Degradation of Cholesterol by Probiotics

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Abstract- *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, according to (Tabbas et al., 2002). Probiotics are live microorgan¬isms that, when administered in ad¬equate amounts confer a health benefit on the host, UNFAO/WHO (2001). Probiotics commonly are isolated from human and animal intestinal tracts. They inhibit the enzymes responsible for producing cholesterol in the body.They bind dietary cholesterol and interfere with its absorption from the intestine which is then excreted from the body (Pan et al., 2011). Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel et al., 1998). There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from excess cholesterol. These mechanisms basically include the inhibiton of some pathogenic microorganisms involved in cholesterol degradation.*

Keywords- Probiotics, cholesterol, Lactobacillus acidophilus, Bifidobacterium animalism, HDL, LDL.

I. INTRODUCTION

Cholesterol is of two types, first high density lipoprotein (HDL) and second low density lipoprotein (LDL). HDL cholesterol is carried away from the heart and back to the liver where it can be reused by the body generally these are known as the "good fat". HDL cholesterol protects against heart attacks because it keeps LDL cholesterol from building up around the heart. LDL cholesterol is carried through the blood stream and the heart when too much LDL cholesterol builds up, which causes blockages in the arteries that carry blood to the heart. This damage the heart and over time can lead to a heart attack or stroke. LDL is known as "bad fat". When LDL cholesterol is too high a person is diagnosed with high cholesterol. The standard test of cholesterol is done after a 9-12 hours fast without food, liquids or pills. It gives information about total cholesterol, LDL, HDL and triglycerides (blood fats). If a person's total cholesterol is 200 mg/dl or more, or his/her HDL cholesterol is less than 40 mg/dl, he/she needs to have a lipoprotein profile done to determine LDL cholesterol and triglyceride levels (Hongbo et al., 2004).

Probiotics as a cholesterol degradation agent: Probiotics are live microorgan¬isms that, when administered in ad¬equate amounts confer a health benefit on the host, UNFAO/WHO (2001). Probiotics commonly are isolated from human and animal intestinal tracts. Dead bacteria, products derived from bacteria or end products of bacte¬rial growth also may impart certain benefits, but these derivatives are not considered to be probiotics, because they are not alive when administered. Native bacteria are not probiotics until the bacteria are isolated, purified and proved to have a health benefit when administered. Probiotics have been studied for both human and animal ap-plications (Ratna et al., 2009).

Lactobacillus as probiotic bacteria for cholesterol degradation: Probiotic cultures have been associated historically with cultures of milks and dairy products, from which there is substantial evidence for positive effects on human health and general well-being. Several in vitro and in vivo experiments on antagonism of different Lactobacillus strains against Helicobacter pylori and Clostridium difficile, Campylobacter jejuni, E. coli were performed.

CLINICAL APPLICATIONS OF PROBIOTICS

Cholesterol assimilation / Hyperlipidemia Probiotic strains, especially lactic acid bacteria have a major role to play in the cholesterol lowering mechanism. As the cholesterol level keeps increasing in the serum, it leads to cardiac diseases. These cholesterol levels can be brought down using probiotics.

Prevention of Allergic Reactions: Probiotic bacteria are important in down regulating inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergy. Probiotics may exert a beneficial effect on allergic reaction by improving mucosal barrier.

II. METHODOLOGY

1 .Isolation of probiotic bacteria:

Sieladie et al., (2011) isolated the lactobacillus bacteria from raw cow milk. Samples were incubated at 37°C

until coagulation. Coagulated samples were then activated in MRS broth (Biolife, Italy) at 37°C for 24h; they also used the MRS agar medium. Cultures were streaked on MRS agar medium and incubated under anaerobic condition using a candle extinction jar with a moistened filter paper to provide a CO2 enriched, water-vapor saturated atmosphere at 37°C for 48h. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium according to Hartemink et al., (1997).

Similarly, Tok et al., (2010) isolated the Lactobacillus delbrueckii subsp. Bulgaricus from home-made yoghurt, cultures were maintained by subculturing 1% inoculation into MRS broth incubating them for 18 hr at 42◦C. All of the Lactobacillus strains had been stored at −20◦C in MRS broth with 10/100 ml glycerol, and subcultured twice until they were used in the experiments.

2. Identification of the isolates :

Different isolates obtained from clinical and environmental samples were cultured on cetrimide agar medium, and then the grown colonies were identified according to Palleroni et al., (2005), by achieving biochemical tests, then full identification using Api 20E system (Jasim et al., 2010).

