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BIOTECHNOLOGICAL APPROACHES TO THE SELECTION AND OPTIMIZATION OF PROBIOTIC STRAINS WITH HYPOCHOLESTEROLEMIC ACTIVITY FOR FUNCTIONAL FOOD APPLICATIONS

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ABSTRACT

The review analyzed biotechnological approaches to the selection and optimization of probiotic microorganisms with hypocholesterolemic activity for their application in functional food products. It summarized current understanding of the role of elevated cholesterol levels in the development of cardiovascular diseases and examined the potential of probiotic bacteria to modulate lipid metabolism. The study described the principal mechanisms of cholesterol reduction, including bile acid deconjugation, microbial assimilation and adsorption of cholesterol, synthesis of short-chain fatty acids, bacterial conversion of cholesterol to coprostanol, and the involvement of exopolysaccharides. Particular attention was given to lactic acid bacteria and bifidobacteria as key probiotic groups. Criteria for strain selection were outlined, including safety, survival under gastrointestinal conditions, probiotic functionality, and technological suitability. The review evaluated primary and secondary screening strategies, approaches to *in vitro* assessment of cholesterol removal, and methods for improving strain performance, including adaptive laboratory evolution. It also examined the advantages of multistrain bacterial compositions and their functional complementarity. The findings indicated that a complex, stepwise selection and optimization strategy enhanced the effectiveness of probiotic cultures. The results provided a methodological basis for the development of functional foods with potential hypocholesterolemic effects.

Keywords: lactic acid bacteria, bifidobacteria, functional products, starter cultures, bacterial compositions, cholesterol, BSH activity

INTRODUCTION

Hyperlipidemia is one of the main risk factors for the development of cardiovascular diseases (CVDs), which in turn account for approximately one-third of all global deaths. The term encompasses conditions characterized by elevated levels of plasma cholesterol, phospholipids, triglycerides (TG), and other lipids **(P *et al.*, 2021)**. Hypercholesterolemia, in contrast, is a subtype of hyperlipidemia and refers specifically to conditions with increased cholesterol levels; however, their etiology and symptomatology may be closely interconnected, making differentiation difficult **(Civeira *et al.*, 2022)**. Hypercholesterolemia associated with the accumulation of low-density lipoprotein cholesterol (LDL-C) is a key factor in the development of atherosclerotic cardiovascular diseases (ASCVDs) **(Civeira et al., 2025)**, the predominant form of CVDs **(Yan *et al.*, 2025)** (approximately 61% of all CVDs **(Mengni *et al.*, 2021)**), for which mortality has recently begun to rise again after a prolonged period of decline and relative stability **(Maniya *et al.*, 2025)**.

To address this problem and reduce the risk of ASCVD, various approaches are employed, aimed both at eliminating the underlying causes of symptoms (dietary modification, weight loss through pharmacotherapy and surgical intervention) and at lowering LDL-C (medications, primarily statins **(Nițu *et al.*, 2025)**, as well as emerging targeted therapies that modulate key pathways of cholesterol metabolism **(Jianu *et al.*, 2025)**). Despite the widespread use and overall effectiveness of medical treatments for hypercholesterolemia, statins are associated with certain serious adverse effects, including liver and kidney diseases, the development of type 2 diabetes mellitus, hemorrhagic stroke, and others **(Khatiwada & Hong, 2024)**. In contrast, newer agents are currently not sufficiently studied, accessible, or widely adopted to constitute a major factor in the treatment of hypercholesterolemia **(Jianu *et al.*, 2025)**, which affects approximately 24.1% of the global population, while an even larger proportion experiences related hyperlipidemic conditions **(Ballena-Caicedo *et al.*, 2025)**.

Meta-analyses of numerous studies indicate that the most effective way to reduce the risk of hypercholesterolemia is prevention based on a healthy lifestyle, with dietary choices playing the most significant role **(Giles, 2024)**. A substantial body of evidence supports the effectiveness of functional foods in preventing various diseases, including hypercholesterolemia and CVDs **(Fekete *et al.*, 2025)**. In this context, probiotics – defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" **(Nutrition Division, 2006)** – occupy a central position in the development and industrial production of functional foods, owing to their ability to produce biologically active compounds and to exert overall beneficial effects on the human body **(Huang *et al.*, 2025)**. They are capable of reducing elevated levels of LDL-C, triglycerides, and other lipids associated, in particular, with unhealthy diets, genetic factors, and comorbidities **(Momin *et al.*, 2023; Seo *et al.*, 2023)**, and are therefore considered not only as preventive agents but also as dietary supplements for patients with clinically manifested hypercholesterolemia **(Sivamaruthi *et al.*, 2021)**.

However, the implementation of probiotics and, especially, functional foods with hypocholesterolemic properties faces a number of challenges **(Li & Ren, 2026)**:

- variability of effects (reduction of cholesterol levels in one individual and no response in another);
- difficulty in standardizing the final product;
- the need to achieve a balance between ensuring bacterial viability for a period sufficient to exert a probiotic effect and maintaining the technological characteristics of the final product;
- complex interactions among microorganisms and with available substrates, etc.

In addition, research is complicated by the fact that results obtained *in vitro* and *in vivo* may differ substantially, both in terms of metabolism and in terms of the wide range of mechanisms affecting cholesterol levels, not all of which can be assessed outside a living organism **(Madani *et al.*, 2013)**. A significant challenge lies in transitioning from empirical data to the development of effective bacterial compositions in which successful strain combinations lead to an enhanced beneficial effect **(Starovoitova & Spivak, 2023)**, as microbial interactions are inherently unpredictable **(Kwoji *et al.*, 2021)**.

The aim of this literature review is to systematize biotechnological approaches to the development of bacterial compositions for functional food products with hypocholesterolemic activity.

MATERIALS AND METHODS

Relevant literature sources were identified through searches of the PubMed, Scopus, Web of Science, ScienceDirect, and Google Scholar databases using combinations of keywords related to probiotics, lactic acid bacteria, bifidobacteria, cholesterol metabolism, bile salt hydrolase, adaptive laboratory evolution, and hypocholesterolemic activity. Priority was given to peer-reviewed publications from recent years; however, earlier studies were also considered when they provided fundamental insights or contained important information regarding the mechanisms underlying the investigated phenomena. The analysis included original research articles, clinical studies, animal experiments, *in vitro* studies, and relevant review papers. Publications were selected based on their relevance to probiotic-mediated cholesterol reduction, strain selection, bacterial interactions, and the mechanisms underlying hypocholesterolemic activity.

BIOLOGICAL BASIS OF CHOLESTEROL-LOWERING ACTIVITY IN PROBIOTIC BACTERIA

Depending on a combination of factors such as strain, formulation, dose, and others, probiotics are capable of exhibiting a wide range of beneficial properties, including hypocholesterolemic, antidiabetic, microbiome-modulating, immunomodulatory, antimicrobial, and even antiviral effects. The vast majority of probiotic microorganisms are lactic acid bacteria (LAB), owing to their well-established safety and long history of successful industrial use, while bifidobacteria represent the second most prevalent group **(Das *et al.*, 2022)**. They exert their activity due to their resistance to the harsh conditions of the gastrointestinal tract (GIT), their ability to adhere to and colonize the intestinal walls, and their effects on the host and surrounding microbiota both through the secretion of specific compounds (enzymes, bacteriocins, organic acids, etc.) and through interactions with cell surface receptors **(Latif *et al.*, 2023)**. A distinct group of mechanisms through which probiotics exert hypocholesterolemic activity has been identified **(Vourakis *et al.*, 2021)**, and understanding and evaluating these mechanisms are essential for further work with these microorganisms. The main ones are illustrated in Figure 1.

