further reduces host cholesterol levels<sup>20</sup>.



**Figure 4.** The process of cholesterol conversion to bile acids (BAs) and the function of bile salt hydrolases (BSHs) in the process

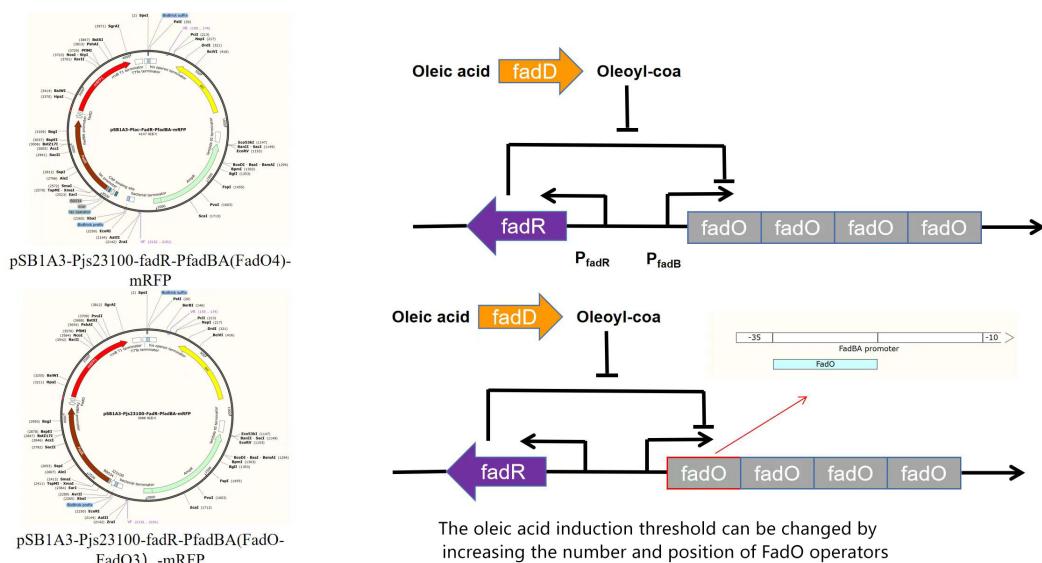
In the liver, cholesterol can be converted into primary BAs. These are conjugated with either taurine or glycine and transported to the small intestine, where conjugated BAs are hydrolyzed to produce primary BAs. The primary BAs can be further converted to secondary BAs by microbial catalyzed reactions. During the transformation from cholesterol to secondary BAs, microbial BSH catalysis is the key step. Increasing the abundance of BSHs in the gut accelerates BA excretion, which hastens the entire process of conversion of cholesterol to BAs. Abbreviation: CYP7A1, cholesterol  $7\alpha$ -hydroxylase.

## Theoretical rationale for the effectiveness of genetically engineered probiotics

In recent decades, advances in宏量生物学 techniques have opened new avenues for understanding how the microbiome can improve human health, of which the link between the microbiome and cardiovascular disease is one of the most widely studied<sup>21</sup>. A study using hypercholesterolemic mice showed that antibiotic-depleted gut microbiota led to elevated cholesterol uptake and synthesis in hepatocytes<sup>22</sup>. It was also shown that transplantation of gut microbiota into human mice with elevated plasma cholesterol levels resulted in an increase in cholesterol levels<sup>22</sup>. This suggests that the gut microbiota can regulate cholesterol metabolism and therefore it is feasible to reduce cholesterol levels by interventions involving the gut microbiota, such as fecal microbiota transplantation, antibiotics, probiotics, and prebiotics.

