Concept and Protocol to Isolate Cholesterol-reducing Bacteria from Carnivores

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Abstract: Cholesterol plays a major role in human heart health. However, the body makes enough cholesterol, so any dietary cholesterol isn't needed. High cholesterol is a leading risk factor for human cardiovascular disease such as coronary heart disease and stroke. Excess cholesterol in the bloodstream can form plaque (a thick, hard deposit) in artery walls. It is necessary for animal to have bacteria to reduce cholesterol in the blood. Lactobacillus bacteria and bifidobacteria decrease blood levels of cholesterol by either increased intestinal absorption and removal through solid excretion, or by limiting the conversion of cholesterol back into bile acids for storage in the liver. Certain bacteria have been known to degrade serum cholesterol in gut of animals. Carnivores (predators) normally eat meat containing high fat and rarely develop atherosclerosis or have cardiovascular events. It is possible to select and characterize strains of bacteria from carnivores that remove cholesterol from blood serum or control blood serum cholesterol level. [Nature and Science. 2004;2(4): 11-17].

Keywords: animal; bacteria; cholesterol; cardiovascular disease; carnivore (predator)

1. Introduction

Cholesterol plays a major role in human cardiovascular pathogenesis. Normally it is needed in the body to insulate nerves, make cell membranes and produce certain hormones. However, the body makes enough cholesterol, so dietary cholesterol intake is not essential for normal adult metabolism. High serum cholesterol level is a leading risk factor for human cardiovascular disease such as coronary heart disease and stroke - America's number one killer (Tabas, 2002). Excess cholesterol in the bloodstream can form plaque in arterial walls. The cholesterol and plaque build-up causes the arteries to become thicker, harder and less flexible, slowing down or/and sometimes blocking blood flow to the heart and other vital organs. When blood flow is restricted, angina (chest pain) can result. A heart attack will result when blood flow to the heart is severely impaired and a clot stops blood flow completely. When too much low-density lipoprotein (LDL) deposits inside the arterial walls, where if is is oxidized, it can build up as hard deposits and cause atherosclerosis, the disease process that underlies heart attacks. There are 102.3 million American adults who have total blood cholesterol values of 200 mg/dl and higher, and about 41.3 million American adults have levels of 240 mg/dl of cholesterol or above. The control of serum cholesterol is the major concern of the modern medicine. Oxidation is important relevant to atheroscelrosis (Steinberg, 2002). However, certain types of bacteria have the ability to inhibit LDL oxidation and, thus, attenuate atherogenesis (Lavy, 2004; Kullisaar, 2003; Terahara, 2000; Terahara, 2001).

Lactobacillus bacteria and bifidobacteria in the host decrease blood cholesterol levels by either decreased total intestinal absorption or removal through solid excretion, or by interrupting the entero-hepatic cycle of bile acids (James, 1999). Investigation at the Shinshu University in Japan demonstrated that *Lactobacillus acidophilus* bacteria suppressed the re-absorption of bile acids carrying cholesterol and enhanced the removal of cholesterol from blood through faeces (Hosono, 2000). Another study demonstrated that increased lactobacillus bacteria in intestine lowered total blood cholesterol by 22% and triglycerides by 33% (Taranto, 1999). Yet, another study demonstrated that lactobacillus bacteria significantly lowered blood pressure in men and women 18 to 55 years of age after eight weeks of supplementation (Agerholm-Larsen, 2000). Subjects in the control group who did not receive the selected strains of lactobacillus bacteria did not experience a drop in their high blood pressure. Therefore, there is significant evidence that specific types of lactobacillus bacteria and bifidobacteria can lower the

three major risk factors for coronary heart disease and stroke: excessive cholesterol, high blood pressure, and high triglyceride levels. However, the cholesterol reducing bacteria have to be carefully selected to achieve the best results. Not all strains of *Lactobacillus acidophilus, Bifidobacteria bifidum*, and *Lactobacillus bulgaricus* bacteria available will lower cholesterol.