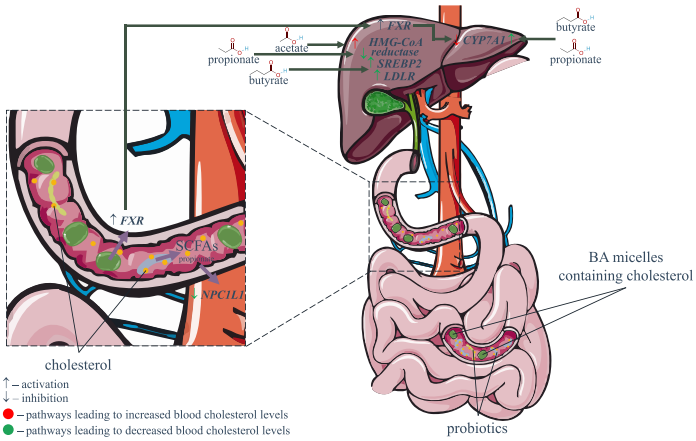


Figure 1 Major mechanisms underlying probiotic-mediated cholesterol reduction

Bile salt hydrolase-dependent mechanisms of hypocholesterolemic activity

It is generally considered that the key mechanism underlying the hypocholesterolemic activity of probiotics is the activity of bile salt hydrolase (BSH) **(Agolino *et al.*, 2024)**. BSH is an enzyme that deconjugates conjugated bile acids (BAs) into free bile acids and amino acids (glycine and taurine) through the hydrolysis of amide bonds within these molecules. Depending on the molecular structure, substrate specificity may vary: some variants act on glycine-conjugated BAs (e.g., glycodeoxycholic acid), while others act on taurine-conjugated BAs (e.g., taurodeoxycholic acid). Due to differences in the amino acid composition of the enzyme's active site, bifidobacteria more often produce BSH capable of hydrolyzing glycine conjugates, whereas LAB more commonly target taurine conjugates **(Liu *et al.*, 2025)**.

In the human body, primary BAs are mostly represented by cholic and chenodeoxycholic acids, which are conjugated with glycine (predominant in humans) and taurine (predominant in rats) to form conjugated BAs that, under physiological conditions, mainly exist as bile salts. These compounds are characterized by high stability, solubility, and efficient reabsorption during intestinal transit. Due to BSH activity, bile salts that pass through the gastrointestinal tract to the distal small intestine and colon (approximately 5% of the total BA pool) are converted into poorly reabsorbed free BAs and subsequently, through a series of metabolic transformations, into secondary BAs (e.g., deoxycholic and lithocholic acids) **(Agolino *et al.*, 2024)**. Reduced reabsorption leads to the excretion of these acids and stimulates the synthesis of new BAs from endogenous cholesterol reserves, thereby lowering its overall level in the body. In addition, deconjugated BAs themselves play a direct role in hypocholesterolemic activity: in their conjugated form, they form micelles with cholesterol, but after amino acid cleavage and under reduced pH, they lose solubility and stability, resulting in the precipitation of insoluble complexes of cholesterol with BAs and phospholipids **(Dong *et al.*, 2025; Tsibranska-Gyoreva *et al.*, 2023)**. BAs can also interact with cellular receptors such as the farnesoid X receptor (FXR), thereby regulating BA homeostasis, cholesterol turnover, and lipid metabolism. In this context, conjugated BAs act as FXR agonists and inhibit BA synthesis, whereas deconjugated and secondary BAs exhibit weak agonistic activity or are inactive **(Samson *et al.*, 2024; Zhang *et al.*, 2024)**.

The set of BSH-dependent mechanisms underlying hypocholesterolemic activity is critically important for many probiotic microorganisms in which the ability to reduce cholesterol has been experimentally demonstrated **(Agolino *et al.*, 2024)**, and therefore often warrants particular attention from researchers and scientists **(Liu *et al.*, 2025; Zhou *et al.*, 2025)**. The presence of BSH activity is a key indicator of bacteria with hypocholesterolemic potential.

Cholesterol assimilation and adsorption on the cell surface

Some of the earliest probiotic mechanisms involved in lowering cholesterol levels in the human body are its adsorption and assimilation into the cytoplasmic membrane of bacterial cells **(Li & Ren, 2026)**. The efficiency of these mechanisms is largely determined by the structural features of the peptidoglycan layer of the cell wall in specific strains, as well as by their ability to synthesize exopolysaccharides (EPS) and the composition of these polymers. Incorporation of cholesterol into the cell membrane leads to an increased content of both saturated and unsaturated fatty acids, enhancing membrane resistance to adverse GIT conditions such as bile salts and low pH **(Reis *et al.*, 2017)**. The degree of cholesterol assimilation partially depends on the growth phase of the cells; accordingly, cultures in the exponential phase exhibit its more intensive uptake **(Li & Ren, 2026)**. At the same time, in certain cases, the presence of cholesterol in culture media simulating GIT conditions may have a positive effect on bacterial growth **(Fernández-Calderón *et al.*, 2021)**. In contrast, non-viable probiotic cells are incapable of cholesterol assimilation but retain the ability to adsorb it on the cell surface. For example, a study by **Shehata *et al.* (2019)** demonstrated that heat-inactivated cells of *Lactococcus lactis* subsp. *lactis* removed 6.35% of cholesterol from the medium over the same period during which resting cells suspended in phosphate buffer removed 12.93%. In the former case, only adsorption was involved, whereas in the latter, both adsorption and assimilation contributed to cholesterol removal.

Theoretically, bacterial cells with adsorbed and assimilated cholesterol are excreted from the human body, thereby preventing the absorption of its molecules by intestinal epithelial cells and providing a hypocholesterolemic effect **(Li & Ren, 2026)**. However, the number of *in vivo* studies addressing these mechanisms remains limited, and the results obtained are inconsistent **(Hatakka *et al.*, 2008; Park *et al.*, 2008)**. Thus, although assimilation and adsorption are described as promising pathways for cholesterol reduction, in the context of food products their contribution remains limited, as they are highly dependent on the growth phase of the cells and do not ensure its consistent removal from the body.

Short-chain fatty acids and host lipid metabolism

As a product of the fermentation of undigested carbohydrate residues, intestinal bacteria produce short-chain fatty acids (SCFAs), the most abundant of which are acetate, propionate, and butyrate. Through their involvement in cellular energy metabolism or interaction with specific receptors, they are able to influence host lipid metabolism, thereby regulating cholesterol levels in the body **(Feng & Xu, 2023)**. Acetate, upon reaching the liver, activates the enzyme HMG-CoA reductase and is directly utilized for cholesterol synthesis. In contrast, propionate acts as an antagonist of this enzyme and inhibits sterol synthesis from acetate molecules; it also activates cholesterol 7α-hydroxylase (CYP7A1), thereby promoting the synthesis of BAs from cholesterol **(Reis *et al.*, 2017)**, and inhibits Niemann-Pick C1-like protein 1 (NPC1L1), which is responsible for cholesterol absorption in the intestine **(Haghikia *et al.*, 2022)**. Butyrate, in turn, acts as an agonist of sterol regulatory element-binding protein 2 (SREBP2) and the low-density lipoprotein receptor (LDLR), which enhance hepatic uptake of circulating cholesterol, as well as CYP7A1, thereby facilitating the conversion and excretion of the absorbed cholesterol **(Feng & Xu, 2023)**.

The influence of SCFAs on lipid metabolism is supported by numerous *in vivo* studies, in which probiotic intake led to changes in the levels of individual acids in the bodies of mice (e.g., acetate or butyrate) and reduced LDL-C levels in the blood of animals, although this mechanism remains insufficiently studied, including due to the limited availability of clinical data in humans **(Yao *et al.*, 2024)**. Given the significant role of SCFAs in the formation and enhancement of the flavor and aroma characteristics of both fermented dairy and other fermented products **(Ali *et al.*, 2025; Li *et al.*, 2025)**, further, more in-depth investigation of this area holds promise for the development of functional food products with hypocholesterolemic properties.

Bacterial transformation of cholesterol to coprostanol

The conversion of cholesterol to coprostanol is one of the least studied mechanisms of hypocholesterolemic activity, despite having been first described nearly a century ago **(Zhang *et al.*, 2025)**. Several biochemical pathways by which this transformation may occur are distinguished: a direct pathway – reduction of cholesterol to coprostanol – and the most studied and experimentally confirmed indirect pathway – via the intermediate compounds cholestenone and coprostanone **(Nafsi *et al.*, 2024; Susanti *et al.*, 2023)**. The progression of these pathways requires the production of two enzymes by the culture: a highly specific cholesterol dehydrogenase and a less specific cholesterol reductase, whose activity may be expressed both intracellularly and on the surface of microbial cells **(Abdi *et al.*, 2022; Susanti *et al.*, 2023)**. The indirect mechanism requires the participation of both enzymes, whereas the direct pathway involves only the reductase **(Nafsi *et al.*, 2024)**. However, the presence of these enzymes is a rare trait: they are characteristic mainly of intestinal bacteria, primarily belonging to the genera *Eubacterium* and *Bacteroides* **(Romero-Luna *et al.*, 2023)**, although there are also reports of their detection in LAB and bifidobacteria **(Zhang *et al.*, 2025)**. The isolation of strains capable of converting cholesterol into coprostanol is a very challenging task, and to date only a few cultures with this property have been obtained, including *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lacticaseibacillus casei* **(Susanti *et al.*, 2023)**.
Since coprostanol, unlike cholesterol, is characterized by extremely low intestinal absorption and is rapidly excreted from the body, the described mechanism exhibits a pronounced hypocholesterolemic effect **(Deng *et al.*, 2023)**. However, its insufficient level of study, the difficulty of isolating cholesterol dehydrogenase–positive strains, and their localization exclusively within the intestine complicate work in this area and indicate low feasibility for consideration in the context of bacterial compositions for food products.