Probiotics were defined in 2014 by the International Scientific Society for Probiotics and Prebiotics as "living microorganisms that, when administered in sufficient amounts, confer health benefits to the *host*"<sup>23</sup>. At present, probiotics have been widely accepted by the public as functional foods or supplements. Certain probiotics containing *Lactobacillus* and *bifidobacterium* species can be used to reduce cholesterol levels in the *host*<sup>24</sup>. Associations between probiotics, gut microbiota, and CVD have been reported. With the rapid development of genetic engineering in recent years, it is possible to engineer probiotics to deliver exogenous therapeutic factors into the body to play their roles more accurately and efficiently<sup>24</sup>. For the programmed host, although a variety of microorganisms can be used as chassis organisms, the *E. coli* strain is a more tractable host, thanks to its clear genetic background and the availability of well-established tools for heterologous expression of a large number of proteins. Among them, *E. coli* Nissle 1917 (ECN) is widely used in targeted therapy of gastrointestinal diseases and tumors because it does not carry any pathogenic factors. Engineered ECN can promote the expression of GLP-1, inhibit the appetite of mice and improve their fat metabolism, thereby reducing the body weight of mice.

Although the great potential of using engineered bacteria to treat diseases is gradually realized, the use of engineered bacteria to regulate human cholesterol level is still rarely reported. Little is known about the mechanism of cholesterol degradation by microorganisms, except that *E. coprostanoligenes* is the only strain that can degrade cholesterol directly. The regulation of cholesterol by microorganisms through the production of SCFAs and BSH is an indirect effect, which may be limited by the patient's own metabolic conditions, and its therapeutic effect may be different. Therefore, in this study, the *IsmA* gene, *BCoAT* gene encoding butyryl-CoA-acetyl-coa transferase and *BSH* gene were used to construct an engineered ECN to directly degrade cholesterol and indirectly regulate cholesterol metabolism. At the same time, alginic nanochitosan was used to coat the strains to reduce their wastage during passage through the GI tract. In order to make cholesterol regulation precise and controllable, the oleic acid inducer was selected as the functional gene control switch. There are two main genes in the oleic acid inducer: *Fad R* and *Fad B*. The expression product of *Fad R* can inhibit the expression of *Fad B* promoter by binding to the operator *Fad O* on *Fad B*. Oleic acid, a common metabolite in the intake of high cholesterol diet, can release the repression of *Fad B*, which provides the possibility of achieving precise cholesterol regulation. Furthermore, based on the characteristic that the activation threshold of oleic acid inducer changes with the position and number of *Fad O* operators (**Figure 5**), this study attempts to find the optimal *Fad O* design set for intestinal cholesterol regulation and design an transformative model for preventing hypercholesterolaemia combined with the functional genes described above.



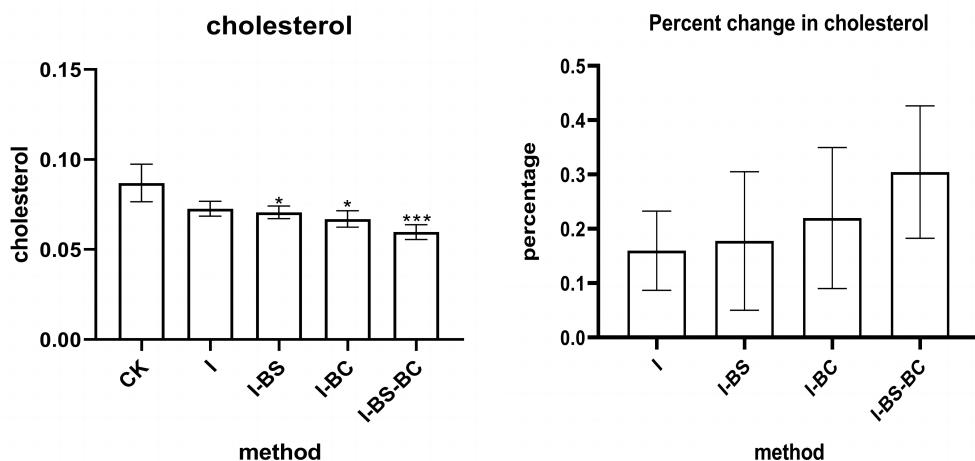
**Figure 5.** The oleic acid induction threshold can be altered by varying the number and position of *FadO* operators