Lactobacillus acidophilus occurs naturally in the human intestinal tract. Hundreds of strains may exist, and the wide degree of variation among strains is an important characteristic in determining their potential for nutrition and health benefits. These benefits include the digestion of lactose (milk sugar) as well as reducing serum cholesterol. *Lactobacillus acidophilus* Gilliland was one of the more effective strains at reducing serum cholesterol from among 123 new isolates tested (Gilliland, 2004). These investigators found two mechanisms that intestinal bacteria can lower serum cholesterol: first, as the bacteria grow in the intestinal tract, they consume cholesterol; second, at least part of the cholesterol actually becomes incorporated into the bacterial cells. Either way, the cholesterol becomes unavailable for absorption from the gut. Numerous studies, however, have demonstrated that *Lactobacillus acidophilus*, *Bifidobacteria bifidum*, and *Lactobacillus bulgaricus* bacteria in animal gut significantly lower cholesterol.

Adjustment of cholesterol level is critical for prevention of cardiovascular events. Carnivores (predators) normally eat meat containing high fat and rarely develop atherosclerosis or have cardiovascular events. If the elevated cholesterol problem in omnivores is linked to a high fat diet, introducing the beneficial bacteria into the omnivorous intestine as a supplement may offer a natural way lower cholesterol levels. There are numerous studies on the cholesterol reducing effects of bacteria in omnivores, but few reports describe cholesterol lowering in carnivores. It is very possible to isolate and characterize bacteria with cholesterol reducing ability in carnivores. These can be tested in an atherosclerotic rabbit model of plaque disruption and thrombosis. This article gives the ideas and methods to do that as a reference for anybody who is interested in this trial.

2. Material and Methods

The purpose of this article is to offer the ideas and methods to isolate and characterize specific strains of bacteria from carnivores that have the ability to reduce cholesterol from the serum of atherosclerotic and hyperlipidemic rabbits. The steps could be:

1. To isolate and culture the bacteria from carnivores (such as tiger, lion and dog) feces or intestine and to screen the specific bacteria strains that reduces cholesterol in serum.

2. To characterize and evaluate the cholesterol-reducing candidate bacteria strains from Step 1 *in vitro*.

3. To determine the bacterial strains if they can be introduced to omnivora or herbivores (such as rabbit) orally then to get the conclusion that the bacteria to function serum cholesterol level reduction without harmful side effect.

4. To test if the bacterial strains and/or their byproducts can be used as an oral medication.

2.1 Isolation of Bacteria

Feces or intestinal contents can be obtained from tigers, lions or dogs, and immediately placed in an aerobe jar, and then transported to an anaerobe container within 20 to 30 min. A 0.5 g portion of feces or intestinal contents is serially ten fold diluted in standard medium to give a bacterial content of 10^{10}/ml. A drop of the 10^2 and 10^3 dilutions is transferred with a sealed pipette to plates and streaked for isolation. The plates and the ten serial diluted tubes are incubated at 35^{o}C. The plates are observed after 3-5 days of incubation. Colonies showing the unusual morphology are inoculated until the medium is clotted, which occurs as the cholesterol-reducing bacteria approaches 10^8/ml. The clotted cultures are streaked to cholesterol agar medium to check purity and analyzed for coprostanol by gas-liquid chromatography. If suspect colonies are not present on the initial plates, the researchers should observe the serial dilution tubes for the clotting. Since large numbers of cholesterol-reducing bacteria will be present when the medium clots, researchers can streak a drop of medium from freshly clotted tubes of the dilution set on cholesterol agar medium. Several subcultures can be made in standard cholesterol agar medium to enhance the numbers of cholesterol-reducing bacteria before streaking to cholesterol agar medium (Brinkley, 1982). The isolation procedures

and biochemical testing are performed in a modified stainless steel Blickmnan chamber under anaerobic conditions (Brinkley, 1978). The gas mixture contains 10% H_2, 10% CO_2 and 80% N_2 (Pereira and Gibson, 2002). The chamber humidity is maintained at 45% relative humidity by a mechanical condenser. The cultures are incubated at 35°C in the chamber. All media are prepared aerobically and passed into the camber at least 24 hours before use.