Exopolysaccharide-related mechanisms of cholesterol modulation

There is substantial evidence of the hypocholesterolemic activity of EPS produced by various LAB species, with such effects observed both for purified EPS and for EPS-producing cultures **(Sørensen *et al.*, 2022)**. The exact mechanisms underlying this effect have not yet been established, but there is sufficient reason to assume that multiple factors are involved **(Prete *et al.*, 2021)**. One of the main experimentally supported mechanisms is the ability of certain EPS to bind BAs, thereby preventing their reabsorption and stimulating the synthesis of new BAs from the body's cholesterol reserves **(Hashemi *et al.*, 2022)**. There are also reports of direct binding of EPS to cholesterol (with EPS acting similarly to dietary fiber) **(Abdalla *et al.*, 2021; Sørensen *et al.*, 2022)**, as well as their involvement in the coprecipitation of cholesterol with unconjugated BAs **(Nguyen *et al.*, 2024)**. Some sources additionally indicate a possible role of EPS in increasing the viscosity of cholesterol micelles and reducing its bioavailability in the intestine; moreover, EPS can serve as a substrate for SCFA synthesis by the intestinal microbiota **(Silva *et al.*, 2021)**.
In the food industry, EPS play an important role due to their ability to improve the organoleptic and technological characteristics of the final product **(Hernández-Figueroa *et al.*, 2025)**. Moreover, a wide range of confirmed and well-studied health-promoting effects has been described for them **(Angelin & Kavitha, 2020)**. This makes this area promising for further investigation and for the development of effective bacterial compositions with hypocholesterolemic activity.

BIOTECHNOLOGICAL STRATEGIES FOR SELECTION AND OPTIMIZATION OF STRAINS WITH HYPOCHOLESTEROLEMIC ACTIVITY

The acquisition of microorganisms with desired characteristics is an extremely important task, as it affects medicine, industry, the food and agricultural sectors, and also plays a role in environmental protection technologies. It involves the work of scientists, engineers, and technologists, and may include both the isolation of new cultures and the genetic modification of existing ones. However, isolation plays a particularly important role, as it provides the foundation for any subsequent work. To obtain microorganisms with desired properties, careful selection is required, along with a well-defined strategy for their isolation, screening, and potential further improvement of their characteristics **(Olicón-Hernández *et al.*, 2022)**.
From a biotechnological perspective, of particular interest are atypical bacteria from unusual or underexplored sources **(Torres-Maravilla *et al.*, 2022)**, as well as those isolated from environments with strong selective pressure for the traits they are expected to possess **(Olicón-Hernández *et al.*, 2022)**. For microorganisms with hypocholesterolemic activity, the intestines of animals and humans represent a promising source of isolation, since the majority of microbiota species there harbor the gene responsible for BSH activity (*bsh*) **(Bourgin *et al.*, 2021)**, which is associated with selective pressure and the need to survive in the constant presence of high concentrations of bile salts. Bacteria carrying the *bsh* gene have an evolutionary advantage and therefore typically dominate in the GIT **(Jones *et al.*, 2008)**. In addition, various studies report the successful isolation of bacteria with pronounced hypocholesterolemic activity, predominantly LAB, from lipid-rich sources: fermented dairy **(Shehata *et al.*, 2016)** and dairy products **(Ostadzadeh *et al.*, 2023)**, milk from animals with high fat and cholesterol content **(Arrichiello *et al.*, 2022; Hameed *et al.*, 2022)**, and colostrum **(Tjandrawinata *et al.*, 2022)**, which contains more fat than regular milk **(Liao *et al.*, 2024)**. Some studies also provide examples of microorganisms with strong cholesterol-lowering capacity isolated from unconventional sources, such as ginseng **(Ahmed *et al.*, 2025)** or boza **(Shehata *et al.*, 2016)** (a non-alcoholic or low-alcohol cereal-based beverage common in the Balkan Peninsula and Turkey **(Khablenko *et al.*, 2025)**). Such cases are likely associated with the harsh conditions faced by bacteria in ginseng (due to the release of ginsenosides **(Luo *et al.*, 2020)**) and with survival during boza fermentation (low pH and ethanol formation **(Ucak *et al.*, 2022)**), which promotes the development of microbiota with more pronounced probiotic properties and cell envelopes resistant to stress factors.

Primary functional screening of strains with hypocholesterolemic potential

Primary functional screening of strains with hypocholesterolemic potential is often based on assessing BSH activity, which is associated with the significant role of BAs in cholesterol metabolism **(Zhang *et al.*, 2025)**. However, although many studies consider this trait as an initial selection criterion **(Koujalagi & Malaviya, 2025; Liang *et al.*, 2020; Lv *et al.*, 2024)**, the opposite approach is also encountered, where authors first evaluate the main probiotic characteristics of cultures (resistance to GIT conditions, antibiotic susceptibility, etc.) **(Ahmed *et al.*, 2025; He *et al.*, 2021)**. For the targeted isolation of cholesterol-lowering microorganisms, the use of BSH-oriented screening is most appropriate. Since the ability to hydrolyze conjugated BAs is a strain-dependent trait **(Liu *et al.*, 2025)**, it is reasonable to perform such analysis even for bacterial groups characterized by a high prevalence of the corresponding genes within the population, such as bifidobacteria or enterococci. In contrast, only about 50% of all lactobacilli and 10% of streptococci exhibit BSH activity **(Song *et al.*, 2019)**; moreover, as shown by **O'Flaherty *et al.* (2018)**, *bsh* genes are predominantly present in 27.65% of *Lactobacillus* (*sensu lato*) genera. This highlights the need to use BSH activity as an initial selection criterion for strains with hypocholesterolemic potential.
There are numerous approaches to assessing BSH activity in the bacteria under study, including qualitative, semi-quantitative, and quantitative methods. These differ in terms of complexity, specificity, and the availability of equipment and reagents. For example, experimental procedures may include incubation of sterile paper discs impregnated with culture on the surface of agar containing BAs and $CaCl_2$, the well diffusion method using the same agar, as well as spectrophotometric and chromatographic techniques **(Daliri *et al.*, 2022; Hernández-Gómez *et al.*, 2021; Ruiz *et al.*, 2021)**. Conjugated BAs are used as substrates, including glycocholic (GCA), glycodeoxycholic (GDCA), glycochenodeoxycholic (GCDCA), taurocholic (TCA), taurodeoxycholic (TDCA), and taurochenodeoxycholic (TCDCA) acids **(Grill *et al.*, 1995; Jiang *et al.*, 2010)**. Since BSH enzymes of potential probiotics often exhibit high substrate specificity, analysis using several BAs may yield a positive result for only one of them **(Hernández-Gómez *et al.*, 2021)**, which suggests the relevance of parallel screening of this trait on multiple substrates. Such an approach is indeed implemented in most studies: assessment is typically performed using one glycine-conjugated and one taurine-conjugated BA **(Frappier *et al.*, 2022; He *et al.*, 2021; Liang *et al.*, 2020)**, as the conjugated amino acid is the main factor determining differences in enzyme activity **(Liu et al., 2025)**. Although cases are possible where a positive reaction is observed for one taurine-conjugated acid but not for another **(Hernández-Gómez *et al.*, 2021)**, the standard approach is generally sufficient.
The frequency of use of different BAs also varies. GCA and TCA are most commonly selected, whereas GCDCA and TCDCA are used very rarely **(Grill *et al.*, 1995; Jiang *et al.*, 2010; Ruiz *et al.*, 2021)**. A tendency toward more frequent use of taurine-conjugated salts in scientific studies has been observed **(Ruiz *et al.*, 2021)**, which reduces the likelihood of reproducing results obtained *in vitro* and even *in vivo* in clinical studies. This is associated with the absence of glycine-conjugated BAs in mice and rats **(Miyazaki *et al.*, 2023)**, which are most commonly used for biological modeling **(Virgo *et al.*, 2025)**. In contrast, in humans the ratio of glycine-conjugated to taurine-conjugated BAs is typically 3-3.5:1, due to the limited capacity for taurine accumulation in the liver **(Miyazaki *et al.*, 2023)**.
It follows from the above that, although the most appropriate approach from the standpoint of selecting strains with hypocholesterolemic activity would be their evaluation using all major substrates, such a process is usually overly complex and impractical. The optimal "cost-benefit" solution is to test two salts – one glycine-conjugated and one taurine-conjugated – while the ability to hydrolyze glycine-conjugated BAs should be considered the primary selection criterion when interpreting the results. However, in cases where only a single compound is used for analysis, preference should be given to GCA/GDCA rather than TCA/TDCA, as is commonly done at present.