## Results

To select the optimal effector engineering strain design, we optimized our effector gene modules by referring to the concept of directed evolution. According to the selected effector genes, we arranged and combined the *IsmA*, *BSH* and

BCoAT genes to obtain the genes IsmA, BSH, BCoAT, isma-bsb, ISmA-Bcoat, ISmA-Bsh-Bcoat. The obtained gene was transformed into ECN, and then the strain containing IsmA gene was cultured in cholesterol medium, and the cholesterol content was detected by OPA method to evaluate the cholesterol-lowering effect of IsmA gene. The strain containing BSH gene was inoculated on filter paper on solid medium containing bile salts, and the effect of BSH gene was characterized by the formation of precipitation circles reflecting the hydrolysis of bile salts. The effect of BCoAT gene was characterized by detecting the content of short chain fatty acids in the culture medium by GC method. Finally, the final effect of different genome pairs was evaluated.

In the cholesterol degradation experiment, as shown in **Figure 6**, all genomes showed cholesterol degradation (**Figure 6**). In the statistical results, the IsmA single gene did not show significant, but the double (ISmA-BSH, ISmA-BCoAT) and triple (ISmA-BSH-Bcoat) showed significant, especially the triple (ISmA-BSH-Bcoat) was extremely significant (**Figure 6**). As shown in Figure 2, in terms of the amount of degradation, the amount of degradation of the triplet strain reached about 30%, which was greater than that of the monogenic strain and the duplex (**Figure 7**). Taken together, the IsmA gene showed the greatest function in the ISmA-BSH-Bcoat triplet.

