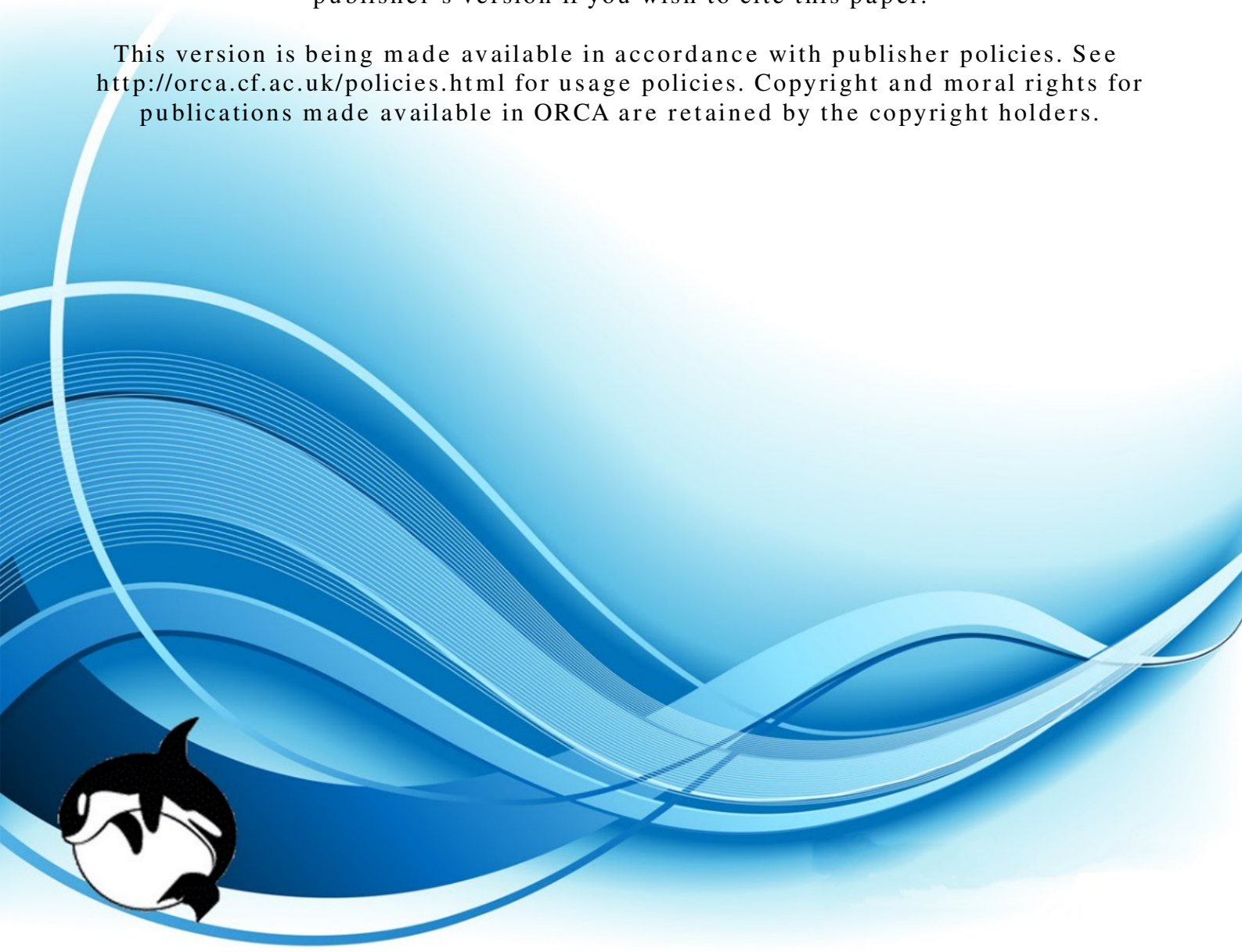

The potential of probiotics in the prevention and treatment of atherosclerosis

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Abbreviations: Apo, apolipoprotein; BET, bromodomain and extra-terminal; BP, blood pressure; BSH, bile salt hydrolase; CETP, cholesteryl ester transfer protein; CRP, C-reactive protein; CVD, cardiovascular disease; CYP7A1, cholesterol 7α-hydroxylase; ECM, extracellular matrix; FGF, fibroblast growth factor; FH, familial hypercholesterolemia; FXR, farnesoid X receptor; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HFD, high fat diet; IL, interleukin; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; LPS, lipopolysaccharide; LXR, liver X receptor; miRNA, microRNA; NF-κB, nuclear factor-κB; NO, nitric oxide; NPC1L1, Niemann-Pick C1-like 1; oxLDL, oxidized LDL; PAMPs, pathogen-associated molecular patterns; PCSK9, proprotein convertase subtilisin/kexin type 9; PRRs, pattern recognition receptors; PUFA, polyunsaturated fatty acids; RCT, reverse cholesterol transport; SCFA, short chain fatty acids; SHP, small heterodimer partner; SR, scavenger receptor; TC, total cholesterol; TG, triacylglycerol; TMAO,

trimethylamine *N*-oxide; TNF, tumour necrosis factor; TLR, toll-like receptors; VSMC, vascular smooth muscle cells

Key words: Atherosclerosis; Cardiovascular disease; Dyslipidemia; Inflammation; Nutraceuticals; Probiotics

Abstract

Atherosclerosis, the underlying cause of cardiovascular diseases such as myocardial infarction, cerebrovascular accident and peripheral vascular disease, is the leading cause of global mortality. Current therapies against atherosclerosis, which mostly target the dyslipidemia associated with the disease, have considerable residual risk for cardiovascular disease together with various side effects. In addition, the outcomes from clinical trials on many promising pharmaceutical agents against atherosclerosis (e.g. low-dose methotrexate, inhibitors against cholesteryl ester transfer protein) have been disappointing. Nutraceuticals such as probiotic bacteria have therefore generated substantial recent interest for the prevention of atherosclerosis and potentially as add-ons with current pharmaceutical drugs. This review will discuss our current understanding of the anti-atherogenic actions of probiotics from pre-clinical and clinical studies together with their potential underlying mechanisms of action.

1. Introduction

Cardiovascular disease (CVD) is responsible for one in three global deaths and poses a substantial economic burden.[1-2] Atherosclerosis is the primary cause underlying CVD-related morbidity and mortality.[1-2] It is a chronic inflammatory disease of the vasculature featuring slow onset with a marked increase in the elderly population.[1-2] The progression of atherosclerosis is largely determined by common modifiable risk factors (e.g. dyslipidemia, smoking, hypertension, diabetes, obesity) and various unmodifiable factors (e.g. age, male gender, ethnicity and genetic predisposition such as familial hypercholesterolemia (FH) and Tangier disease).[3-4] The most important causal agents of atherosclerosis are apolipoprotein (apo) B-containing lipoproteins of which low-density lipoprotein (LDL) has long been regarded as the principle driver for the initiation and progression of atherosclerotic plaques.[1-4] Indeed, a more recent evaluation of evidence from a range of meta-analyses of genetic, epidemiological and clinical studies demonstrated an unequivocal causality between LDL-cholesterol (LDL-C) and atherosclerosis-associated CVD.[5] The majority of current therapies therefore aim to reduce plasma LDL-C; however, they are associated with considerable residual risk for CVD together with various side effects.[1-2] This, together with many promising pharmaceutical leads failing at the clinical level, has fuelled substantial interest in harnessing the potential of nutraceuticals in the prevention of atherosclerosis and their use as add-ons with current pharmaceutical agents.[1-2,6] In this regard, many recent studies have highlighted the promise of probiotic bacteria. This review will discuss the pathogenesis of atherosclerosis, current therapies and their limitations, and probiotics as anti-atherogenic agents together with the mechanisms underlying their actions.