Identification of the isolates at genus level was carried out by Sieladie et al., (2011) following the criteria of Sharpe (1979) using morphological, phenotypic and biochemical methods. The cultures were examined microscopically for gram staining and catalase production (Harrigan and MacCance, 1976). In addition, all isolates were tested for growth at 10°C for 10 days, 45°C for 48h and CO2 production from glucose.

3. Production of cholesterol oxidase:

Production of cholesterol oxidase by the locally isolated P.aeruginosa was carried out in the production medium (Doukyo et al., 1999) by inoculating 100 ml of this medium with 1 ml of fresh culture each isolate separately and incubated in shaker incubator at 30°C for 24 hrs

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4. Determination of Protein concentration:

Protein concentration of the enzyme sample was determined according to the method described by Bradford et al., (1976), using bovine serum albumin as a standard protein solution.

5. Purification of Cholesterol Oxidase:

Cholesterol oxidase was purified first by ammonium sulphate precipitation at a saturation ratios ranged in between 25% to 85% (Jasim et al., 2010). They were then subjected to dialysis and then ion exchange chromatography through CM Cellulose column (22×1.5cm) equilibrated with phosphate buffer (0.25M, pH 7.0).

6. Characterization of purified cholesterol oxidase:

Purified cholesterol oxidase was characterized by determining the optimum pH and temperature for both activity and stability according to (Whitaker et al., 1972).

7. Acid tolerance:

Preliminary selection of acid tolerant lactobacilli using rapid method was determined according to slightly modified methods as described by Pelinescu et al., (2009) to simulate gastric conditions. Tested lactobacilli isolate cultures were grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6h old culture was inoculated into 100ml MRS broth whose pH had been adjusted to 2, 3 or 7 using 1N HCL or NaOH (Sieladie l., 2011).

8. Bile salt tolerance:

An aliquot of 1ml of the 6h old culture was inoculated into 100ml MRS broth with 0.2 or 0.4% (w/v) bile salts (Sigma, USA). Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24h incubation period at 37°C (Sieladie et al., 2011).

9. Measurement of cholesterol removal:

Cholesterol removal by Lactobacillus delbrueckii subsp. bulgaricus strains was studied in freshly prepared MRS broth supplemented with 0, 1, 2, and 3 mg/ml concentration of oxgall as a bile source (Sigma, St Louis, MO, USA) by Tok et al., (2010). A filter-sterilized cholesterol solution (10 mg/ml in ethanol) was added to the broth to a final concentration of 100 µg/ml, inoculated with each strain (at 2%), and incubated at 42◦Cfor 19 and 48 hr. After the incubation period, cells were removed from the broth by centrifugation for 20 min at 10 000 × g and 1◦C. A modified colorimetric method as described by Rudel and Morris (Rudel et al., 1973) was used to determine the amount of cholesterol in the resuspended cells and spent broth. The amount of cholesterol removed was estimated by subtracting the cholesterol amount in the spent broth from that in the uninnoculated control broth.

10. Resistance to antibiotics:

The antibiotic susceptibility of selected acidotolerant and bile tolerant lactobacillus isolates was determined towards nine antibiotics, namely, penicillin G (10 units), ampicillin (10µg), amoxicillin (10µg), erythromycin (15µg), tetracycline (30µg), chloramphenicol (30µg), Doxycycline (25µg), cotrimoxazole (25µg) and ciprofloxacine (5µg). Strains

selection was based on their performance toward acid and bile salts (Sieladie et al., 2011).

11. Antimicrobial activity:

Antimicrobial activity of the selected probiotic isolates was checked by using the agar-spot test (Mami et al., 2008). Isolates were screened for production of antimicrobial against Listeriainnocua ATCC 33090, Staphylococcus aureus ATCC 25923, S. aureus ATCC 25922, S. aureus (MDR, clinical isolate), Streptococcus mutans DSM 20523. The agar well diffusion technique was also used to discriminate antimicrobial activity of the selected probiotic isolates due to organic acid production.

12. Cholesterol removal by dead and resting cells:

Overnight cultures of the Lactobacillus delbrueckii subsp. bulgaricus strains were inoculated into 10 ml of MRS broth and incubated at 42C for 19 hr. After incubation, the cells were harvested by centrifugation for 15 min at 1800 × g, washed twice with sterile distilled water, and resuspended in 10 ml of distilled water. The suspension was divided into two portions. The first portion was autoclaved for 15 min at 121◦C to prepare heat-killed cells whereas the other portion was not processed (i.e. resting cells) (Tok et al., 2010).