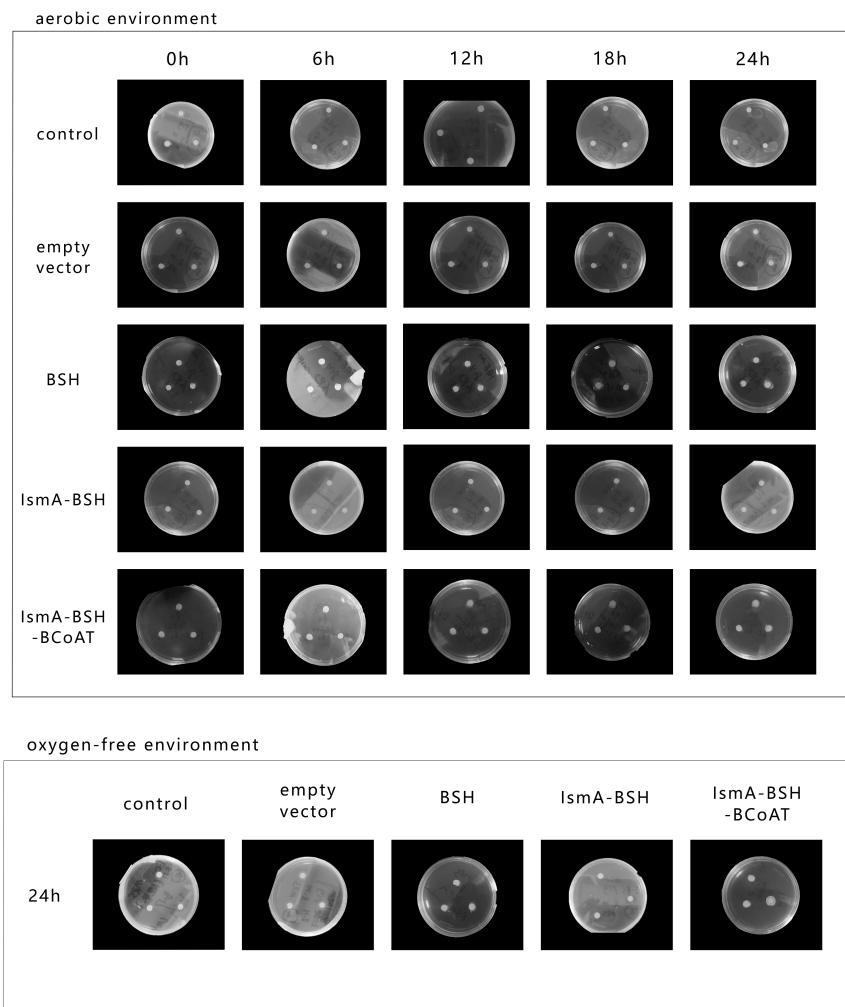


**Figure 6.** Strains carrying the IsmA gene were grown in cholesterol medium (BCM), and the cholesterol content in the medium was measured by the phthalaldehyde method after 48 hours of culture

**Figure 7.** Strains carrying the IsmA gene were grown in cholesterol medium (BCM), and the cholesterol content in the medium was measured by the phthalaldehyde method after 48 hours of culture

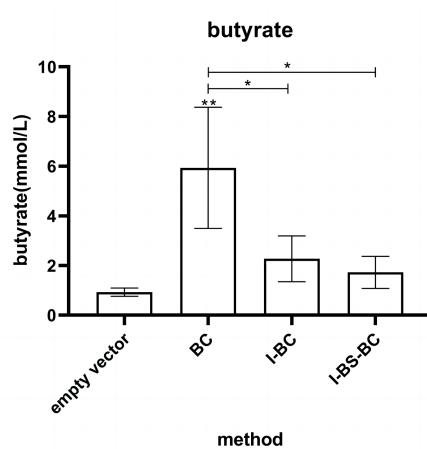
In the evaluation of the effect of bile saline hydrolysis, as shown in **Figure 8**, all effector gene combinations showed good bile saline hydrolysis, with obvious precipitation rings appearing around the filter paper on the medium (**Figure 8**). In the dynamic observation of the whole process of precipitation ring formation, we found that the activity performance of the BSH gene was generally better under hypoxic conditions than under aerobic conditions, and in the triplet strain (IsmA-BCoAT-BSH), the activity performance of BSH was higher than that of the BSH monogenic strain(**Figure 8**).

In the SCFA yield assessment, a total of three indicators were examined: butyrate, acetate and propionate. In the butyrate assay, all effector gene combinations showed butyric acid production (**Figure 9**). However, in the statistical analysis, only the butyric acid production of the BCoAT single gene showed statistical significance compared with the control group and also with the other combinations of effector genes (**Table 1**).



**Figure 8.** Results of qualitative detection of bile saline hydrolase

- (a) Under aerobic condition, the BSH gene carrying strain was cultured in MRS Medium containing sodium taurocholate for 24h, and the precipitation ring formation was recorded at 0h,6h,12h,18h, and 24h
- (b) Under hypoxia, BSH gene carrying strains were cultured in MRS Medium containing sodium taurocholate for 24 hours, and the formation of precipitation rings was recorded at the end of 24 hours of culture

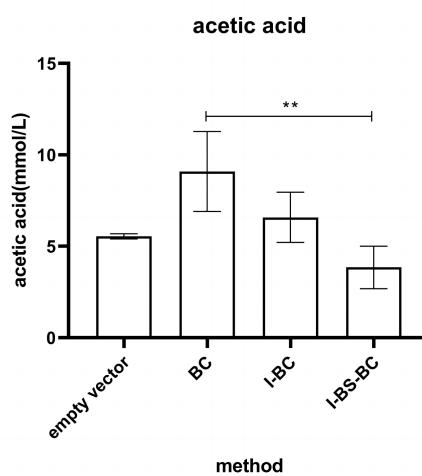


**Figure 9.** Butyric acid production measured after 24h of incubation of the strain carrying the BCoAT gene grown in MRS medium