2.2 Growth of Bacteria

Standard technique of bacteria culture is used. Briefly, bacteria sample is dissolved in 0.3 ml LB plus tetracycline (2 mg/ml) medium, transferred into a tube containing 5 ml LB plus tetracycline (2 mg/ml) medium, kept at 37°C overnight with shaking, then the bacteria frozen at –70°C. These can be divided into several tubes before freezing.

2.3 Harvesting Bacteria

A. Streak an inoculum across one side of a plate using sterile technique. Re-sterilize an inoculating loop and streak a sample from the first streak across a fresh part of the plate, and then incubate at 37°C until colonies appear (overnight).

B. Transfer a single bacterial colony into 2 ml of LB medium containing tetracycline (2 mg/ml) in a loosely capped 15-ml tube. These should be kept at 37°C overnight in Elmer flasks with vigorous shaking to enhance bacterial growth.

C. Pour 1.5 ml of the culture into a micro-centrifuge tube. Centrifuge at 12,000 rpm for 30 seconds at 4°C in a micro-centrifuge. Store the remainder of the culture at 4°C.

D. Remove the medium by aspiration.

2.4 LB Medium (Large-Bertani Medium, per liter)

To 950 ml of deionized H_2O, add: bacto-tryptone 10 g, bacto-yeast extract 5 g, and NaCl 10 g. Shake until the solutes have dissolved. Adjust to pH 7.0 with 5 N NaOH (about 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilized by autoclaving for 20 minutes in a liquid cycle at 1 kg/cm^2.

2.5 Cholesterol and Plasmalogen Requirements

All isolates are streaked on supplemented cholesterol agar medium to test their ability to grow without cholesterol and plasmalogen. Isolates that grow are repeatedly subcultured on cholesterol agar medium to ensure that cholesterol and plasmalogen in the inoculum are not responsible for the growth. All isolates are inoculated into lecithin-cholesterol medium to test for cholesterol deduction in the absence of plasmalogen. Each isolate is also inoculated into lecithin-cholesterol medium containing 0.5 mg of PLE as a positive control. Cultures which reduce cholesterol in the lecithin-cholesterol medium without PLE are repeatedly subcultured to confirm the results.

2.6 Bacteria Characterization

All isolated strains could be tested for reduction of nitrate, production of indole, hydrolysis of starch, gelatin, esculin, fermentation of amygdalin, arabinose, cellobiose, erythritol, fructose, glucose, glycogen, inositol, lactate, lactose, maltose, mannitol, mannose, melezitose, melibiose, pyruvate, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, threonine, trehalose, and xylose with the standard methods. The final volume of the sterile solutions of substrate to sterile base medium is 2 ml. The test media are inoculated with 0.05 ml of cultures of the isolates grown in base medium. All cultures are incubated at 35°C for 7 days and observed for clotting as an indicator of growth. Fermentation of carbohydrates is determined by a change in pH compared with uninoculated controls and cultures in base medium without carbohydrate. Fermentation of lactate, pyruvate, thronine, reduction of nitrate, production of indole, hydrolysis of starch, gelatin, and esculin are tested by the method of Holdeman (1977).

2.7 Induction of Atherosclerosis in Rabbits and Bacteria Administration

New Zealand white rabbits are fed on a 1% cholesterol enriched diet (Harlan-Sprague Dawley, Inc., Indianapolis, IN, USA) alternating with regular diet every month for a total of 6 months (Ma, 2002). Two weeks after the starting of 1% cholesterol diet, balloon-induced deendothelialization of the aorta is performed using a 4F Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Irvine, CA, USA) introduced through the right femoral artery cutdownunderwent under general anesthesia (ketamine 50 mg/kg and xylazine 20 mg/kg, i.m.). The catheter is advanced in a retrograde fashion to the ascending aorta and pulled back three times by 1 ml

balloon volume (Hage-Korban, 1999; Ma, 2003; Ma, 2004a). Atherosclerotic rabbits will be fed with the selected bacteria by adding to the rabbit chow starting at the time of atherosclerosis induction. This has been shown to be effective in previous reports (Gilliland, 2004). Serum cholesterol of atherosclerotic rabbits at six months ranges from 2000 - 4000 mg/dl (Abela, 1995; Ma, 2003).