III. RESULT AND DISCUSSION

1. Isolation and identification of Probiotic bacteria:

Results showed that these isolates gave a positive result for production of oxidase, catalase, and gelatinase, growing on king A and king B medium, and at 42°C , while they are negative for the other biochemical tests.

Similarly Martin et al., (2003) identified the bacteria isolated from human milk as Lactobacillus gasseri, Lactobacillus fermentum and Enterococcus faecium. These species are considered among the probiotic bacteria (Holzapfel, et al. 1998, Collins, et al.,. 1998) and contain strains that are used in commercial probiotic products.

2. Acid tolerance

Sieladie et al., (2011) performed the screening and selection of the 107 lactobacilli isolates under the acidic conditions using rapid selective method resulted in four groups. Sixty-six isolates out of the 107 tested demonstrated poor tolerances to acidic condition, 34 isolates showed good tolerance, 1 isolate demonstrated very good tolerance and 6 isolates presented excellent tolerance. Among the 41 lactobacilli isolates demonstrating at least good tolerance under the acidic conditions using rapid selective method, 18 best isolates were screened for their ability to tolerate acidic condition in citric acid, pH 3 after 5h.

3. Cholesterol removal

All five strains of L. delbrueckii subsp. bulgaricus showed a capacity for removing cholesterol from MRS broth with and without oxgall. The amount of cholesterol removed by the cultures during the 48 hr incubation ranged from 8% to 40%. Minimum cholesterol removal was observed in the medium without bile whereas maximum cholesterol removal was determined in the medium supplemented with 1 mg/ml bile. In addition, it was confirmed that in the mediums containing 2 and 3mg/ml oxgall, cholesterol removal was higher compared to the medium that did not contain oxgall, but it was lower compared to the medium supplemented with 1mg/ml oxgall. For all the strains used in this study, except B2, higher cholesterol removal was observed during the 19-hr incubation period; however, very little cholesterol was removed after 19 hr. However, it was determined that maximum cholesterol removal was exhibited at the end of 48 hr (Tok et al., 2010).

4. Bile salt tolerance

After exposure to acidic conditions, 15 selected acidotolerant lactobacilli isolates were assayed by Sieladie et al., (2011) for bile salt tolerance. All isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C. These isolates were further investigated for their safety properties including sensitivity to antibiotic, haemolysis and gelatinase activity.

5. Purification of Cholesterol oxidase

Cholesterol oxidase produced by P.aeruginosa H48 was purified first by ammonium sulphate precipitation using gradual saturation ratios ranging between 25 and 85%. Enzyme obtained from ammonium sulphate precipitation and dialysis was further purified by ion exchange chromatography using carboxymethyl cellulose. Results indicated showed that cholesterol oxidase was eluted by 0.3M NaCl with specific activity of 10.46U/mg protein. This step demonstrated approximately 1.99 fold of purification with 30.21 % overall yield (Jasim et al., 2010).

6. Resistance to antibiotics

Fifteen potentially probiotic lactobacilli isolates were subjected to antibiotic susceptibility testing

using the agar diffusion method (Sieladie et al., 2011). All of them were sensitive to penicillin, ampicillin, amoxicillin, erythromycin, tetracycline, chloramphenicol, and doxycycline. Three isolates (20RM, 48RM, 53RM) demonstrated intermediate resistance to cotrimoxazole. Notable observation is the resistance towards ciprofloxacin expressed by all isolates.

7. Characterization of purified cholesterol oxidase enzyme

The purified enzyme was characterized by determining the optimum pH and temperature for both activity and stability respectively Jasim et al., (2010). Results indicated in (figure 2) showed that pH7.0 was the optimum for cholesterol oxidase activity, when the purified enzyme was added to substrate (cholesterol solution) incubated previously at a range of pH between 5.0 and 9.0 for 10 minutes at 32°C. At this pH, enzyme activity was 3.8 U/ml, and the activity was decrease at the acidic and basic pH values because of the conformational changes in enzyme configuration due to the changes in the ionizable groups located in the active sites of the enzyme. On the other hand, it was found that the optimum pH for the stability of cholesterol oxidase was pH 6.5 when the purified enzyme was incubated in test tubes containing 1ml of buffer solutions at a pH range between 5.0 and 9.0. At this pH, the enzyme activity was 3.62 U/ml, and the remaining activity was 100% as it was shown in (figure).