2. Pathogenesis of atherosclerosis

Atherosclerosis is an inflammatory disease of the medium and large arteries occurring predominantly at sites of low shear stress and disturbed laminar flow.[4] Endothelial cells of the arteries are particularly sensitive and susceptible to shear stress induced by laminar blood flow, especially at sites of arterial branching and curvature where disturbed flow contributes in part to the subendothelial accumulation of apoB-containing lipoproteins and lesion initiation.[3-4,7] Many recent reviews have discussed the pathogenesis of atherosclerosis and the readers are therefore directed to these for more details.[1-4] Briefly, the accumulation of LDL in the subendothelial space, either via passive diffusion or scavenger receptor (SR)-B1-driven transcytosis,[8] triggers an inflammatory response and activation or dysfunction of the endothelium.[1-4] The endothelial cells in such a state express a range of adhesion molecules and chemokines which aids in the recruitment of circulating leukocytes, particularly monocytes, to the site of LDL accumulation.[1-2] Through a process of adherence and rolling, monocytes transmigrate into the intima, where they differentiate into macrophages.[4] A wide spectrum of macrophage phenotypes has been identified, such as pro-inflammatory M1 and anti-inflammatory M2, with polarization influenced by the intimal micro-environment.[3-4] M1 polarized, pro-inflammatory macrophages represent the most abundant immune cells residing in atherosclerotic plaques, originating either from the transmigration and differentiation of circulating monocytes[1-4] or from local proliferation, which has recently been shown to significantly contribute to lesional macrophage accumulation.[9-10]

The LDL trapped in the subendothelial space undergoes both enzymatic and non-enzymatic modifications by processes such as glycation, aggregation or oxidation.[3] Oxidation represents one of the most common modifications leading to the

formation of oxidized LDL (oxLDL), a highly pro-inflammatory and pro-atherogenic molecule and a key instigator of atherogenesis.[3] During the oxidation of LDL, oxidation-specific neoepitopes are generated, which are not only immunogenic but prominent targets of pattern recognition receptors (PRRs).[11] Under atherosclerotic conditions, monocyte-derived macrophages exhibit many morphological changes, including decreased ability to migrate, a feature that contributes to the failure of inflammation resolution and to plaque progression, and increased expression of cell surface PRRs, such as SRs A and CD36, that are able to uptake modified LDL.[3,12] Macrophage SRs are classic PRRs, which readily recognize these oxidation-specific epitopes on oxLDL particles.[11] Macrophage uptake of native LDL via LDL receptors is negatively regulated by an increase in intracellular cholesterol levels.[3] In contrast, the uptake of modified LDL via macrophage SRs is unregulated, rapid and excessive.[3] In addition, other processes such as macropinocytosis, a form of fluid-phase endocytosis, contributes significantly to the uptake of LDL and modified LDL.[13] The cholesterol efflux machinery normally functions to transport excess intracellular cholesterol out of the cell either by high-density lipoprotein (HDL)-mediated passive diffusion, or to extracellular lipid acceptors for hepatic removal via reverse cholesterol transport (RCT).[3] RCT is a multi-step process responsible for the transport of excess cholesterol from peripheral tissues to the liver where it may be excreted via the bile system (see Section 11).[3] However, cholesterol efflux and associated RCT are compromised during atherosclerosis resulting in the formation of lipid-laden foam cells – the hallmark of atherosclerosis.[3]

Cholesterol-induced cytotoxicity results in increased apoptotic and necrotic cell death.[1-4] Under normal conditions, apoptosis occurs at a very high rate and apoptotic cells are rapidly cleared via efferocytosis (clearance of apoptotic cells by phagocytes,

including macrophages).[14] In early lesions, the numbers of apoptotic macrophages balance with effective efferocytosis leading to reduced plaque cellularity.[14] However, in advanced lesions, efferocytosis is ineffective resulting in an accumulation of apoptotic and necrotic cells and associated debris.[14] As the atherosclerotic plaque advances, an inflammatory response regulated by the actions of several cytokines[3,4,15,16], together with continuous accumulation of apoptotic cells and debris, pro-atherogenic lipoproteins and lipoprotein remnants, leads to secondary necrosis and the formation of a lipid-rich necrotic core.[1-4] Vascular smooth muscle cells (VSMCs) migrate from the media to the intima and contribute to extracellular matrix (ECM) remodelling and formation of a protective fibrous cap between the necrotic core and the lumen, which functions to stabilize the plaque.[1-4] VSMC also make significant contribution to foam cell formation.[17] In advanced disease with an enhanced inflammatory setting, protease action degrades the ECM, compromising the integrity of the protective cap.[1-4] Plaque vulnerability and eventual rupture results in the release of plaque contents into the lumen, thrombosis and subsequent clinical complications.[1-4]

3. Current anti-atherogenic therapies, their limitations and the potential of nutraceuticals

Statins often represent the first-line lipid-lowering therapy in global treatment guidelines.[2,18] Statins belong to a class of cholesterol-lowering pharmaceutical agents and are known for their ability to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme involved in the rate limiting step of cholesterol biosynthesis.[2,18] Inhibition of this enzyme results in a reduction of circulating LDL-C, subsequently lowering cardiovascular risk.[2,18] In addition to this LDL-C-dependent activity, statins have been

reported to exert LDL-C-independent (pleiotropic) effects, including anti-inflammatory actions.[18] However, owing to the over-shadowing effect of cholesterol reduction on cardiovascular risk, the clinical significance of these pleiotropic effects remains controversial.[18] The maximum reduction in cardiovascular mortality that can be attributed to statin therapy is approximately 22% per 1 mmol/L reduction in LDL-C, and a substantial residual cardiovascular risk is therefore associated with statin therapy as reported by a large number of studies.[2,18] Furthermore, of those patients receiving statins, a small subset is unable to achieve target plasma cholesterol levels even at the highest possible dose, while further subsets suffer intolerable statin-associated side effects such as myopathy.[19] Due to the limitations of statin therapy, a number of statin co-therapies with non-statin agents have been developed. One co-therapy involves statins in combination with the lipid-lowering agent ezetimibe, designed to reduce cholesterol absorption in the intestine by modulating the action of Niemann-Pick C1-like 1 (NPC1L1) protein.[20] In IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial), the addition of ezetimibe to statin therapy in the long-term treatment of patients following acute coronary syndrome led to a significantly lower risk of cardiovascular events than that achieved with statin monotherapy.[20] No differences were seen in cardiovascular mortality or the rate of death from any cause though there were significant reductions in the rates of myocardial infarction and ischemic stroke.[20] Furthermore, a recent comparative meta-analysis reported that statin-ezetimibe co-therapy was more effective in reducing the incidence of CVD in comparison to statin monotherapy.[21] Proprotein convertase subtilisin/kexin type 9 (PCSK9)-inhibitors have also shown potential as efficient lipid-lowering agents.[22] PCSK9 is a serine protease which binds to the LDL receptor, inducing its intracellular degradation and thereby reducing the clearance of plasma