Secondary phenotypic validation and technological suitability

Microbial strains that have passed primary screening will possess BSH activity, which implies not only greater effectiveness as cholesterol-lowering cultures but

also increased probiotic potential, a higher likelihood of resistance to stressful GIT conditions, and improved ability to colonize the human intestine. Thus, **Yang *et al.* (2019)** demonstrated that a wild-type strain of *Lactiplantibacillus plantarum* and its genetically modified variant carrying an additional set of *bsh* genes differed significantly in probiotic characteristics: the emergence of new properties contributed to enhanced survival and increased adhesion of bacterial cells to intestinal epithelial cells. **Ertürkmen *et al.* (2023)** confirmed that the production of BSH enzymes is closely associated with hypocholesterolemic effects and tolerance to bile salts. However, the presence of BSH activity does not show a direct correlation with bile resistance in microorganisms **(Kang *et al.*, 2025)**, which is related both to the complex composition of bile **(Taylor *et al.*, 2025)** and to the numerous mechanisms by which probiotics adapt to its detergent action (active efflux of BAs from cells, alterations in cell wall and membrane composition, general stress response mechanisms, global reorganization of carbohydrate metabolism, etc.) **(Bustos *et al.*, 2018)**. Moreover, due to the greater toxicity of unconjugated BAs compared to conjugated ones and the lack of understanding of the principles underlying BSH-mediated cellular protection against bile **(Foley *et al.*, 2021)**, the practical evaluation of strain survival under GIT conditions *in vitro* and *in vivo* becomes even more important.

However, before proceeding to the evaluation of probiotic characteristics of the studied microorganisms, it is necessary to determine their ability to remove cholesterol *in vitro*. Conducting parallel experiments with actively growing cells, resting cells, and heat-inactivated cells allows differentiation between cholesterol assimilation and adsorption. The first reflect the combined effect of assimilation and adsorption, the second serve as a control in which continuous biomass increase does not affect assimilation parameters, and the latter demonstrate exclusively the adsorption capacity of the selected bacteria **(Tjandrawinata *et al.*, 2022)**. In addition, the inclusion of BAs in the medium with live cultures, as recommended by most protocols, also reveals the effect of cholesterol precipitation by deconjugated BAs **(Reis *et al.*, 2017)**, thereby enabling a more comprehensive assessment of cholesterol removal under *in vitro* conditions. A comparison of the results reported in different studies (Table 1) indicates that strains capable of removing at least 50% of cholesterol represent a relatively small subset of isolates exhibiting comparatively high cholesterol-removal capacity. Based on the analyzed literature data, a cholesterol removal rate of ≥50% may be considered a practical benchmark for the secondary screening of cultures. Although this value should not be regarded as a universal threshold, it corresponds to a relatively high level of activity among the probiotic strains described in the literature and may be useful for identifying the most promising candidates for further investigation.

Table 1 Comparative overview of the ability of bacterial strains to remove cholesterol under *in vitro* conditions

Number of tested strains	Max. removal, %	Min. removal, %	Number of strains with ≥ 50% removal	Share of strains with ≥ 50% removal	Reference
9	43.75	8.40	0	0.00	**(Shehata *et al.*, 2016)**
10	75.00	29.16	5	50.00	**(Ostadzadeh *et al.*, 2023)**
11	84.67	21.72	5	45.45	**(Tjandrawinata *et al.*, 2022)**
8	66.20	11.97	2	25.00	**(He *et al.*, 2021)**
7	79.00	51.20	7	100.00	**(Daliri *et al.*, 2022)**
58	55.00	0.00	3	5.17	**(Albano *et al.*, 2018)**
50	71.73	0.05	5	10.00	**(Ali *et al.*, 2022)**
5	55.80	32.30	2	40.00	**(Sui *et al.*, 2021)**
24	50.64	6.53	1	4.17	**(Liu *et al.*, 2022)**
6	51.45	0.82	1	16.67	**(Mojgani *et al.*, 2024)**
9	82.15	51.04	9	100.00	**(Bhat & Bajaj, 2020)**
6	53.60	19.34	1	16.67	**(Umeh & Achi, 2024)**
10	68.75	22.08	2	20.00	**(Yusuf *et al.*, 2020)**
12	69.02	14.02	7	58.33	**(Syakila *et al.*, 2019)**
Total:					
225	84.67	0.00	50	22.22	-

Although the studies summarized in Table 1 employed broadly similar experimental approaches, certain methodological differences should be taken into account when interpreting the reported cholesterol removal values. These differences included cholesterol concentration (100-300 $\mu g \cdot mL^{-1}$), bile supplementation (0.2-0.5% bile, bile salts, or no bile addition), incubation time (18-24 h), and, to a lesser extent, cultivation conditions. Such factors may influence the absolute cholesterol removal values obtained for individual strains. Therefore, the data presented in Table 1 should primarily be viewed as an overview of the range and distribution of reported activity levels rather than as a basis for precise comparisons of strain performance. Nevertheless, the overall trends remain sufficiently consistent to support comparative assessments during the screening and preliminary selection of strains.

The investigation of the most promising microorganisms for potential probiotic properties constitutes the second stage of rigorous selection, as only a small proportion of strains meet the established criteria **(Hassanzadazar *et al.*, 2012)**. Although evaluation methods vary considerably, the requirements include survival under conditions of the oral cavity, stomach, and intestine **(Wendel, 2022)**, including resistance to enzymes present therein, particularly lysozyme **(Uhegwu & Anumudu, 2025)**; high levels of autoaggregation and cell surface hydrophobicity, as these ensure adhesion to intestinal epithelial cells **(Elnar *et al.*, 2025)**; absence of hemolytic and DNase activity; susceptibility to antibiotics **(Borthakur *et al.*, 2025)**. However, as noted by **Ayyash *et al.* (2021)**, variability in experimental parameters complicates the comparison of results across different studies. For example, resistance to gastric conditions may be tested at pH values ranging from 2.0 to 4.0 for 1-4 hours, while the medium may consist of MRS or phosphate-buffered saline, as well as more specific mixtures designed to simulate physiological digestive processes. Accordingly, some studies include digestive enzymes (pepsin, pancreatin, etc.), whereas others do not. **Ayyash *et al.* (2021)** recommend applying as stringent GIT simulation conditions as possible for evaluating probiotic properties, with the mandatory inclusion of digestive enzymes at the gastric stage, which improves the reliability of reproducing *in vivo* survival rates. This process is further complicated by substantial differences in probiotic requirements across countries, making it necessary to consider regional regulations and restrictions **(Kim *et al.*, 2026)**. However, since the most effective probiotic strains often exhibit poorer technological properties and adaptability to food matrices **(Gueimonde *et al.*, 2024)**, a less stringent selection may be appropriate for certain cultures, depending on their role in supporting key fermentation processes. For example, in the development of a starter culture for fermented dairy products with a symbiotic thermophilic culture, it may be acceptable to select *Streptococcus thermophilus* with suboptimal probiotic characteristics due to its significant role as a starter organism **(Han *et al.*, 2022)**.