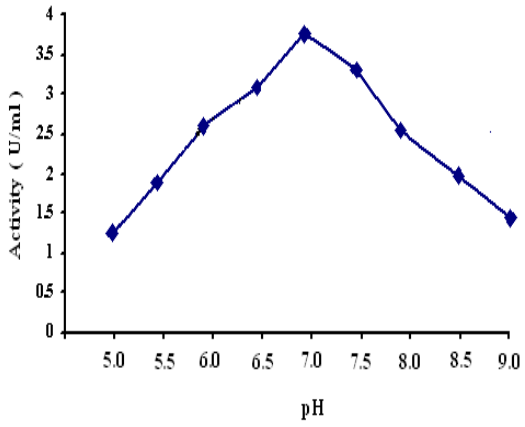

fig: Effect of pH on the activity of the purified cholesterol oxidase from P.aeruginosa H48

Optimum temperature for cholesterol oxidase activity and stability was also determined. Results indicated in (figure) showed that the optimum temperature for enzyme activity was 35 °C when the enzyme was added to substrate (cholesterol solution) and incubated at different temperatures ranged between 25°C and 65°C for 10 min. At this temperature, the activity was 3.48 U/ml, and represents the optimum for enzyme activity because of the high effect on the reaction energy for both enzyme and substrate which leads to formation of enzyme–substrate complex and this will result in increasing the reaction speed, hence enzyme activity was decreased above and below this temperature.

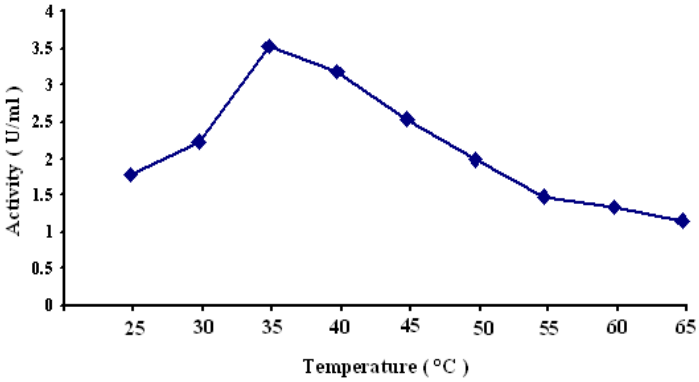

Fig Effect of different temperatures on the activity of purified cholesterol oxidase from P.aeruginosa.(Jasim et al., 2010)

Antimicrobial activity

Results for antimicrobial activity of fifteen safe probiotic lactobacilli isolates were tested by Sieladie et al., (2011). All isolates inhibited the growth of all pathogenic strains when agar spot method was used. The free-cell neutralized supernatant of 14 out of the 15 lactobacilli tested did not inhibit the growth of the tested pathogenic indicators. It was also noticed that, the neutralized free-cell supernatant from the culture of the isolate 29V inhibited the growth of all pathogenic indicator.

IV. CONCLUSION

Probiotics are gaining importance because of the innumerable benefits, e.g. treating lactose intolerance, hypercholesterol problem, and cardiac diseases and managing cardiac problems like atherosclerosis and arteriosclerosis. Current evidence supports the concept that oral administration of probiotic therapies may be beneficial in a multitude of disorders both inside and outside the gastrointestinal tract. Probiotic organisms can have a significant influence on the treatment and prevention of disease. The Lactobacillus strains associated with dominant micro flora that people from Mbororo's tribe in the western highlands of Cameroon use to manufacture fermented milk contain new potentially safe probiotic strains with antimicrobial and cholesterol-lowering property.

The use of probiotics prevents the people from using drugs which are generally accompanied with vaiety of

side effects. For example, Statins drug (3-hyroxy-3-methylglutaryl coenzyme A reductase inhibitors) is generally able to inhibit cholesterol synthesis in the liver and peripheral tissues. They have been extensively studied and found to possess better therapeutic effects than other lipid lowering drugs (Martin et al., 2013). Statins reduce the risks of major cardiovascular events and overall mortality (Baigent et al., 2010). Yet, another meta-analysis of 11 studies showed no reduction in mortality with the use of statins (Ray et al., 2010). Statins are critical in patients with increased cardiovascular risk as opposed to low-risk patients. This is probably due to the adverse effects related to the use of this class of drugs, which include myopathy (Redberg et al., 2011) and cognitive impairment (Muldoon, 2000, and Fernandez, 2011). Other pharmacological agents that are used in the management of hypercholesterolemia are bile acid sequestrants, cholesterol absorption inhibitors, niacin, and fibrates.

Therefore more and more studies should be done on the isolation of cholesterol degrading microorganisms from different natural sources and their use as probiotic agents.

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