LDL-C.[22] Monoclonal antibodies targeting PCSK9, namely alirocumab and evolocumab, have shown success in lowering LDL-C and are currently approved for use in hypercholesterolemic patients who otherwise fail to respond to statin therapy.[22] Thus, in the ODYSSEY OUTCOMES (Evaluation of Cardiovascular Outcomes After an Acute Coronary Syndrome During Treatment With Alirocumab) clinical trial of 18,924 participants, the risk of recurring cardiovascular events was reduced in patients with previous acute coronary syndrome who were on high intensity statin therapy when they received alirocumab compared to the placebo control.[23] In the FOURIER (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk) trial involving 27,564 participants with CVD and LDL-C of 70 mg/dL (1.8 mmol/L), evolocumab reduced both LDL-C and the risk of cardiovascular events compared to those receiving placebo.[24] However, due to the expensive nature of these treatments, they are restricted to high risk patients such as those with homo- or hetero-zygous FH.[22]

In addition to lipid-lowering agents, a number of alternative therapies have been investigated, including HDL elevating agents and anti-inflammatory treatments.[25-32] Low levels of HDL are known to be associated with high cardiovascular risk as demonstrated in patients with Tangier disease, while increasing levels of HDL-C is known to lower the risk of CVD.[2,25] The beneficial effects of HDL and its negative correlation with CVD is thought to be due to its role in RCT of excess cholesterol from foam cells to the liver for biliary excretion.[2,3,25] In prospective epidemiologic studies, every 1 mg/dL increase in HDL was associated with a 2-3% decrease in cardiovascular risk, independent of LDL-C and triacylglycerol (TG) levels.[25] HDL-C represents a promising target for pharmacological intervention; however, studies report conflicting results.[25] For example, niacin has been shown to reduce CVD risk

by both lowering LDL-C and elevating HDL-C in pre-clinical studies.[25] However, in the Heart Protection Study 2—Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) trial, niacin treatment did not significantly reduce major vascular events and was even associated with adverse effects.[26] Cholesteryl ester transfer protein (CETP) is responsible for the movement of esterified cholesterol from HDL to VLDL and LDL, in exchange for TG.[27] Although lower CETP levels are known to promote HDL formation, clinical trials on several CETP inhibitors have failed.[27]

Given the inflammatory nature of atherosclerosis disease, anti-inflammatory agents represent a promising therapeutic strategy for the reduction of cardiovascular risk; however, many promising candidates have failed in clinical trials.[28] In patients with high levels of the inflammatory marker, C-reactive protein (CRP), treatment with canakinumab, a monoclonal antibody that inhibits inflammation by blocking the cytokine interleukin (IL)-1β, resulted in significantly reduced incidence of atherosclerotic events than placebo in the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) trial.[29] However, patients also became prone to infection and so treatment will have to be restricted to high risk patients.[29] Despite the promising success of IL-1β blockers to date, trials of alternative anti-inflammatory agents have reported fewer encouraging outcomes. For example, one potential anti-inflammatory that is currently used in the treatment of rheumatoid arthritis is methotrexate.[4] However, the CIRT (Cardiovascular Inflammation Reduction Trial) trial of low-dose methotrexate failed to reduce the incidence of cardiovascular events in patients with hyperglycemia and high levels of CRP.[28,30] Additionally, the highly anticipated lipoprotein-associated phospholipase A2 inhibitor darapladib failed to reduce cardiovascular risk in two separate clinical trials; STABILITY (Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy) and SOLID-TIMI 52 (The

Stabilisation Of pLaques usIng Darapladib-Thrombolysis In Myocardial Infarction 52).[31] The potential of anti-inflammatory agents to reduce the incidence of CVD remains the subject of investigation as the search continues for effective anti-inflammatory therapies.[4] For example, bromodomain and extra-terminal (BET) proteins are epigenetic regulators of inflammation, lipoprotein metabolism and thromogenesis and the BETonMACE trial is investigating whether a selective BET protein inhibitor, apabetalone, improves cardiovascular outcomes in patients with acute coronary syndrome and diabetes.[32]

An alternative approach for atherosclerosis intervention involves the use of nutraceuticals, defined as foods or dietary supplements with health benefits beyond their basic nutritional value.[1-2] A number of nutraceuticals have demonstrated anti-atherogenic effects in preclinical studies and in human trials (reviewed in detail in [1]). Unlike pharmaceuticals, nutraceuticals are derived from natural compounds and are therefore considered safe for use over an extended period of time.[1-2] Among the most studied in human trials are omega-3 polyunsaturated fatty acids (PUFAs), polyphenols, phytosterols and vitamins.[1-2,33] Recently, the clinical trial -The Reduction of Cardiovascular Events with Icosapent Ethyl–Intervention Trial (REDUCE-IT), which was designed to address the residual cardiovascular risk in statin-treated patients with elevated TG, demonstrated success in further reducing cardiovascular risk.[34] In this study, icosapent ethyl, a highly purified ethyl ester of eicosapentaenoic acid omega-3 PUFA, was found to significantly reduce the risk of major adverse cardiovascular events by 25%.[34] In addition to these nutraceuticals, recent studies have revealed an association between atherosclerosis-associated CVD and gut microbial dysbiosis,[35] and probiotic bacteria have been highlighted as potential candidates for atherosclerosis intervention.

4. Probiotics in atherosclerosis

Composed of approximately 1 x 10^{14} bacteria, the gut microbiota is an essential mediator in health and disease and can be influenced by many factors, including host genetics, diet, stress and disease state.[36] The intestinal barrier is an epithelial monolayer which forms a primary interface preventing the diffusion of potentially injurious factors from the intestinal lumen into the tissue and systemic circulation.[36] Dysbiosis in the gut compromises the intestinal barrier function leading to the leakage of lipopolysaccharide (LPS) and other bacterial components (e.g. peptidoglycans) into the circulation, triggering an inflammatory response that drives atherosclerosis.[35-36] LPS is able to promote monocyte recruitment to the activated endothelium and subsequent macrophage foam cell formation by stimulating the uptake of modified LDL and reducing the efflux of cholesterol from foam cells.[35,37] LPS is also able to induce vascular inflammation either directly or via the production of pro-inflammatory factors from immune cells.[37] Toll-like receptors (TLRs) are a family of recognition receptors for pathogen associated molecular patterns (PAMPs), activated in response to bacterial components such as LPS.[35-37] Upon activation of TLRs, an inflammatory response is orchestrated via intracellular signaling cascades that induces the expression of many pro-inflammatory cytokines and chemokines.[35-37] Furthermore, microbiota-derived metabolites, including atherogenic molecules such as choline and trimethylamine *N*-oxide (TMAO) link the gut microbiome to disease (see Section 10).[37]