2.8 Reduction of Serum Cholesterol by Bacteria

The serum cholesterol level in the atherosclerotic rabbits can be measured with a cholesterol diagnostic kit (Sigma Chemical Co., St. Louis, MO, USA). Serum samples will be obtained monthly and for four months then bi-weekly for two months. This can be done via an ear lobe vein stick. This measurement will determine if the bacteria can reduce serum cholesterol of the atherosclerotic rabbits. Cholesterol levels in the bacterial treatment group will be compared to controls (normal rabbits). Rabbits will be observed closely for any potential adverse effects from the administration of bacteria. In these studies, no reports of gastrointestinal symptoms (i.e. diarrhea or vomiting) have been reported.

2.9 Evaluation of Cholesterol Reduction in Cells

Both human aorta smooth muscle and endothelial cells (ATCC, USA) are cultured in a high cholesterol medium (1%). Bacteria with potentially cholesterol-reducing ability will be added to compare the amount of cholesterol in the medium. Two groups of cell cultures are evaluated, one with bacteria that has the cholesterol reducing effects and another with a related strain that does not demonstrate cholesterol reducing effects. These two cultures will be compared with respect to the amount of cholesterol in the cells as well as the amount of cholesterol remaining in the medium.

2.10 Tissue Cholesterol Measurement

To determine the effect of the bacteria on the animal tissue cholesterol concentration, the rabbit (with and without bacteria feeding) liver and arterial cholesterol levels are measured. One cm^2 mid-thoracic and mid-abdominal aortic tissue samples are obtained. Total cholesterol (free and individual ester) in the tissue is measured by high-performance liquid chromatography (HPLC) (Kim, 1984). Each sample of the tissue is ground to a fine powder with anhydrous sodium sulfate and extracted twice with 5 ml of chloroform and methanol mixture (2:1). The extract is dried under nitrogen and re-dissolved in 5 ml of isopropanol. A portion of isopropanol extract is filtered, dried and re-dissolved in the mobile phase. The sample (0.1 ml) is injected into the HPLC column and separated by using a Waters Radial-Pack C18 column eluted isocratically with acetonitrile:isopropanol (45:55 by volume) at 2 ml/min. The absorbance of the eluate is measured at 210 nm with a UV detector. Total cholesterol concentration is calculated by comparing the peak areas of samples with those obtained from the standard (Sigma Chemical Co., St. Louis, MO) (Witztum, 1985).

2.11 Gross Examination

The extent of atherosclerosis is assessed by surface planimetry after sacrifice and exposure of the intimal surface of the aortas. This can be performed on digitized images of the intimal surface obtained by a digital camera. The images can be then downloaded to a computer and the plaque area can be outlined using a custom software package for analysis.

2.12 Light Microscopy

Histology by light microscopy is done to observe the effect of the bacteria on the cholesterol rabbit tissue growth. Arterial and liver tissue specimens are embedded in paraffin, cut and mounted on glass slides. The sections are then stained with hematoxylin and eosin or Masson's trichrome stains. These will be then examined under a light microscope.

2.13 Electron Microscopy

Histology by electron microscopy are done for evaluation of cellular responses. The tissue samples are fixed overnight in 4% glutaraldehyde (Fisher Scientific, Pittsburgh, PA, USA) with 0.1 M phosphate buffer (pH 7.4). Arterial segments (5 mm long) are subjected to critical point drying in liquid CO_2, mounted on stubs and gold-coated in a sputter coater. The intimal surface is examined using a JEOL scanning electron microscope (JEOL Ltd, Model JSM-6400V, Tokyo, Japan). Tissue sections are obtained and processed routinely for ultrastructural examination. Thin sections are stained with uranyl acetate and lead citrate and then examined with a

transmission electron microscope (BEI preamplifier, Au Evirotech Company, Germany).