It is also appropriate to consider the numerous additional beneficial properties characteristic of probiotic microorganisms, including antioxidant activity **(Saleem *et al.*, 2025)**, anti-inflammatory effects, the ability to produce bacteriocins and inhibit pathogenic microorganisms **(Xiong *et al.*, 2025)**, and resilience under stress conditions, such as exposure to phenolic compounds that may form in the stomach or elevated NaCl concentrations, which indicate high osmotolerance of the cultures **(Borthakur *et al.*, 2025)**, among others. Osmotolerance is of considerable importance for the use of probiotics, especially LAB, in the food industry, as salt is frequently used as a food additive but affects cell membranes, their morphology, and adhesion capacity **(Zhang *et al.*, 2020)**. Consequently, prior determination of the ability of strains to function effectively at NaCl concentrations characteristic of the target product – for example, a specific type of cheese **(Mbye *et al.*, 2020)** – is an extremely important task. The same applies to other characteristics of the target environment. After confirming the probiotic status of bacteria *in vitro*, their compatibility with the food matrix and preliminary technological suitability should be evaluated.

Technological suitability includes the determination of resistance to a range of key technological stresses: identification of the threshold and optimal temperature and pH values that support culture growth and may also influence the shelf life of the final product; osmotolerance; and enzymatic activities such as proteolytic, lipolytic, and amylolytic activities **(Borthakur *et al.*, 2025; Rapoo & Thaoge-Zwane, 2024)**. Where possible, full control over the selection of optimal strains for a composition can be achieved by evaluating the aromatic compounds produced by the cultures, as they play a key role in most fermented food products. For example, more than 90 different aroma compounds contribute to the characteristic flavor of yogurt, but only a few have a significant impact on the final result – and can be analyzed and quantified **(Kouakou-Kouamé *et al.*, 2023)**. It is also important to consider the ability of the studied strains to produce EPS and the SCFA profile they generate – as noted earlier, these characteristics are essential both for the manifestation of hypocholesterolemic properties of the final product and for its organoleptic attributes.

Establishing the compatibility of probiotic microorganisms with the selected food matrix plays a significant role. Different substrates are characterized by different conditions, composition, and levels of carbon and nitrogen sources, dissolved oxygen content, acidification during cultivation, and other factors. Thus, dairy substrates typically provide more favorable conditions for growth than non-dairy ones, as they lack antimicrobial compounds (e.g., organic acids such as benzoic acid) and are richer in nutrients **(Shori, 2016)**. In contrast, plant-based matrices often contain dietary fiber and polysaccharides that promote bacterial survival during storage and passage through the GIT **(Uhegwu & Anumudu, 2025)**, and may also act as prebiotics **(Meral *et al.*, 2024)**, provided that the selected strains are compatible with them. Microorganisms isolated from plant sources have a higher likelihood of survival and successful proliferation in such matrices **(Holubchyk *et al.*, 2025; Khablenko *et al.*, 2025)**. Optimal compatibility between the food matrix and the culture ensures not only good growth and viability of cells in the final product but also desirable organoleptic properties after fermentation **(Uhegwu & Anumudu, 2025)**.

Overall, after establishing BSH activity in microorganisms, further strain selection may be based on their ability to remove approximately 50% of cholesterol from the medium within 24 hours in the presence of BAs, as this level of activity was observed only among a minority of the strains described in the literature. At the next stage, among the selected cultures, it is advisable to retain only probiotic strains and to determine optimal matrices and preliminary technological parameters for them, or to assess their compatibility with target substrates and food products.

Adaptive laboratory evolution and culture-based optimization

One of the promising yet fundamental biotechnological approaches is adaptive laboratory evolution (ALE), whose foundations date back to the time of Charles Darwin. Its concept is based on the ability of microbial cultures to adapt to stress factors, which can be achieved through gradual intensification of selective pressure, selection of the best-performing colonies after short-term exposure to a stressor, or other methods. Strains obtained in this way are not considered genetically modified microorganisms and can be freely used in the food industry, making this approach of interest to numerous experts in microbiology and biotechnology **(Fernandes *et al.*, 2023; Schöpping *et al.*, 2022)**. Independent studies of experimental work **(Mavrommati *et al.*, 2022; Tirloni *et al.*, 2023)** have demonstrated the effectiveness of ALE, including its application for improving probiotics such as LAB and bifidobacteria, with enhancements observed both in general industrial performance (e.g., yield of organic acids) and in specific characteristics – primarily stress resistance to GIT conditions or oxidative stress. However, most studies have examined only a limited number of strains and experimental conditions, making it difficult to generalize the obtained results across different probiotic species. The use of ALE can also activate existing *bsh* genes during adaptation to corresponding bile salts; for example, **Ma *et al.* (2024)** increased the BSH activity of *Lactobacillus kefir* M20 by 2.1-fold, resulting in a 10.8% increase in total cholesterol (TC) removal *in vivo* and a 25.2% increase in LDL-C reduction. At the same time, cultivation in the presence of bile or BAs does not induce BSH activity in strains lacking the relevant genetic machinery or possessing only the ability to hydrolyze other types of BAs **(Horackova *et al.*, 2020)**.

Cultures that have passed primary and secondary screening and have demonstrated strong potential for use in bacterial compositions with hypocholesterolemic activity for selected food products under *in vitro* conditions may achieve improved performance through the application of ALE. The possibilities are not limited to optimizing their BSH activity, including enhancing the ability to hydrolyze those BAs that are relatively poorly degraded by the selected microorganisms **(Boucard *et al.*, 2025)**. If necessary, it is also possible to increase the thermal resistance of strains and their overall technological suitability **(Bommasamudram *et al.*, 2023)**, as well as their resistance to GIT conditions **(Ming *et al.*, 2016)**, among other traits – changes that may theoretically exert a positive effect on hypocholesterolemic activity. To a large extent, this is due to the interconnection of stress response mechanisms, where adaptation to one adverse factor often leads to the development of a more resistant bacterial phenotype toward another **(Gao *et al.*, 2022)**. For example, adaptation to low pH may increase strain hydrophobicity **(Nyabako *et al.*, 2020)** or its resistance to bile acids and simulated intestinal fluid **(Ming *et al.*, 2016)**. This mechanism is referred to as cross-protection. Among the most common responses to stress factors in LAB and bifidobacteria are changes in cell membrane composition and the production of stress proteins **(Gao *et al.*, 2022)**. When LAB adapt to highly acidic conditions, this leads to an increased proportion of unsaturated fatty acids in their membranes **(Zhu *et al.*, 2024)**, and findings from some studies **(Johnson *et al.*, 2003; Ziar & Riazi, 2022)** suggest that this process may promote cholesterol assimilation and adsorption by increasing membrane fluidity and permeability. However, clear experimental confirmation or refutation of these mechanisms remains lacking, and further research in this area is required.

A drawback of ALE is the unpredictability of genetic and phenotypic changes in microbial strains. The problem is further exacerbated by the limited cellular resources – it is not possible to obtain a strain that simultaneously exhibits excellent probiotic characteristics, including high hypocholesterolemic activity, rapid accumulation of large amounts of biomass, and a unique flavor-aroma profile in the final product. Improvement of certain properties may lead to the loss of others **(Kim *et al.*, 2020)**. In this regard, the use of ALE is not always appropriate and should be determined by the researcher for each specific strain, depending on the combination of its traits. If these characteristics are already significantly above average, applying such approaches may be unnecessary and even detrimental.

The available evidence makes it possible to propose a stepwise framework for the selection and optimization of microorganisms with hypocholesterolemic potential (Figure 2). This framework summarizes the key stages discussed in this review, ranging from the initial screening of strains to their validation, subsequent incorporation into functional bacterial formulations, and the assessment of interactions between cultures.



Figure 2 Stepwise process for the selection and optimization of probiotic strains with hypocholesterolemic activity

FROM SINGLE STRAINS TO FUNCTIONAL BACTERIAL COMPOSITIONS

Probiotic microorganisms that have passed all required evaluation stages can be used in the food industry to produce desired products. Although the use of mono-strain starter cultures is possible in some cases **(Akabanda *et al.*, 2014)**, the resulting products are generally inferior in organoleptic properties and technical characteristics compared to those obtained using complex bacterial compositions. This is due to the wide range of properties exhibited by microbial cultures, differences in acidification rates, and variation in the spectrum of aroma compounds produced **(Chen *et al.*, 2024; Wang *et al.*, 2021; Ziarno *et al.*, 2025)**. Multistrain starters are functionally closer to the established microbiota of

spontaneously fermented products that have been consumed by humans for thousands of years; however, their composition is formed through targeted selection of strains with desired characteristics rather than through natural selection **(Holubchyk *et al.*, 2024)**.