Probiotics are defined as microorganisms that when ingested in adequate amounts, confer a health benefit to the host.[35] Probiotics are ‘good’ bacteria that may be exploited for their ability to combat dysbiosis and promote gut health.[35] Probiotic bacteria have beneficial effects on the host by producing vitamins K and B2, together

with short chain fatty acids (SCFAs) such as acetate, butyrate or propionate, which are used as fuel by the intestinal flora and colonocytes.[38] Indeed, bacterial butyrate has been shown to prevent atherosclerosis in mouse model systems.[39] Importantly, probiotics are known to improve gut barrier function via strengthening of the epithelial tight junctions.[40] By reducing gut leakage, probiotic bacteria strengthen immunological and non-immunological gut barrier function, and reduce the translocation of microbial immunogens.[41] The implication of host gut microbiota in disease and the ability of probiotics to promote overall gut health has led to an explosion of research showing therapeutic benefits in a vast range of disease states. Indeed, probiotics are currently used for the prevention and treatment of inflammatory bowel diseases, irritable bowel syndrome, gluten intolerance, gastroenteritis and antibiotic-associated diarrhoea.[40] More recent data implicates the gut microbiota in a diverse range of diseases via the gut-brain axis, gut-lung axis, gut-liver axis and gut-vascular axis.[35,37,40,41]

Probiotic supplementation has been shown to beneficially modify a number of major atherosclerosis-associated cardiovascular risk factors, including hypercholesterolemia, dyslipidemia, hypertension and chronic inflammation (Figure 1). The anti-atherogenic effects of several different probiotic strains, as reported in human and animal studies, are summarized in Table 1. It should, however, be noted that not all probiotics are anti-atherogenic. For example, *Lactobacillus reuteri* had no effect on atherosclerosis in apolipoprotein E deficient mice ($ApoE^{-/-}$; a widely used model of atherosclerosis) fed a high fat diet (HFD).[64]

5. Probiotics and lesion formation

Only a few studies have investigated the effect of probiotic supplementation on atherosclerotic plaque formation (Table 1). VSL#3 is a consortium of 8 lyophilized lactic acid bacterial strains (*Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus plantarum,* and *Streptococcus salivarius subsp. thermophilus*).[42] Chan and colleagues have demonstrated significantly reduced HFD-induced lesion development in ApoE$^{-/-}$ mice when supplemented with the VSL#3 consortium, in addition to reduced vascular inflammation and significant reductions in plasma levels of s-intercellular adhesion molecule-1, s-vascular cell adhesion molecule-1 and s-E-selectin.[42] Another study investigated the effect of two *Lactobacillus* strains (*L. acidophilus* ATCC 4356 and 4962) on atherosclerosis development in ApoE$^{-/-}$ mice.[48] The authors reported a dramatic reduction in atherosclerotic lesion area in the L.4356 group; however, no significant effect was observed in the L.4962 group.[48] In the same study, the plasma levels of total cholesterol (TC) and non-HDL-C-containing lipoproteins were significantly reduced and a significant decrease in cholesterol absorption was observed.[48] In a separate study, *L. acidophilus* ATCC 4356 was again shown to reduce atherosclerosis lesion development in ApoE$^{-/-}$ mice, in addition to reduced plasma oxLDL and tumour necrosis factor (TNF)-α levels, and increased plasma IL-10 indicating a beneficial effect on inflammatory markers.[47]

6. Probiotics and dyslipidemia

The beneficial effects of probiotic supplementation on plasma lipids is well documented and a large number of meta-analyses concur that probiotics are associated with a significant reduction in TC and LDL-C.[65-67] One meta-analysis of the effects of probiotic supplementation on lipid profiles of normal and

hypercholesterolemic individuals included 26 clinical studies utilizing fermented milk products and probiotic supplements.[65] Probiotic supplementation resulted in a significant reduction in plasma TC and LDL-C, with no change in HDL-C or TG. Subgroup analysis revealed a statistically greater reduction in TC and LDL-C with long-term (>4 weeks) probiotic intervention. The authors highlighted *Lactobacillus acidophilus* as the strain most effective in reducing TC and LDL-C.[65] A further meta-analysis included 15 clinical studies with 788 participants.[66] Significant pooled effects of probiotics were achieved for the reduction of TC, LDL-C, body mass index and inflammatory markers.[66] Subgroup analysis revealed statistically greater reductions in TC and LDL-C with long-term (>8 weeks) intervention, and with multiple versus single probiotic strains. Again *Lactobacillus acidophilus* was highlighted as the most effective strain in reducing LDL-C.[66] A more recent meta-analysis of 32 clinical trials and 1971 patients also reported a significant reduction in TC with probiotic supplementation.[67] Subgroup analysis suggested that a difference in baseline TC as well as the duration of intervention may significantly impact results; however, the probiotic strain and the dose were found to have no significant influence.[67] Although there is evidence that probiotic bacteria are able to influence host lipid profiles, the exact mechanisms of action remain poorly understood (see Section 11).[65-67]

7. Probiotics and endothelial dysfunction

Endothelial dysfunction mediated by various CVD risk factors is a critical early event in the pathogenesis of atherosclerosis.[1-4] Probiotics have been shown to attenuate several pro-atherogenic changes associated with endothelial dysfunction via multiple mechanisms, including increasing availability of nitric oxide (NO), improving oxidative stress, restoring endothelial architecture, recruitment of endothelial progenitor cells and improving vascular inflammation.[68] For example, ingestion of

VSL#3 attenuated endothelial dysfunction that was associated with improved vascular oxidative stress in the mesenteric artery of rats following common bile duct ligation.[69] In addition, *Lactobacillus coryniformis* CECT5711 reversed endothelial dysfunction in obese mice by increasing NO bioavailability.[70] The probiotic kefir also improved endothelial dysfunction in spontaneously hypertensive rats by decreasing ROS production, increasing NO bioavailability and restoring the recruitment of endothelial progenitor cells.[71] Similarly, *Lactobacillus fermentum* improved tacrolimus-induced endothelial dysfunction by reducing vascular oxidative stress and inflammation.[72] The beneficial actions of probiotics on endothelial dysfunction has also been seen in some human studies.[68,73] Thus, a clinical trial of 81 women showed that multispecies probiotic supplementation improved several parameters of endothelial dysfunction [e.g. systolic blood pressure (BP), vascular endothelial growth factor, pulse wave velocity, inflammatory cytokines).[73] However, no significant changes in endothelial dysfunction were observed in a study of 30 subjects with metabolic syndrome that received *Lactobacillus casei Shirota*.[74] It is therefore essential that further research is carried out on the impact of probiotics on endothelial dysfunction especially because of its importance not only in atherosclerosis but other diseases such as diabetes, obesity and chronic renal failure.