2.14 Potential Mechanisms of Cholesterol Reduction

In order to detect specific enzymes expressed in the cholesterol-reducing bacteria, which human body cannot produce, it needs to isolate these enzymes. Briefly, selected bacteria are homogenized with physiological buffered solution at 0°C and centrifuged at 10,000 rpm 20 minutes at 0°C. The supernatant is purified by low pressure liquid chromatography or HPLC using gel filtration, ion-exchange and other suitable columns. The solutions at the peaks are collected and the cholesterol-reducing activity is detected in vitro. With these isolates, the specific partials or molecules are characterized and the construction of the molecules is determined. These can be tested in the cell culture set up as a bioassay for cholesterol reduction.

2.15 Rab7 gene regulation

Rab7 may be involved in the process of atherogenesis (Kim, 2002). To identify genes responding to the cholesterol-rich diet and cholesterol-down bacteria treatment, differentially expressed somatic genes are searched from the diet-induced hypercholesterolemic bacteria by differential display reverse transcription-polymerase chain reaction (DDRT-PCR). To visualize the location of elevated Rab7 expression in tissues, patterns of the gene expression are monitored within rabbit aortic tissues by *in situ* hybridization and immunohistochemistry. To find out a potential relationship between the Rab7 and the atherogenesis, the same experiments are conducted with the atherosclerotic plaques obtained from the rabbits.

2.16 Pharmacological Triggering

In this study, the effect of the selected bacteria on the thrombus formation can be also observed. Thrombus is triggered by Russell's viper venom (0.15 mg/kg, i.p., Sigma Chemical Co., St. Louis, MO, USA) and histamine (0.02 mg/kg, i.v., Sigma Chemical Co., St. Louis, MO, USA) given at 48 and 24 hours prior sacrifice (Abela, 1995; Ma, 2003).

2.17 Quantitation of Thrombosis

The total surface area of the aorta, the surface area of aorta covered with atherosclerotic plaque, the surface area of aorta covered with *ante mortem* thrombus are measured to observe the effect of the selected bacteria on the thrombosis. The surface area is measured from images obtained by a color charge-coupled device camera (TM 54, Pulnix, Sunnyvale, CA, USA) and digitized by an IBM PC/AT computer with a color image processing subsystem. The digitized images are calibrated by use of a graticule. Surface area is measured by using of a customized quantitative image analysis package. Also, the number of thrombi on the aortic arch to the distal common iliac branches is counted.

2.18 Glucose and Glucose-6-phosphatase

To observe if glucose metabolism is involved in the bacteria's invading into animal body, rabbit serum glucose concentration and glucose-6-phosphatase activity are detected. Glucose concentration is measured with Sigma Glucose Diagnostic Kit (Sigma Chemical Co., St. Louis, MO, USA). Glucose-6-phosphatase measurement is followed Harper method (Harper, 1965). Briefly, 0.1 ml of tissue homogenate (100 mg tissue/ml) in citrate buffer (0.1 M, pH 6.5) is added into a test tube and incubated at 37°C for 5 minutes. 0.1 ml of glucose-6-phosphate (0.08 M) is added and the sample is incubated at 37°C for 5 minutes, then 5 ml of trichloroacetic acid (10%, w/v) is added and centrifuged at 9,000 rpm at 4°C for 5 minutes. 1 ml of the supernatant is taken into a test tube and 5 ml of ammonium molybdate solution (2 mM) then 1 ml of reducing solution (42 mM 1-amino-2-naphthol-4-sulphonic acid, 560 mM SO_3) is added. The sample is incubated at room temperature for 30 minutes then absorption is measured at 660 nm.

2.19 C-reactive Protein, PAI-1, Interleukin-6 and Tissue Factor Detection

After atherosclerotic rabbits are fed the selected bacteria, inflammation factors C-reactive protein, PAI-1 and interleukin-6 are measured with ELISA method, and tissue factor is measured with immunohistology method (Ma, 2004a; Virk, 2004). This will observe if the bacteria could reduce the inflammation induced by atherosclerotic syndrome of rabbits.

2.20 Heat Shock Proteins Detect

After atherosclerotic rabbits are fed the selected bacteria, heat shock proteins (such as heat shock proteins 25, 60, 70 and 90) are measured by Western Blotting method to observe if the bacteria induce stress response on the rabbits (Ma, 2004).