Rationale for multi-strain compositions

Bacterial compositions generally exhibit higher growth rates, greater capacity to utilize nutrient substrates, and improved productivity compared to single-strain starter cultures. The main reasons for this effect lie in the limited ecological adaptability and metabolic functionality of a single culture – whereas combining multiple microorganisms allows a balance between their requirements, resulting in enhanced performance **(Canon *et al.*, 2020)**. A classic and most extensively studied example is the interaction between *L. bulgaricus* and *S. thermophilus* in yogurt production: the former hydrolyzes the majority of milk proteins into amino acids and peptides, which are then assimilated by the latter, which possesses weak proteolytic activity **(Markakiou *et al.*, 2020; Song *et al.*, 2021; Xin *et al.*, 2021)**. In turn, *S. thermophilus* provides *L. bulgaricus* with optimal growth conditions in terms of the physicochemical parameters of the medium **(Angelov *et al.*, 2009)**.

There are also significant advantages to using multistrain compositions in terms of the effects of probiotic cultures on human health. Their application promotes synergistic and additive effects that enhance immunoregulatory, anti-inflammatory, antimicrobial, neuromodulatory, and other beneficial properties of microbial strains **(Kwoji *et al.*, 2021)**. Individual dietary habits, lifestyle, gut microbiota composition, and many other factors play a substantial role in determining the effectiveness of specific cultures **(Healey *et al.*, 2017)**. In contrast, when bacterial compositions are used, a decrease in the activity of one microorganism may be compensated by others, and such variation does not significantly affect the final outcome.

The impact of mono- and multistrain probiotics on hypocholesterolemic effects in randomized clinical trials can be traced using the data presented in Table 2.

Table 2 Hypocholesterolemic effects by single- and multi-strain probiotics

Strain/Composition	Reduction in TC, %	Reduction in LDL-C, %	Reduction in TG, %	Form of administration	Reference
Monostrain probiotics					
***Lpb. plantarum* ECGC 13110402**	37,60	13,90	53,90	Capsule	**(Costabile *et al.*, 2017)**
***Lactobacillus gasseri* BNR17**	n. s.*	n. s.	n. s.	Capsule	**(Kim *et al.*, 2018)**
***Limosilactobacillus fermentum* PCC®**	n. s.	7,00	n. s.	Capsule	**(Simons *et al.*, 2006)**
***L. acidophilus* LA-1**	n. s.	n. s.	n. s.	Capsule	**(Lewis & Burmeister, 2005)**
***Enterococcus faecium* M-74**	12,12	19,74	n. s.	Capsule	**(Hlivak *et al.*, 2005)**
***Bifidobacterium breve* B-3**	n. s.	n. s.	n. s.	Capsule	**(Minami *et al.*, 2018)**
***Limosilactobacillus reuteri* NCIMB 30242**	4,81	8,92	n. s.	Yogurt	**(Jones *et al.*, 2012)**
***L. acidophilus* L1**	2,9	n. s.	n. s.	Fermented milk	**(Anderson & Gilliland, 1999)**
***Lpb. plantarum* A7**	6,49	8,21	8,57	Soy milk	**(Abbasi *et al.*, 2018)**
Multistrain probiotics					
***Lpb. plantarum* CECT 7527, CECT 7528, CECT 7529**	10,95	12,30	18,52	Capsule	**(Fuentes *et al.*, 2016)**
L. acidophilus*, *Bifidobacterium bifidum	10,33	n. s.	n. s.	Capsule	**(Rerksuppaphol & Rerksuppaphol, 2015)**
***Bifidobacterium animalis* subsp. *lactis* MB 2409, *B. bifidum* MB 109B, *Bifidobacterium longum* subsp. *longum* BL04**	3,4	3,8	1,9	Capsule	**(Guardamagna *et al.*, 2014)**
VSL#3: *B. longum*, *Bifidobacterium infantis*, *B. breve*, *L. acidophilus*, *Lacticaseibacillus paracasei*, *L. bulgaricus*, *Lpb. plantarum*, *S. thermophilus*	5,47	n. s.	5,38	Capsule	**(Rajkumar *et al.*, 2014)**
Ecologic®Barrier: *B. bifidum* W23, *B. lactis* W51, W52, *L. acidophilus* W37, *Levilactobacillus brevis* W63, *Lcb. casei* W56, *Ligilactobacillus salivarius* W24, *L. lactis* W19, W58: а) $1 \cdot 10^{10}$ CFU·day^{-1}; б) $2{,}5 \cdot 10^{9}$ CFU·day^{-1}	а) 7,32; б) 4,85	а) 3,99; б) 6,36	а) 7,05; б) n. s.	Capsule	**(Szulińska *et al.*, 2018)**
Ecologic®Barrier	19,00	22,20	48,00	Capsule	**(Sabico *et al.*, 2019)**
L. acidophilus*, *B. lactis	5,51	n. s.	n. s.	Yogurt	**(Ataie-Jafari *et al.*, 2009)**
***L. acidophilus* La5, *B. lactis* Bb12**	4,16	n. s.	n. s.	Yogurt	**(Sadrzadeh-Yeganeh *et al.*, 2010)**
***L. acidophilus* La5, *B. lactis* Bb12**	4,54	7,45	n. s.	Yogurt	**(Ejtahed *et al.*, 2011)**
***E. faecium* CRL 183, *Lactobacillus helveticus* 416**	13,80	13,5	n. s.	Fermented soy product	**(Cavallini *et al.*, 2016)**

**Note: n.s. at $p > 0.05$*

It can be seen from the above that the actual numerical outcomes vary considerably depending on the strains used in the studies and the conditions under which they were conducted. Direct comparison of the results reported across different studies is hindered by variations in study design, intervention duration, probiotic dosage, and baseline lipid profile characteristics. For example, **Szulińska *et al.* (2018)** demonstrated that changing the probiotic dose while keeping other variables constant leads to different results. At the same time, the use of the same preparation at the same dosage ($2.5 \cdot 10^{9}$ CFU·day^{-1}), but in a different clinical group of patients and over a longer period, resulted in markedly different quantitative outcomes **(Sabico *et al.*, 2019; Szulińska *et al.*, 2018)**. This complicates – and within the scope of this review, makes it practically impossible – the comparison of the hypocholesterolemic effects of mono- and multistrain probiotics. Nevertheless, several studies indicate that multistrain probiotics may be more likely to produce statistically significant effects than monostrain formulations; however, this observation should be interpreted cautiously due to the considerable heterogeneity of the available evidence. This may be related to the previously mentioned interaction between individual patient characteristics across different clinical groups and specific microbial strains – when multistrain probiotics are used, the combined action of multiple cultures may contribute to a more consistent beneficial effect on health outcomes.

Moreover, the collected data indicate a lack of understanding of the relationship between the mechanisms underlying the hypocholesterolemic effects of specific strains, their effective doses – which may vary depending on these mechanisms – and the manifestation of the final hypocholesterolemic outcome. This highlights the need for further research in this area, focusing not only on establishing the efficacy of individual strains or compositions, but also on determining the relationship between the characteristics of microbial cultures and their ultimate impact on the human body.

Functional complementarity and metabolic cross-feeding

The optimal selection of strains within a bacterial composition makes it possible to maximize positive interactions and minimize negative ones among the cultures **(Luo *et al.*, 2024)**, thereby enhancing cell viability and growth rate, as well as improving the organoleptic properties of the final product **(Ma *et al.*, 2025)**. In addition, this enables the achievement of optimal hypocholesterolemic activity

through the combination of different cholesterol-lowering mechanisms. Despite the selection of BSH-positive strains with high *in vitro* cholesterol removal capacity, certain mechanisms may vary substantially among them. First, as noted earlier, the ability to hydrolyze conjugated BAs is strain-dependent – thus, some cultures may complement others. Second, bacteria may differ considerably in many technological and probiotic characteristics, yet lack or exhibit only weak expression of a particular cholesterol removal mechanism, such as the ability to produce EPS **(Khan *et al.*, 2025)**. The absence of a desired trait in some strains can be compensated by its presence in others.