8. Probiotics and inflammation

Probiotics are known to modify the host immune responses.[75-76] However, interactions between probiotic bacteria, the gut and the host immune systems are highly complex and despite increasingly growing clinical evidence, remains poorly understood.[75-76] In a recent study, a reduction in atherosclerotic lesion development was accompanied by the suppression of interferon-γ-producing $CD4^+$ T cells and pro-inflammatory cytokine production in *Pediococcus acidilactici* R037-treated mice.[45] In

addition to a reduction in pro-inflammatory T cells, probiotic bacteria have been shown to decrease inflammation via an increase in regulatory T cells.[77] DNA from the VSL#3 consortium has been shown to limit epithelial proinflammatory responses *in vivo* and *in vitro*, and to attenuate systemic release of TNF-α in response to *Escherichia coli* DNA injection.[78] In addition, VSL#3 was found to reduce vascular inflammation, including the expression of adhesion proteins, in ApoE$^{-/-}$ mice fed a HFD.[42] In a separate study, VSL#3 DNA was shown to exert anti-inflammatory effects via TLR9 signaling;[79] interestingly, the authors concluded that the protective anti-inflammatory effects of the probiotics were mediated via their own DNA rather than metabolites, and that TLR9 signaling is essential in mediating this effect.

9. Probiotics, hypertension and hyperglycemia

In a meta-analysis of the effect of probiotics on hypertension, the authors reported a significant reduction in systolic and diastolic BP.[80] Subgroup analysis revealed a greater reduction achieved with long-term treatment duration (>8 weeks), where durations of <8 weeks showed no significant changes.[80] Additionally, the inclusion of multiple compared to single strains, and daily consumption of doses ≥10^{11} colony forming units, were associated with significant reductions in both systolic and diastolic BP.[80] Furthermore, a recent meta-analysis of 11 clinical studies also reported beneficial effects of probiotic supplementation on both hypertension and dyslipidemia in diabetic patients.[81] Pooled data demonstrated significantly reduced systolic and diastolic BP in addition to plasma LDL-C, TC and TG.[81] Similarly, some studies investigating probiotic supplementation in relation to type 2 diabetes and insulin resistance have shown success.[82-84] In a meta-analysis of 22 cohort studies and 579,832 individuals, total dairy consumption was inversely associated with type 2 diabetes risk, where yogurt consumption was reported to be particularly effective.[82] A

separate meta-analysis of 10 clinical trials reported significantly reduced fasting blood glucose, systolic and diastolic BP, and plasma TC, LDL-C and TG in type 2 diabetic patients with probiotic supplementation.[85] Further studies have demonstrated probiotic-associated reduction in blood glucose levels and/or insulin resistance with various strains of *Lactobacillus, Bifidobacterium* and *Streptococcus*.[51,52,62] However, the association between probiotic treatment and diabetes is less clear with a small number of studies showing no such association.[86] For example, in a recent clinical trial, type 2 diabetic patients receiving a consortium of 8 different probiotic strains exhibited significantly higher median glucose levels, in addition to higher circulating TC and LDL-C.[86]

10. Probiotics and TMAO

In addition to their effects on atherogenic risk factors, probiotic bacteria have been shown to affect the production of potentially atherogenic metabolites.[35,37] A key metabolite currently receiving attention for its strong association with atherosclerosis is TMAO.[87] The bacterial metabolite trimethylamine is produced by the gut microbiota from dietary choline, phosphatidylcholine and L-carnitine, then oxidized to TMAO in the liver and released into the circulation.[87] It has been suggested that TMAO contributes to atherosclerosis in part by promoting macrophage foam cell formation in atherosclerotic lesions as well as ineffective RCT and disruption of lipid homeostasis.[76,87-88] For example, TMAO increased the macrophage expression of SR CD36, thereby promoting the uptake of modified LDL, and reduced the expression of enzymes involved in the synthesis of bile acids that are involved in RCT.[35] In addition, TMAO has been shown to promote vascular inflammation, activation of arterial endothelial cells and thrombosis.[37] Probiotic treatment has been shown to reduce levels of TMAO in correlation with decreased atherosclerosis development;[89]

however, this effect is strain specific. A recent study investigated potential TMAO-lowering property of five different probiotic strains.[49] Only *Lactobacillus plantarum* ZDY04 was able to significantly reduce plasma TMAO, and it was suggested that this effect was achieved via remodelling of the gut microbiota.[49] Furthermore, *Lactobacillus plantarum* ZDY04 significantly attenuated the development of TMAO-induced atherosclerosis in ApoE$^{-/-}$ choline-fed mice[49] In a similar study, plasma TMAO was significantly reduced in choline-fed mice treated with *Enterobacter aerogenes* ZDY01, and a similar remodelling of gut microbiota was demonstrated.[90] However, the beneficial effect of probiotic bacteria on TMAO production is strain specific and a human study investigating *Streptococcus thermophilus* (KB19), *Lactobacillus acidophilus* (KB27), and *Bifidobacteria longum* (KB31), reported no change in TMAO levels.[91] Similarly, supplementation with *Lactobacillus casei Shirota* for 12 weeks had no effect on TMAO levels in patients with metabolic syndrome.[82]

11. Potential mechanisms underlying the anti-atherogenic actions of probiotics

The molecular mechanisms underlying the anti-atherogenic actions of probiotics are not fully understood. However, what is clear is that probiotics act at multiple steps (Figure 2). For example, probiotics combat gut dysbiosis by strengthening the epithelial tight junctions, which then prevents the leakage of microbial immunogens (e.g. LPS) and other pro-atherogenic factors such as TMAO.[35] This is achieved in part via production of glucagon-like peptide 2 that modulates the expression of intestinal tight junction proteins.[35] The role of TMAO in atherosclerosis has been described above (Section 10). Leakage of LPS into systemic circulation is a major pro-atherogenic contributor during dysbiosis.[35] LPS is known to promote

inflammation by binding to cell surface TLR4 receptor that are expressed at high levels in myelomonocytic cells.[35] Signaling by LPS involves a cell surface complex of TLR4 and its co-receptors cluster of differentiation 14 and myeloid differentiation protein-1. The intracellular domain of TLR4 then activates many signaling pathways leading to increased synthesis of pro-inflammatory molecules such as cytokines and chemokines, hence promoting inflammation in plaques.[35] Thus, the chemokines stimulate the recruitment of monocytes to the activated arterial endothelial cells, thereby increase plaque macrophage burden and associated inflammation.[35] LPS and pro-inflammatory cytokines produced by its actions also promote macrophage foam cell formation in atherosclerotic plaques.[35] LPS inhibits the actions of liver X receptors (LXR), which stimulate macrophage cholesterol efflux.[35] In addition, pro-inflammatory cytokines produced by the action of LPS inhibit the expression of key transporters (ATP-binding cassette transporters A1 and G1) that also stimulate cholesterol efflux from foam cells.[35] The increased production of pro-inflammatory cytokines also affects risk factors such as hypertension by promoting oxidative stress and the levels of oxLDL.[35] Thus, high levels of oxLDL inhibit the action of nitric oxide synthase, thereby reducing the levels of the vasodilator NO, and increase the production of the vasoconstrictor endothelin-1.[35]