**Table 1.** Chi-square analysis of butyric acid production detected after 24h of culture of the strains

Serial Number	1	2	3	4	5
Plasmid type	BC	I-BC	I-BS-BC	empty vector	EcN
Mean concentration ofbutyric acid (mmol/L)	3.1723	2.27	1.8583	1.8555	1.443
Homogeneity-of-variance			0.862		
ANOVO			0.012		
Duncan analysis grouping	a	ab	b	b	b

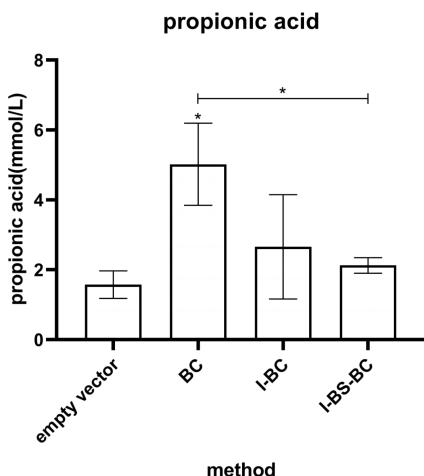
In the acetic acid assay, the BCoAT single gene could be seen to have an acetic acid-producing capacity, but it was not statistically significant (**Figure 10,Table 2**). In contrast, the other functional gene combinations showed essentially no acetic acid-producing capacity (**Figure 10**). In a functional genome comparison, there was a significant difference between the acetic acid-producing capacity of the BCoAT gene and the triplex (IsmA-BSH-BCoAT) gene (**Table 2**). This partly shows the more prominent acetate-producing capacity of the BCoAT single gene, but is not entirely convincing.

**Figure 10.** Acetic acid production measured after 24h of incubation of the strain carrying the BCoAT gene grown in MRS medium**Table 2.** Chi-square analysis of acetic acid production detected after 24h of culture of the strains

Serial Number	1	2	3	4	5
Plasmid type	BC	I-BC	I-BS-BC	empty vector	EcN
Mean concentration ofbutyric acid (mmol/L)	9.0927	6.5837	3.8453	5.5463	5.699
Homogeneity-of-variance			0.042		
Welch			0.179		
Brown-Forsythe			0.053		
Duncan analysis grouping	a	ab	b	b	b

In the detection of propionic acid, the BCoAT gene showed good propionic acid production characteristics with statistical significance compared with the control group, and in the comparison between effector genomes, it showed significant difference with the triplet (IsmA-BSH-BCoAT). "Other effector gene composition types, however, did not reflect this propionic acidogenic property (**Figure 11,Table 3**).

Combined with the SCFAs test results, the single BCoAT gene performed better than the combined effector gene.



**Figure 11.** Propionic acid production measured after 24h of incubation of the strain carrying the BCoAT gene grown in MRS medium