2.21 Artery Diameter Respond Evaluation

After a period that atherosclerotic rabbits are fed the selected bacteria, the rabbits are sacrificed and both carotid arteries are isolated from each rabbit and placed

in a dual organ chamber then perfused with oxygenated physiologic buffered solution (PBS) (NaCl 119 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 22.6 mM, glucose 11.1 mM and Na_2EDTA 0.03 mM) at 60 mmHg and 2.5 ml/minute flow rate at 37°C. Baseline vasodilation is determined using norepinephrine (NE, 1×10^{-6} M) preconstriction and pharmacological challenge is then performed with acetylcholine (Ach, 1×10^{-5} M) and sodium nitroprusside (SN, 1×10^{-5} M) successively. Vessel diameter is measured by a computer planimetry system (Figure 1). The data are calculated according to the formulas: Ach-NE (%)=(Ach-NE)/NE×100 and SN-NE (%)=(SN-NE)/NE×100 separately, where Ach, NE and SN represented the diameter (mm) of the arteries that will be perfused by the PBS containing a corresponding chemical (Figure 1).

2.22 Statistical Analysis

SigmaStat (Sigma Chemical Co., St. Louis, MO, USA) or Microsoft Excel software is used for data statistical analysis. $P<0.05$ is considered statistically significant difference. Measured data are reported as mean±SD or mean±SE. The student t-test is used for comparison.

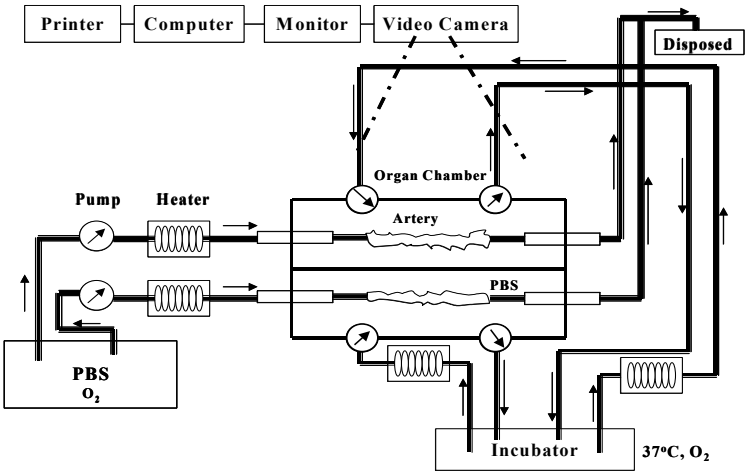

Figure 1. Dual organ chamber with separate perfusion using oxygenated physiological buffered solution at 37°C. Vessel diameter is measured by a computer planimetry system.

2.23 Potential Difficulties and Limitations and the Alternative Approaches to Achieve the Aims

The selection of the bacteria stains is difficult and it is labor intensive. However, bacteria could play role in cardiovascular diseases (Kuramitsu, 2001). There is a potnetial that the rabbits may not tolerate the oral bacteria. This could be addressed by using lower amounts of bacteria given or to treat more intermittently. Veterinary physicians could be consulted to help address these issues.

3. Results and Discussions

Bacteria from dog intestine were cultured in the medium with different cholesterol content (0, 50, 100, 200, 300, 400, 500, 600, 800, 1000 mg/dl). We noted the growth of bacterial colonies in culture that was inversely related to cholesterol concentration (i.e. fewer colonies in high cholesterol concentration media).

The purpose of this article is to offer the idea and techniques of selecting bacteria from carnivores that will reduce cholesterol level in human. There are many reports to show that bacteria in animal host decrease blood cholesterol levels by either decreased total intestinal absorption and removal through solid excretion, or by interrupting the entero-hepatic cycle of bile acids, such as lactobacillus bacteria and bifidobacteria (James, 1999). As our study results, we selected bacteria from dog gut that decrease cholesterol level in the human cell culture dish. As this article is to describe the possibility and technique to isolate and characterize bacteria from carnivores that inducing cholesterol level in animal blood, it will not give the detail of bacteria selection results here. We hope that the information in this article could help readers in do further studies in the cardiologic medicine that will be benefit the medical research and health improvements.

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