Primary bile acids are synthesized from cholesterol in the liver and secreted into the small intestine via the gall bladder where they aid in the digestion of lipids via emulsification.[93] About 95% of the bile acids are reabsorbed back from the small intestine though some pass through the feces, thereby providing a route for the body to eliminate cholesterol.[35,37] The enterohepatic circulation of bile acids is tightly regulated via the hepatic farnesoid X receptor (FXR) as high levels of bile acids are toxic to the cells.[93] FXR modulates the transcription of many enzymes involved in the

synthesis, conjugation, detoxification and transport of bile acids.[93] Thus, activation of hepatic FXR by bile acids inhibits the expression of cholesterol 7α-hydroxylase (CYP7A1), a rate limiting enzyme in the *de novo* biosynthesis of bile acids from cholesterol.[93] FXR does not directly bind to the CYP7A1 promoter but acts via induced expression of small heterodimer partner (SHP), which then inhibits the transcription of the CYP7A1 gene by suppressing the activity of liver receptor homologue 1, a transcription factor involved in transactivation of CYP7A1[93] FXR also suppresses CYP7A1 expression via mouse fibroblast growth factor-15-c-Jun N-terminal kinase (FGF15-JNK) axis (FGF19-JNK axis in humans).[93] FGF15/19 is secreted by the intestine and returns to the liver via enterohepatic circulation and acts via FGF receptor 4.[93] These pathways are affected by certain probiotics with bile salt hydrolase (BSH) activity, which makes a major contribution to their cholesterol lowering activities.[35,37,94,95] BSHs deconjugate primary bile acids forming deconjugated secondary bile salts, which are less soluble and are less efficiently reabsorbed from the intestine.[94-97] Deconjugated bile salts are therefore excreted in the feces, which creates greater demand for the *de novo* synthesis of bile acids to replace those lost in the feces.[33,35, 96-97] As cholesterol is a precursor of bile salts, this deconjugation and loss of bile acids results in a cholesterol-lowering effect in part via increased mobilization of plasma cholesterol by the liver for *de novo* bile acid synthesis via inhibition of FXR and thereby increased transcription of the CYP7A1 gene.[35,37, 96-97] Deconjugated bile acids are also less efficient in the solubilization and absorption of lipids in the gut leading to reduced absorption of cholesterol from the intestinal lumen.[35,37,96-97]

Some probiotics are also able to inhibit the expression of FXR and/or SHP, and thereby increase the expression of CYP7A1, independent of their BSH activity.[35,37,96-

[97] This together with increased expression of the LDL receptor induces the liver uptake of cholesterol and its subsequent metabolism into primary bile acids. However, other mechanisms have also been identified.[98-101] For example, *Lactobacillus acidiphilus K301* inhibited atherogenesis in HFD-fed ApoE$^{-/-}$ mice via a mechanism involving increased macrophage cholesterol efflux and associated RCT via production of 24(S), 25-epoxycholesterol, an endogenous ligand for LXRs, which induces an anti-foam cell and anti-inflammatory transcriptional program.[98] *Lactobacillus acidophilus* ATCC 4356 also prevented atherosclerosis in HFD-fed ApoE$^{-/-}$ mice by stimulation of macrophage cholesterol efflux and RCT via activation of the LXR pathway.[48] In addition, intestinal cholesterol absorption was inhibited by modulation of NPC1L1 expression.[48] This correlates well with *in vitro* studies in Caco-2 enterocytes that demonstrated reduced absorption of cholesterol by *Lactobacillus rhamnosus* BFE5264, *Lactobacillus plantarum* NR74 and *Lactobacillus acidophilus* via suppression of NPC1L1 expression.[99-100] Promotion of cholesterol efflux in enterocytes via induced expression of ABCG5/8 also represents another mechanism for probiotic-mediated decrease in hypercholesterolemia as demonstrated by studies on Caco-2 cells with *Lactobacillus rhamnosus* BFE 5264 and *Lactobacillus plantarum* NR74.[101] As detailed above, some probiotics can also induce the expression of LXR that is involved in transcriptional activation of the ABCG5/8 genes.[48,98] Probiotics have also been shown to bind to or even use cholesterol from the intestine by incorporation into cellular membranes.[96] In addition, probiotics are able to metabolize cholesterol to coprostanol, which can ultimately be lost in the feces and thereby reduce intestinal cholesterol absorption.[96]

Mechanisms of immunomodulation have also been investigated and thought to be achieved via changes in cytokine production and modulation of associated

signaling pathways in intestinal epithelial and immune cells.[38,102-103] This has been demonstrated via probiotic release of bioactive metabolites and immunomodulatory factors.[38,102-103] SCFAs, such as butyrate, propionate and acetate, produced by the probiotic bacteria have multiple anti-atherogenic actions.[38] Butyrate in particular has been shown to decrease adhesion of monocytes to the activated endothelium and the expression of pro-inflammatory cytokines and adhesion proteins by inhibiting the cytoplasmic to nuclear translocation of nuclear factor-κB (NF-κB).[38-39] Many of the actions of SCFA are mediated via binding to cell surface G-protein coupled receptors and associated signaling pathways in target cells.[38] In addition, histamine derived from *Lactobacillus reuteri* suppressed pro-inflammatory TNF production via transcriptional regulation through protein kinase A and extracellular signal-regulated kinase signaling.[104] Furthermore, S-layer protein A produced by the probiotic *Lactobacillus acidophilus* NCFM has been shown to bind to the intestinal dendritic cell surface receptor (dendritic cell-specific ICAM-3-grabbing nonintegrin) to induce the production of anti-inflammatory IL-10 in a dose-dependent manner.[105] Probiotics can also exert anti-inflammatory actions by modulating the expression of key transcription factors or microRNAs (miRNAs) implicated in pro-inflammatory signalling.[47,106] For example, *Lactobacillus acidophilus* ATCC 4356 attenuated the HFD-induced levels of TNF-α and markers of oxidative stress, and reversed the reduction in IL-10 levels, in ApoE$^{-/-}$ mice via a mechanism involving inhibition of NF-κB translocation from the cytoplasm to the nucleus.[47] In relation to miRNAs, *Lactobacillus acidophilus* was found to protect apoptosis and necrosis of human endothelial cells induced by LPS stimulation and this was associated with decreased expression of pro-inflammatory miR-155 and increased expression of anti-apoptotic mIR-21.[106] In addition, in dextran sodium suphate model of mouse colitis, the probiotics *Lactobacillus fermentum*,

Lactobacillus salivarius or *Saccharomyces boulardii* reduced the expression of pro-inflammatory miR-155 and miR-223 and this was associated with intestinal anti-inflammatory effects.[107,108] However, further research is required on the impact of probiotics on the expression of miRNAs associated with atherosclerosis and the impact of such changes on key cellular processes associated with the disease.

