Fayoum Journal of Agricultural Research and Development
ISSN:1110- 7790

DEGRADATION OF UNDESIRABLE CHOLESTEROL BY *LACTOBACILLUS SP.*

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ABSTRACT:

Cholesterol is a heterogeneous compound in its chemical composition. It is very important both on a medical and nutritional health level. Low-density lipoprotein (L.D.L) poses some danger to human life and deserves more research on it to clarify the idea of its specific or safe behavior in human life. 68 of 162 isolates were lead to the degrade of cholesterol present in the cholesterol-agar medium of microbial Basel medium.The samples were collected from 17 sources, the most promising one was from local Rayeb followed by yoghurt and cow ghee. *Lactobacillus sp.* was one of the most effective in degrading cholesterol. The optimal environmental conditions for cholesterol degradation were generally found at pH 7, temperature 37, cholesterol concentration 1.5 g/L and incubation period 48 hours.

INTRODUCTION:

It is well known that microorganisms harbor a lot of enzymes, some are constitutive and others are induced in their nature. Among enzymes which produced inside and outside bacterial cell wall and of the most important position in medical and food safety is cholesterol oxidase (ChO), which plays a major part in getting rid of cholesterol, by decomposing to safe by-product. Cholesterol is an essential structural component of the cell membrane and precursor of vitamin D. it is absent among prokaryotes (bacteria and archaea).

Cholesterol is an essential structural component of the animal cell membrane; however, elevated serum cholesterol is associated with a major risk for coronary heart diseases. Consumption of probiotics (like LAB) products has been proposed to lower serum cholesterol. It has also been mentioned by many researchers that probiotic microbes assimilated directly cholesterol (**Gyawali and Ibrahim, 2012, Ghada, 2015, Anila et al, 2016, Vaishnavi et al, 2015 and 2016). Kulkarni et al, 2013** reported that therapy that could decrease cholesterol without any side effect is strongly needed because it is unavailable now. **Gyawali and Ibrahim, 2012** found isolated lactic acid bacteria from lamb meat which displayed the high ability to degrade cholesterol (84.6 – 86.4 %) .

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Received: 27/10/ 2021
Accepted:1/11/ 2021

Mesclenious organisms were isolated by versatile researchers capable of degrading the compound with high capacity. **Yazdi *et al*., 2001 (*Rhodococcus* sp.) Ouf et at., 2012 (S. prodiae), Saranya et al., 2014 (*Pseusomonas* sp., *Bacillus* sp. and *Streptomyces* sp.),**. Many factors influencing the degradation potential of cholesterol by different microorganisms (in vitro studies) were done by many researches and were served mostly in pH, temperature, incubation period and cholesterol concentration in the medium, cholesterol degradation as affected by temperature prevailing was found variable depending on the nature of the organism examined. different range of temperature was recorded **Jayachitra et al**., **2012** tested cholesterol degradation by different bacterial isolates at 30, 37 and 57 ˚C, while **Vaishnavi et al., 2015** employed 37, 40 and 50˚C for their study in the same direction **Kokila and Amutha, 2016** tested the degradation at 20, 25, 30, 35 and 45 ˚C by different bacterial isolates. Also, the pH of the growth medium affects the degradation process. **Jayachitra *et al*., 2012**, served PH 6, 7 and 8 when tested various bacterial isolates. While **Vaishnavi et al., 2015** used three pH mainly 4, 7 and 9 and their effect on the degradation process. In the same line **Kokila and Amutha, 2016**, examined to somewhat different pH.s which were 2, 4, 5, 7, and 8 in their study.

As to cholesterol concentration **Jayachitra et al., 2012**, tested 0.05, 0.075 and 0.1 mg/ml concentrations on the activity of ChO producers. But **Kokila and Amutha 2016,** implemented 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. The incubation period was also studied by the same workers which were variable between 1 to 45 hours incubation time (**Kokila and Amutha, 2016**).

Finally, the target of this work is dealing with a survey on the occurrence of different cholesterol degradation microorganisms in various food samples. Isolation and identification of selected strain active in this respect, and at the same time are safe when used in human diets and uses. Factors affecting microbiological degradation of cholesterol were also included such as pH, temperature, cholesterol concentration and incubation period which were discussed in this work.

MATERIAL AND METHODS:

Collection and preparation of tested samples

Different fresh and preserved food samples were collected from local retail and wholesale markets in Fayoum governorates, Egypt. Namely: Fresh raw cow's milk, Fresh raw buffalo's milk, Rayeb (local), yogurt (local), mesh, white cheese, Cow butter, Buffalo butter, Buffalo ghee, Cow ghee, lamb meat, brain, liver, fresh salmon fish, egg yolk, soil and Serum samples of hypercholesterolemia patients, Collected from Clinical Laboratory, Fayoum governorates, Egypt .The collected samples were packed aseptically in plastic bags and transferred to the laboratory, and were aseptically transported to the laboratory of Agric. Microbiology Department, Faculty of Agricultural, Fayoum University.

Medium

MRS agar medium **De Man et al., (1960)** use to isolation of Lactobacillus from samples. Basal medium **Kumar *et al*., (2004)** and **Yehia,. et al 2015** contained which was as follows (g/l): Ammonium chloride 0.5, Dipotassium hydrogen phosphate 0.3, Magnesium sulfate 0.2, Potassium di hydrogen phosphate 0.001, Sodium chloride 0.5, Agar 20.0, Cholesterol 1, pH 7 ± 0.2, use for test the ability of degradation of cholesterol.