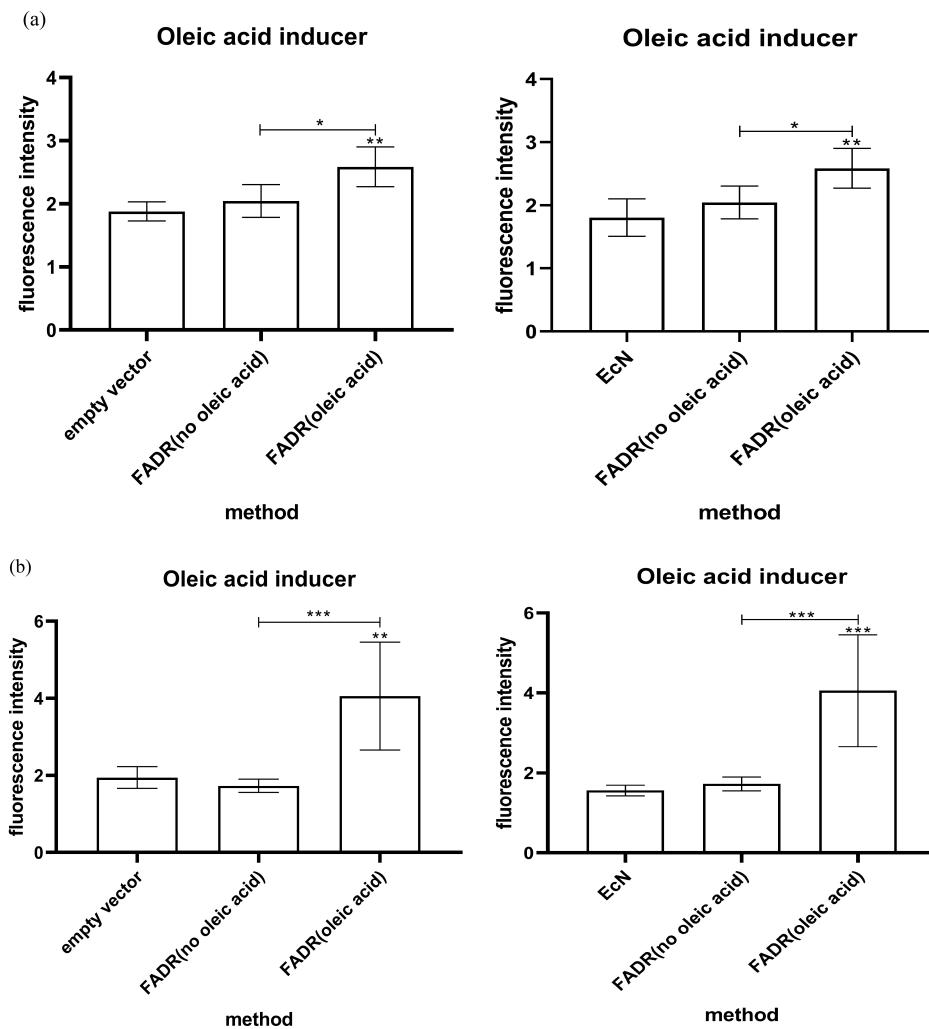
**Table 3.** Chi-square analysis of propionic acid production detected after 24h of culture of the strains

Serial Number	1	2	3	4	5
Plasmid type	BC	I-BC	I-BS-BC	empty vector	EcN
Mean concentration of butyric acid (mmol/L)	5.019	3.9017	2.2143	1.575	2.372
Homogeneity-of-variance			0.021		
Welch			0.047		
Brown-Forsythe			0.07		
Duncan analysis grouping	a	ab	bc	c	bc

In the analysis of the results of directed evolution, the gene composition corresponding to the optimal expression of different genes is not the same. In general, the IsmA and BSH genes were most effective in the triplet engineering (isma-Bsh-Bcoat), while the BCoAT gene was most effective in the monogenic engineering.

Targeting the oleic acid inducer, we first verified its function. To characterize the expression effect of the oleic acid inducer, mRFP was added and expression was characterized by mRFP fluorescence. The engineered bacteria that had been transformed into oleic acid inducer gene were cultured under both aerobic and anaerobic conditions, and under two media conditions with or without oleic acid. As shown in **Figure 12.** the results show that the oleic acid inducer is functional and can be highly expressed in the presence of oleic acid. In addition, the oleic acid inducer has a better performance under anaerobic conditions, which undoubtedly indicates that the oleic acid inducer can perform well in the intestinal anaerobic environment.

In order to achieve intelligent regulation of oleic acid inducer, we need to modify the number and position of Fad O operators on the Fad B promoter to modify the oleic acid priming threshold, and screen the most appropriate intestinal cholesterol regulation threshold interval and strain design set by directed evolution. However, in the process of modifying the Fad O operator, it is always a build failure result. In future experiments, we will explore the reason and find new methods to try to complete the directed evolution of oleic acid inducer.