In addition to cross-feeding among bacteria within a composition – which includes the exchange of released amino acids, modification of the growth medium toward more favorable conditions, and related processes **(Luo *et al.*, 2024)** – there are also interactions between typical probiotics (LAB, bifidobacteria) and gut microbiota that indirectly contribute to the reduction of cholesterol levels in the body (Fig. 3). These interactions typically proceed via several metabolic pathways. According to the first pathway, LAB produce large amounts of lactate (and, depending on the bacterial species, small amounts of acetate), whereas bifidobacteria produce large amounts of acetate and smaller amounts of lactate. Lactate, in the presence of acetate, is utilized by gut bacteria that are unable to degrade complex substrates (e.g., *Anaerostipes caccae*, *Anaerobutyricum hallii*, *Megasphaera indica*) to produce butyrate **(Moens *et al.*, 2017; Zhao *et al.*, 2023)**, which, as mentioned earlier, affects enzymes and proteins, thereby contributing to the reduction of cholesterol levels.

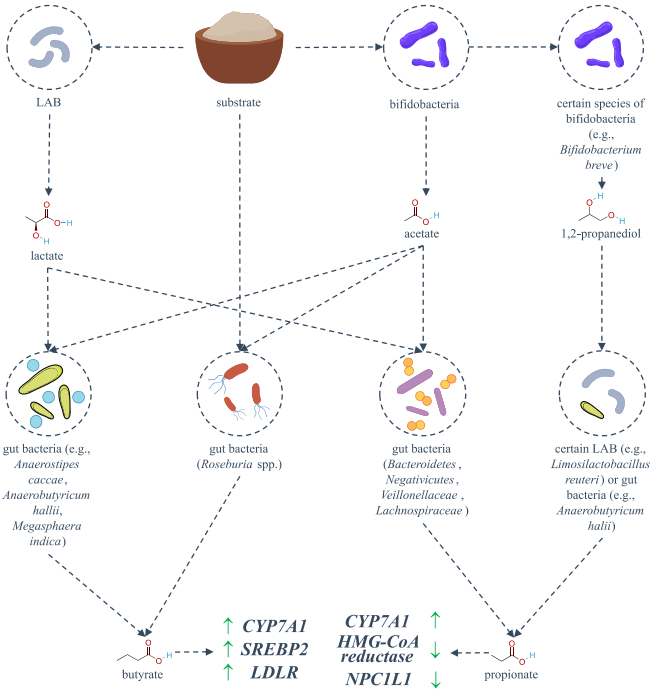


Figure 3 Metabolic cross-feeding contributing to hypocholesterolemic effects via SCFAs

The second pathway is mediated by gut bacteria that are unable to metabolize lactate (e.g., *Roseburia hominis*, *Roseburia intestinalis*, *Roseburia inulinivorans*). These bacteria utilize simple degradation products of complex substrates (monosaccharides, oligosaccharides, etc.), as well as acetate produced by LAB and bifidobacteria, and convert them into butyrate **(Belenguer *et al.*, 2006; De Vuyst & Leroy, 2011)**.

The third pathway involves the conversion of lactate and acetate produced by LAB and bifidobacteria into propionate via either the succinate pathway (in *Bacteroidetes*, *Negativicutes*) or the acrylate pathway (in certain *Veillonellaceae* and *Lachnospiraceae*) **(Louis & Flint, 2017; Ríos-Covián *et al.*, 2016)**, which also contributes to cholesterol reduction. In addition, a fourth pathway exists: certain species of bifidobacteria (e.g., *B. breve*) can produce 1,2-propanediol from rhamnose or fucose but are unable to further metabolize it. Instead, the conversion of this compound into propionate is carried out by some LAB (e.g., *Lm. reuteri*) or specific gut bacteria such as *A. hallii* **(Cheng *et al.*, 2020)**.

However, the quantitative contribution of cross-feeding mechanisms to cholesterol-lowering effect has not yet been fully characterized.

Considering the information presented above, as well as the differences in the metabolic organization of LAB and bifidobacteria and their mutual interactions **(Altieri *et al.*, 2024; Nogacka *et al.*, 2023)**, a promising approach in the development of starter cultures with hypocholesterolemic properties for food products is the formulation of bacterial compositions that include not only LAB but also bifidobacteria, which would contribute to achieving a more pronounced and multifaceted effect.

Stability and interaction within mixed cultures

When combining individual strains into an integrated bacterial composition, it is essential to consider the potential for antagonistic interactions between cultures – such as the production of bacteriocins to which some microorganisms in the starter are sensitive, the formation of organic acids in amounts that may inhibit their growth **(Ouwehand *et al.*, 2018)**, or even more specific compounds capable of altering gene expression in surrounding cells **(Kwoji *et al.*, 2021)**. To prevent this, strain compatibility should be assessed in model fermentations, and various strategies for combining cultures should be implemented when their simultaneous growth is detrimental to the process or technologically challenging. These include sequential inoculation (e.g., first cultivating a strain with an optimum at 37 °C, followed by lowering the temperature and introducing a strain with an optimum at 30 °C), microencapsulation (primarily used to ensure the long-term stability of probiotics in food products, especially dairy), and prevention of bacteriocin production by rapidly increasing lactic acid levels in the medium **(García *et al.*, 2019)** (typically achieved by increasing the CFU of strains with high acidification rates **(Asimakoula *et al.*, 2021)**). Such an approach inevitably affects the ratio of cultures in the inoculum; therefore, optimal inoculation levels are determined empirically **(Yang *et al.*, 2024)**. Based on these data, mathematical modeling can be performed, followed by validation of the obtained results **(Dulf *et al.*, 2022)**.

Once the strains and their ratios in the bacterial composition have been definitively established, it is necessary to ensure the long-term and reliable preservation of the original cultures. Experimental studies show that maintaining microorganisms under laboratory conditions with regular subculturing leads to relatively frequent genotypic and phenotypic changes in strains **(Feng *et al.*, 2020)**. In contrast, storage under industrial conditions – where cryopreservation and low-temperature storage are predominantly employed **(Tsutsaeva *et al.*, 2008)** – has a minimal impact on culture stability **(Brunelli *et al.*, 2025)**. This is supported by a separate study **(Sardar *et al.*, 2025)**, which found that, in order to preserve the full spectrum of bacterial phenotypic traits, including probiotic properties, cultures should be stored in collections at ultra-low temperatures with an optimal combination of cryoprotectants. However, the selection of a specific temperature regime and cryoprotective agents should be made only after individual testing of each culture due to strain-dependent variability in outcomes **(Fan *et al.*, 2024)**.

CURRENT LIMITATIONS AND FUTURE PERSPECTIVES

Current challenges

One of the major challenges in the field of microorganisms with hypocholesterolemic activity is the reproducibility and extrapolation of results obtained *in vitro* to *in vivo* studies, and even more so the reproducibility and extrapolation of *in vivo* findings to clinical trials. There is a substantial body of research **(Starovoitova *et al.*, 2024; Tsai *et al.*, 2014; Wang *et al.*, 2019)** demonstrating that strains performing well *in vitro* can effectively reduce cholesterol levels *in vivo*, and **Liu *et al.* (2025)** were even able to demonstrate a direct impact of a BSH-positive LAB strain on the gut microbiota. However, there are also studies reporting directly opposite results, which – together with the incomplete understanding of hypocholesterolemic mechanisms – raise questions and fuel ongoing debate. For example, **Madani *et al.* (2013)** investigated *Lpb. plantarum* A7, which was capable of lowering cholesterol *in vivo* but showed no ability to assimilate it *in vitro*. It should be noted, however, that their study examined only a single characteristic of the strain, and cholesterol removal was assessed without the addition of BAs to the medium – thereby completely excluding the potential contribution of BSH activity to the observed outcome.