Conclusions

A large body of evidence suggests a beneficial role for probiotic bacteria in the management of many atherogenic risk factors. *Lactobacillus acidophilus* in particular has shown promise in many human and animal studies where a variety of strains have had significant beneficial effects on atherosclerotic plaque development, plasma lipid profile, pro-inflammatory markers, and even insulin resistance and blood glucose levels (Table 1). This makes probiotics a promising nutraceutical in the prevention and treatment of atherosclerosis. However, many anti-atherogenic effects are subject to strain specific variation and a number of further studies have shown no effect, or even pro-atherogenic effects of probiotic treatment.[35-37,64,96,97] Differences in experimental design, such as concentration and duration of treatment, together with model systems used may have contributed to the discrepancy in the literature. Moreover, little is understood about the mechanisms underlying the observed effects of probiotics on host health. Future studies should focus on more mechanism-based animal studies using several different concentrations of various probiotics using a consistent experimental design. In addition, large clinical trials as REDUCE-IT[34] detailed in Section 3 are required. We are indeed entering an exciting phase in probiotic research.

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Conflict of Interest

None

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Figure Legends

Figure 1. Probiotics beneficially modulate a number of atherosclerosis-associated cardiovascular risk factors.

Abbreviations: HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TG, triacylglycerol; TMAO, trimethylamine *N*-oxide.

Figure 2. The actions of probiotics are mediated via multiple mechanisms.

Many mechanisms are utilized by probiotic bacteria to mediate beneficial anti-atherogenic actions, including combating gut dysbiosis and leakage of microbial immunogens, modulation of hepatic bile acid and cholesterol biosynthesis through pathways initiated by their bile salt hydrolase activity and/or direct actions on the expression and activities of key enzymes, or by production of beneficial metabolites (see text for more details). ↑, increase; ↓, decrease. Abbreviations: ABCA1/G1, ATP-binding cassette transporter A1/G1; ABCG5/8, ATP-binding cassette transporter G5/8; BSH, bile salt hydrolase; FXR, farnesoid X receptor; IL-10, interleukin-10; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; LXR, liver X receptor; mIR, micro RNA; NF-κB, nuclear factor-κB; NO, nitric oxide; NPC1L1, Niemann-Pick C1-like 1; oxLDL, oxidized LDL; RCT, reverse cholesterol transport; SCFA, short chain fatty acids; SHP, small heterodimer partner; TMAO, trimethylamine *N*-oxide; TLR4, toll-like receptor 4.

Table 1. The athero-protective effects of probiotic bacteria

Probiotics	Anti-atherogenic effects	Study group	References
VSL#3	Reduced lesion development; decreased vascular inflammation	ApoE$^{-/-}$ mice	42
L. rhamnosus GG	Reduced lesion development; decreased plasma cholesterol, sE-selectin, sICAM-1, sVCAM-1 and endotoxin	ApoE$^{-/-}$ mice	43
E. faecium CRL183	Increased HDL-C; reduced TG; no change in plaque size	Hypercholesterolemic rabbits	44
P. acidilactici R037	Reduced lesion development; decreased production of pro-inflammatory cytokines and CD4^{+} T cells	ApoE$^{-/-}$ mice	45
L. acidophilus 145 *and B. longum* 913	Increased HDL-C; reduced LDL:HDL ratio	Human	46
L. acidophilus ATCC 4356	Reduced lesion development; decreased plasma cholesterol, oxLDL and TNF- ; increased plasma IL-10	ApoE$^{-/-}$ mice	47-48
L. plantarum ZDY04	Reduced TMAO-induced lesion development; decreased plasma TMAO	ApoE$^{-/-}$ mice	49
L. acidophilus and *B. bifidum*	Reduced TC, HDL-C and LDL-C	Human	50
VSL#3	Reduced TC, LDL-C, TG, hsCRP; increased HDL-C and improved insulin sensitivity	Human	51
S. thermophiles, *L. bulgaricus*, *L. acidophilus* LA5, *B. lactis* BB12	Reduced TC, LDL-C, insulin resistance, postprandial blood glucose and fasting insulin	Human	52
L. plantarum LRCC 5273	Reduced TC and LDL-C; induced expression of LXR-	C57BL/6 mice	53
S. cerevisiae ARDMC1	Reduced TC, LDL-C, TG	Wister rats	54
L. plantarum ECGC 13110402	Reduced TC, LDL-C, TG; increased HDL-C	Human	55

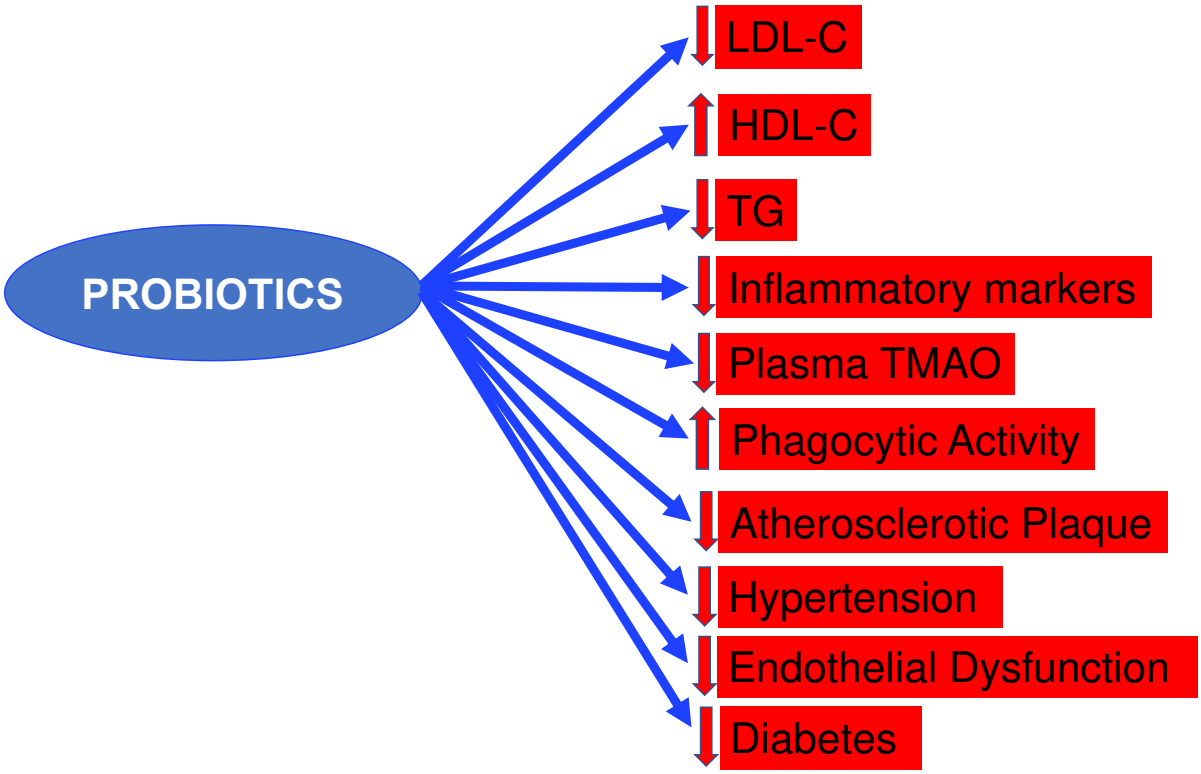

PROBIOTICS
LDL-C
HDL-C
TG
Inflammatory markers
Plasma TMAO
Phagocytic Activity
Atherosclerotic Plaque
Hypertension
Endothelial Dysfunction
Diabetes