**Figure 12.** Expression of the oleic acid inducer

- (a) Expression of the oleic acid inducer in the presence or absence of oleic acid induction under aerobic conditions
- (b) Expression of the oleic acid inducer in the presence or absence of oleic acid induction in the absence of oxygen

## Discussion

Our experimental design was designed to obtain the optimal combination of effector genes for cholesterol regulation and the optimal oleic acid inducer design set for human intestinal cholesterol regulation by directed evolution, respectively. We hope to combine the products of these two directed evolution genes to obtain an ingenious and intelligent human gut cholesterol regulation system. This system can be designed according to different people's constitution to take different oleic acid inducer to fit each person's physical condition, so that the system starts when the user consumes more than the required amount of cholesterol, and adjusts the cholesterol so that the user's cholesterol absorption is always within a reasonable range.

We performed the combination and effect verification of different effector genes, hoping to complete the directed evolution screen and screen the best combination of cholesterol regulatory effector genes. However, from the point of view of the results, the gene combinations corresponding to the maximum function of different genes are not the same, and some experiments do not show significance to verify the gene function. The first question requires a more comprehensive test to complete the directed evolutionary screen. The second problem implies inaccuracies in our experimental procedure or flaws in our experimental design. To address this inaccuracy, we will repeat the experiment and try to improve the protocol to complete the validation. We will not only repeat and improve the original protocol, but also conduct further validation experiments to further verify the gene effect and complete the directed evolution screening. For the IsmA gene, we will start with the downstream products of cholesterol degradation by UHPLC to complete the effect verification and directed

evolution screening. At the same time, for BSH gene and IsmA gene, we will also extract their enzyme solutions and corresponding proteins, and characterize the enzymatic hydrolysis efficiency and protein expression to verify the expression and complete the screening.

In order to realize the purpose of intelligent regulation, we try to carry out directed evolution of the oleic acid inducer, and modify the number and position of Fad O operators of the oleic acid inducer to change the starting threshold of the oleic acid inducer. However, we failed in this step, and there are still some technical problems to be solved. In the future experiments, we will try to solve this problem as much as possible to complete the directed evolution of oleic acid inducer. After we solve the problem of modifying the Fad O operator, we will conduct fluorescence characteristics through the mRFP after the oleic acid inducer, select the most suitable oleic acid threshold interval for intestinal regulation of cholesterol according to the data analysis, and model the relationship between the operator state and the expression degree to obtain the intelligent oleic acid inducer design model. The expression at temperature was detected and modeled, and the intelligent oleic acid inducer design model was integrated to screen out the best design scheme set of human intestinal cholesterol regulating oleic acid inducer.

After we complete the validation and directed evolution of the oleic acid inducer and effector genes, we plan to connect the oleic acid inducer with effector genes according to the optimal gene composition type and construct the engineered bacteria. Based on the engineered bacteria, we will conduct the effector gene detection experiment mentioned above to verify the effect of the engineered bacteria, and in the process of detection, we will try to simulate the intestinal environment as much as possible to realize the verification of our final engineered bacteria. We hope that our engineered bacteria will eventually be developed into an intelligent cholesterol regulatory system to improve the quality of life, prevent hypercholesterolemia, and provide an adjuvant therapeutic effect for the treatment of hypercholesterolemia.

## Materials and Methods

### 1. Construction of engineered bacteria

Information on IsmA, BCoAT, BSH, FadR and FadB was obtained from NCBI, iGEM and BioCyc databases and amplified from E. coli DH5 $\alpha$  DNA by PCR. Or synthesized by Weizhi Kim (Genewiz, China) or Twist Bioscience (USA). mRFP was stored and provided by the laboratory. E. coli Nissle 1917 (EcN) was purchased from Biobw (China, Beijing). E. coli DH5 $\alpha$  was from the laboratory collection. All plasmids were based on the pSB1A3 plasmid backbone. pSB1A3 is ampicillin resistant and carries a high-copy replicon ColE1. All recombinant plasmids were transformed into E.coli DH5 $\alpha$  for preservation and amplification, and verified by DNA sequencing (GENERAL Biol, Chuzhou, China) before being transformed into EcN. Among them, pSB1A3-FadR and pSB1A3-FadB were formed corresponding reporter strains with mRFP, and the purpose was to verify the in vitro response of FadR and FadB using fluorescent protein mRFP.