A significant issue is also the difference in BA composition between mice/rats and humans (the absence of glycine-conjugated BAs in the former and their predominance in the latter), which reduces the reliability of the most commonly used *in vivo* models and the reproducibility of their results in clinical studies. Despite this, the substantial similarity in metabolism between mice/rats and humans, as well as the multifactorial nature of the hypocholesterolemic activity of bacteria themselves, contributes to a generally acceptable level of model effectiveness. However, alternative *in vivo* models have also proven effective, including hamsters, guinea pigs, and pigs **(Ooi & Liong, 2010)**. A distinguishing advantage of these models over mice/rats is the presence of glycine-conjugated BAs. In hamsters, the ratio of glycine- to taurine-conjugates varies considerably depending on age, sex, and diet, but averages approximately 1.5:1 **(Trautwein *et al.*, 1999)**; in guinea pigs, 3.65:1 **(Tavoloni, 1986)**; and in pigs, 6.3:1 **(Hafkenscheid & Hectors, 1975)**, although it may sometimes exceed 9.0:1 **(Kuramoto *et al.*, 2000)**. Despite having a BA composition that differs from humans due to an excessively high proportion of glycine conjugates, the overall characteristics of lipid metabolism in pigs make them one of the most suitable animal models for studying the hypocholesterolemic activity of probiotics **(Bergen & Mersmann, 2005)**. However, their use is significantly more expensive than that of other models **(Koopmans & Schuurman, 2015)** and is not always justified, considering that probiotics rarely exhibit adverse or side effects **(Alsuwaylihi & McCullough, 2023)** that would necessarily need to be identified in advance. Accordingly, guinea pigs represent an optimal choice for *in vivo* studies of probiotic hypocholesterolemic activity, as they combine similarity to humans in lipid metabolism, general metabolism, and BA composition with relatively low

cost of use. Studies involving hamsters are also a strong alternative, still offering substantial advantages over mice/rats, which remain the most commonly used models at present **(Ooi & Liong, 2010)**. Similar results supporting our conclusions were also obtained by **Guo *et al.* (2018)**: in their study, they experimentally demonstrated the greater reliability of hamsters as a model for assessing the hypocholesterolemic activity of LAB compared to rats.

Bacterial compositions with hypocholesterolemic activity are also subject to the typical issues associated with probiotics that are frequently highlighted in the literature. For example, manufacturers of probiotic products in various forms often incorporate higher CFU counts into the final product than declared, in order to compensate for losses in viability during storage and to ensure the presence of a minimum guaranteed number of viable cells at the end of the stated shelf life. **Da Silva *et al.* (2024)** argue that in such cases, the total cell count, in addition to the viable count, should be indicated, since a higher number of live bacteria may exert a greater impact on the host or on the technological process. Moreover, the effects of non-viable cells on inflammatory processes in the body remain insufficiently studied and cannot be definitively ruled out **(Adams, 2010; Noh *et al.*, 2022)**.

Another issue is the lack of sufficiently specific regulatory requirements, which vary across countries and result in high heterogeneity of studies, limited empirical support for the efficacy of individual formulations, and inconsistency in their composition **(Liang *et al.*, 2024)**. As noted by **Hakim *et al.* (2023)**, the presence of recommendations rather than strict regulatory provisions leads to situations where some products on the market do not even provide information on the minimum daily dose required to achieve a beneficial effect. Consequently, studies focused on hypolipidemic and hypocholesterolemic activities are characterized by a lack of standardization, making comparison of their results difficult **(Yilmaz *et al.*, 2025)**. Overall, these factors highlight the need for comprehensive studies that take into account all potentially relevant aspects, the conduct of further meta-analyses, and the development of clearer and more standardized regulatory frameworks for probiotics based on such evidence. The establishment of such a regulatory basis would facilitate the emergence of unified research protocols, enabling more reliable comparison of results and reducing their ambiguity.

Emerging technologies and future perspectives

With the continuous growth of data volumes, one of the promising directions in probiotic research is the use of machine learning (ML) algorithms and models to reduce the scope of experimental tasks, simplify data processing, and accelerate the generation of results with potential practical applications. This is because traditional approaches based on logical reasoning and classical bioinformatics are significantly limited compared to the capabilities of artificial intelligence in handling complex datasets commonly encountered in modern biotechnology **(Asar *et al.*, 2025; Zhou & Zhao, 2025)**. For example, **Westfall *et al.* (2021)** successfully applied an ML algorithm to predict synergistic interactions among probiotic microorganisms coexisting within an artificial GIT model, which may be useful both for the development of bacterial compositions and in personalized medicine. A similar objective was achieved by **Brunner and Chia (2024)** through computational modeling using the Lotka-Volterra model (predator-prey model). After analyzing a patient's microbiota, they were able, with fairly high accuracy, to predict whether a particular strain would successfully colonize the gut or fail to persist.

The capabilities of ML are not limited to predicting interactions or strain behavior. **McCoubrey *et al.* (2022)** implemented a novel active learning approach to identify probiotic-excipient interactions aimed at improving the delivery of microbial cells to the human gut. Using data on the interactions of the studied bacterium with six excipients and introducing three additional ones for model training, they were able to predict interactions with a further 111 compounds with an accuracy of 67.70%. As noted by the authors, the moderate predictive performance of the model is likely related to the small initial dataset and its specific composition; however, they suggest that the model can be further trained and refined if needed.

Despite promising findings supporting the feasibility of this approach, the application of ML in probiotic research remains largely limited by small datasets, heterogeneous experimental designs, and limited validation of models using independent datasets.

Significant progress is being made in the targeted selection of probiotics for the treatment of specific diseases **(Matera, 2024)**, particularly inflammatory bowel diseases and metabolic disorders. For the strains selected, not only the presence of hypocholesterolemic activity is important, but also the ability to produce the BSH enzyme **(Gadaleta *et al.*, 2022; Maqsood *et al.*, 2025)**. Since different variants of the *bsh* gene exist, leading to distinct phenotypic traits in bacteria and exerting different effects on the microbiota and human health **(Foley *et al.*, 2021; Zhou *et al.*, 2025)**, it is possible to obtain strains with specific desired properties using various genetic engineering techniques – from homologous recombination to CRISPR-Cas9. Thus, despite regulatory constraints, low public acceptance, and safety concerns, the use of genetically modified probiotic microorganisms is being considered and implemented in the food industry, as modern technologies enable the development of strains with desired characteristics, and their introduction into production requires less time than the isolation and selection of new cultures **(Ma *et al.*, 2022)**.

From the perspective of regulating hypocholesterolemic activity or influencing the organoleptic properties of the final product, genetic engineering approaches can also be applied to modulate SCFA production by LAB or bifidobacteria. Despite the challenges and limitations associated with implementation, this approach holds considerable promise for development as both clinical solutions and functional food products in the near future **(Nami *et al.*, 2025)**.

However, most genetically engineered probiotic systems have been evaluated only under experimental conditions, whereas evidence from human studies remains limited.

Thus, despite numerous obstacles, methodological constraints, regulatory shortcomings, and the lack of harmonized standards, the market for probiotics with hypocholesterolemic activity has substantial potential for expansion, advancement, and even positive transformation, provided that appropriate strategies are adopted and effective cooperation is established between researchers and regulatory authorities across different countries.

CONCLUSIONS

The formulation of a bacterial composition with hypocholesterolemic activity for food products should be based on clearly defined criteria, among which BSH activity is of primary importance (preferably toward glycine-conjugated BAs, which predominate in the human body), as well as the ability to remove cholesterol *in vitro* (in the presence of BAs) at a level of no less than 50%. Promising taxonomic groups remain LAB and bifidobacteria, whose combination within the final product may ensure functional complementarity and a potential synergistic effect.

A comprehensive evaluation of strains should encompass all key probiotic parameters, including resistance to GIT conditions, antibiotic susceptibility, autoaggregation ability, and the absence of hemolytic and DNase activities. Additional parameters and the choice of food matrix should be determined by the intended purpose of the final product. Adaptive laboratory evolution represents a promising tool for optimizing strain properties; however, its application requires a clear scientific rationale and a cautious approach.

The development of a bacterial composition with hypocholesterolemic activity requires a thorough assessment of its stability, interstrain compatibility, and impact on the food matrix, which can be achieved through model fermentations and the experimental determination of optimal storage conditions. For *in vivo* validation of efficacy, guinea pig and hamster models are the most relevant due to their combination of biological similarity to humans and practical feasibility, demonstrating higher predictive value compared to traditional mouse and rat models.

At the same time, unclear regulatory requirements, the lack of harmonized protocols for probiotic evaluation, and insufficient standardization of research methodologies remain major barriers to the advancement of the field. These limitations complicate comparisons across studies and hinder the development of a robust evidence base for the clinical efficacy of hypocholesterolemic probiotics. Coordinated collaboration between the scientific community and regulatory authorities could facilitate the development of standardized methodological approaches and create a foundation for further progress in this field, particularly through the integration of emerging technologies such as machine learning and genetic engineering.

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