Figure 1

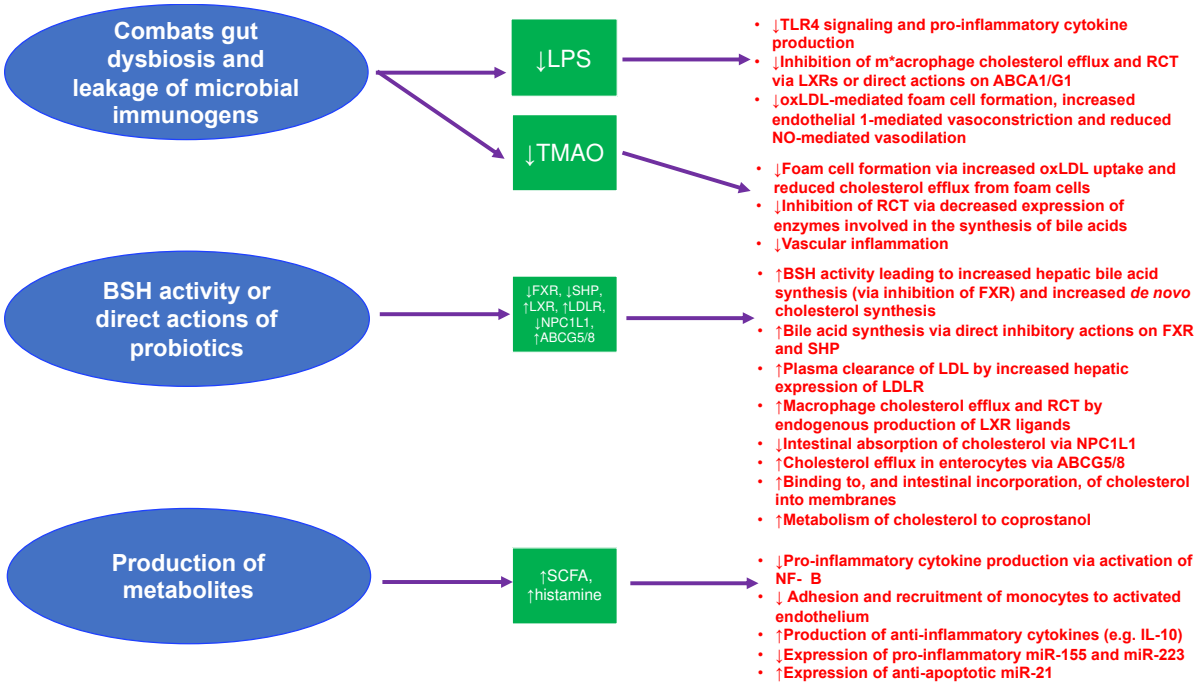
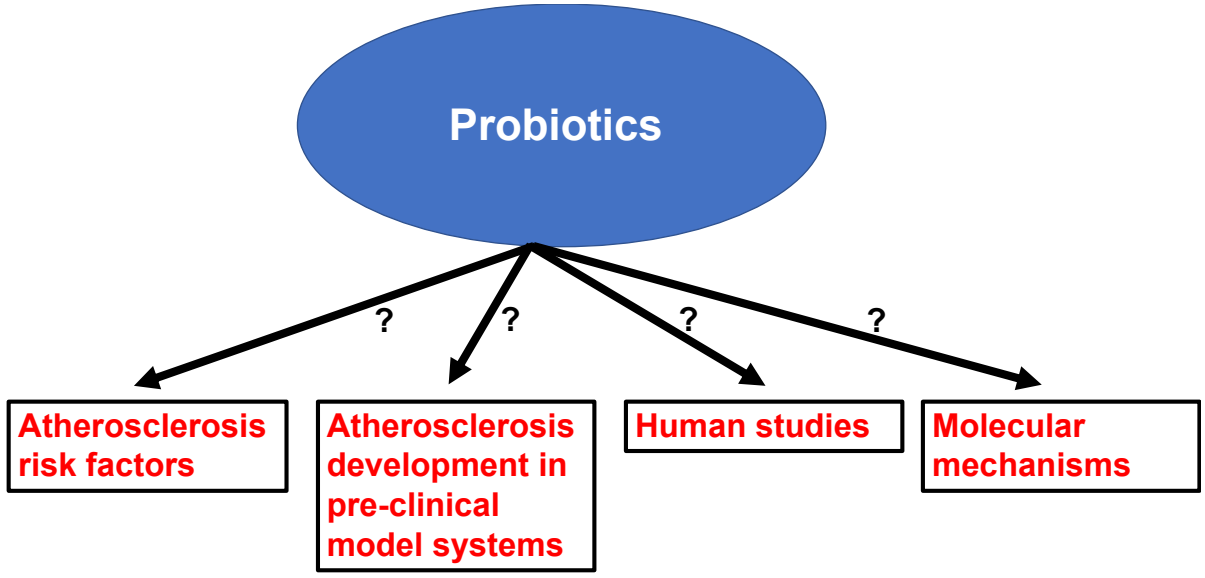

Combats gut dysbiosis and leakage of microbial immunogens
↓LPS
↓TMAO
• ↓TLR4 signaling and pro-inflammatory cytokine production
• ↓Inhibition of m*acrophage cholesterol efflux and RCT via LXRs or direct actions on ABCA1/G1
• ↓oxLDL-mediated foam cell formation, increased endothelial 1-mediated vasoconstriction and reduced NO-mediated vasodilation
• ↓Foam cell formation via increased oxLDL uptake and reduced cholesterol efflux from foam cells
• ↓Inhibition of RCT via decreased expression of enzymes involved in the synthesis of bile acids
• ↓Vascular inflammation
BSH activity or direct actions of probiotics
↓FXR, ↓SHP, ↑LXR, ↑LDLR, ↓NPC1L1, ↑ABCG5/8
• ↑BSH activity leading to increased hepatic bile acid synthesis (via inhibition of FXR) and increased *de novo* cholesterol synthesis
• ↑Bile acid synthesis via direct inhibitory actions on FXR and SHP
• ↑Plasma clearance of LDL by increased hepatic expression of LDLR
• ↑Macrophage cholesterol efflux and RCT by endogenous production of LXR ligands
• ↓Intestinal absorption of cholesterol via NPC1L1
• ↑Cholesterol efflux in enterocytes via ABCG5/8
• ↑Binding to, and intestinal incorporation, of cholesterol into membranes
• ↑Metabolism of cholesterol to coprostanol
Production of metabolites
↑SCFA, ↑histamine
• ↓Pro-inflammatory cytokine production via activation of NF- B
• ↓ Adhesion and recruitment of monocytes to activated endothelium
• ↑Production of anti-inflammatory cytokines (e.g. IL-10)
• ↓Expression of pro-inflammatory miR-155 and miR-223
• ↑Expression of anti-apoptotic miR-21

Figure 2



Graphical Abstract