### 2. Cholesterol degradation test

All the engineered bacteria were first removed from the -80°C refrigerator, and then activated in LB medium for 12h and cultured in detection medium for 24h. For strains containing the IsmA gene, basal cholesterol medium containing cholesterol was selected as the assay medium. For strains containing BSH gene, M9 medium without glucose but containing bile salts and cholesterol was used for detection. The control group consisted of blank control, EcN naked bacteria, and empty plasmid transformed bacteria.

The OPA method was used at the time of detection. The standard curve was obtained first: 0.1mg/ml cholesterol working solution was prepared and mixed with acid (concentrated sulfuric acid: acetic acid =1:1). A total of 9 gradients of 0ml-0.4ml cholesterol working solution were taken in turn, and 4ml mixed acid and 0.2ml phthalaldehyde were added to each gradient, and the volume of each gradient was supplemented to 4.6ml with methanol. The order of sample addition was working solution, methanol, phthalaldehyde, mixed acid. After adding the sample, the mixture was mixed and allowed to stand for 20min, and then the absorbance value was measured at 550nm.

Sample detection: The sample was diluted with methanol, and the initial amount of cholesterol in the assay medium was used as a reference, so that it was less than the maximum cholesterol concentration of the standard curve. 0.4ml of the sample was taken, 0.2ml of phthalaldehyde was added, and 4ml of mixed acid was added, mixed evenly, left for 20min, and the absorbance was measured at 550nm.

### 3.BSH plate inoculation experiment

Qualitative plates were obtained by adding bile salts to MRS Solid medium and placing three small round sterile filter papers in the plates. BSH containing strains were first cultured in MRS Medium for more than 12 hours, and then 10ul of bacterial solution was inoculated on one filter paper. The control group was set as EcN null bacteria and empty plasmid transformation bacteria. After the completion of inoculation, each strain was cultured in anaerobic and aerobic environment for 24 hours, and the formation of precipitation rings was recorded.

#### 4.GC method was used to detect short-chain fatty acids

The strain containing BCoAT gene was activated in LB for 12h and then transferred to MRS Medium for 24h to obtain the sample solution. Take 1ml of the sample solution into a 1.5ml centrifuge tube, centrifuge at 10000g, take the upper solution, filter the liquid with 0.4um organic filter membrane to get the liquid to be tested, and freeze it at -20 °C for later use. After the test solution was prepared, the short-chain fatty acids were detected by gas chromatography. EcN naked bacteria were selected as controls, and empty plasmid transformed bacteria were used.

#### 5. Functional validation of the oleic acid inducer

The mutant strains containing pSB1T3-FadR-FadB genes were inoculated into 24-well plates supplemented with oleic acid-M9 medium and basal M9 medium, respectively. After 12-16 hours of incubation, the fluorescence and absorbance of all bacteria were measured using a microplate reader (Thermo Fisher Scientific, USA). To test the oleic acid inducer function.

#### 6.Statistical analysis

All statistical analyses were performed using the Prism 8.0.2 software (GraphPad, La Jolla, CA). All data were derived from at least three independent experiments. Results were presented as a mean with either standard deviation (SD) or standard error of mean (SEM), and sample numbers are indicated unless otherwise noted in the figure legends. Statistical significance calculations comparing two conditions were performed using chi-square test. The criterion of statistical significance level was denoted as follows: \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.

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### Author contributions

XK.L, Q.Y, YB.Y designed experiments. XK.L ZY.Y and Q.Y provided advice and expertise on experimental design, data analysis and manuscript editing. YB.Y, MLL, MY.W, ZC.L and ZH.L performed experiments. WT.W, YB.Y, ZY.S, WH.K, ZC.L analysed data. ZY.S, YB.Y, WT.W, MW.X,

ZY.Y writing and editing of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Supplementary information (optional)**

Supplementary materials submitted online with the manuscript.