Isolation of Lactobacillus degrading cholesterol

A volume of 1 ml of the above mentioned peptone water were inoculated with the previous samples and added to test tube containing 9 ml of saline solution (0.9% NaCl) and 10-fold serial decimal dilutions The media used to isolate lactic acid bacteria . The petri dishes were incubated at 37 for 2 days, and incubated in anaerobic conditions because lactic acid bacteria are a micro aerophilic. After incubation for 48 hours, all the colonies which grew on the medium were picked up and plotted on media containing cholesterol as the sole source of carbon, the medium did not contain a carbon source expect (cholesterol) which was purchased from (**Sigma Aldrich)**, and separately sterilized. The ability of bacteria to decompose cholesterol was estimated by the appearance of clear zones of translucency around colonies on agar medium after incubation at 37 °C for 48 h.

Identification of Potential Probiotics.

Isolates were characterized based on Gram's stain reaction, cell morphology, motility according to **Lalam et al. (2015)**. Putative Lactobacilli were identified to species level based on the API 20 strip (bioMérieux, Marcy l'Etoile, France) according to the instructions of the manufacturer, and by the database provided by Biomerieux (Kim et al., 2006). All the isolates were overnight cultured in MRS broth, and then added individually to the wells of the API strips. The inoculated strips were incubated at 37°C and then monitored for

5 changes in the color of the medium after 24 h. Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

Factors influencing microbial growth and cholesterol degradation

Strain Count has been measured to relate the microbial growth with cholesterol oxidase production and thus cholesterol degradation.

Effect of pH on bacterial growth and cholesterol degradation

The bacterial activity was checked at different pH which were 2, 3, 6 ,7 and 8 used in 10 ml of Basal Medium **(Yehia et al,. 2015)** broth containing cholesterol (1mg /ml). A 0.1 ml of microbial broth was inoculated and incubated at 37°C for 12, 24, 48 hours. The microbial growth intensity was measured by colorimetric reading, at optical density 560 nm (VWR. UV-6300PC Double Beam Spectrophotometer). **(Kokila and Amutha 2016).**

Effect of cholesterol concentration on microbial growth and cholesterol degrading activity.

The cholesterol at different concentrations which were 0.5, 1, 1.5 and 2 mg/ml in 10 ml Basal Medium **(Yehia et al ,.2015)** broth were prepared and 0.1 ml broth of selected isolates were inoculated and incubated at 37°C for 12, 24, 48 hours. The microbial growth intensity was observed by colorimetric reading at Optical density 560 nm **(Kokila and Amutha 2016).**

Effect of incubation period on bacterial growth and cholesterol degrading ability.

Different incubation periods, (12, 24 and 48 hr.) were used to test microbial growth intensity in 10 ml Basal Medium **(Yehia,. et al 2015)** broth containing cholesterol (1 mg/ml) . A .01 ml broth of microbial isolates were inoculated and incubated at 37°C for 12 , 24 , 36 hours. Colorimetric reading, at optical density 560 nm was used **(Kokila and Amutha 2016)**.

RESULTS AND DISCUSSION:

Screening and isolation of microorganisms degrading Cholesterol

Cholesterol is a vital substance in the human body. Long- standing elevated levels of blood cholesterol may lead to atherosclerosis and may therefore pose a major risk for developing cardiovascular diseases (CVDs). Despite the proven cholesterol-lowering ability of certain pharmacological agents, unwanted side effects can occur in some cases, such as gastrointestinal discomfort .Probiotics are defined by the Food and Agriculture Organization (FAO) and WHO as living microorganisms which when administered in adequate amounts confer upon the host a health benefit. In the 1970, fermented milk containing a wild Lactobacillus strain was reported to have a hypocholesterolemic effect in humans. Since then, many experiments have been conducted in vitro or in vivo to investigate the cholesterol-lowering effect of lactic acid bacteria (LAB), especially strains of Lactobacillus and Bifidobacterium (**Tsai et al., 2014).**

Therefore, The First part of this study concerned with Screening and isolation for the most efficient and safe microorganisms can degrade cholesterol, isolates with unique characteristics that could determine their usefulness as cholesterol degradation. In order to achieve this objective, Lactobacilli was isolated from retail samples of different foods. Some authors recorded higher counts of cholesterol degrading organisms with different species **(Ghada, 2015, Kulkarni et al., 2013 and Vaishnavi et al., 2016)**. Some food counts and species **(Saranya *et al.*, 2013 and Ouf et al., 2012)** found many organisms prevailed in many foods that resistant cholesterol .The result showed that a total of 162 isolates were isolated from different local food samples **Table (1).**

Carbohydrate fermentation patterns of selected *Lactobacillus* sp. isolates determined by API 50 CHL system

The API test was carried out for lactobacillus sp. strains to find out more properties about them for the fermentation of sugar sources. The ability of selected strains to ferment different sugar sources and their enzyme activities were investigated using API 50CHL. It was noticed that, these isolates able to utilize a waid range of organic substrates. All isolates were positive for Galactose, D-Glucose D-Fructose, D-Mannose, Mannitol, N-Acetyl-glucosamine, Amygdalin, Arbutin, Esculin, Salicin, Cellobiose.

Effect of pH and cholesterol concentration on cholesterol degradation.

The present study found that the effect of pH on Lactobacillus sp. isolated and selected from viable plate counts technique were resistant to low ph. This research applied and tested different pH where it were 2, 3, 6, 7 and 8 to obtain the most appropriate one for degradation of cholesterol, with high efficiency . In general, as illustrated in Table (2) which figure show of the optical density resulting from the turbidity due to decomposition of cholesterol by the microorganism used. As the value of O.D. increases cholesterol, degradation increases

Table (1): Source and percentage of cholesterol degrading isolates.

No.	Isolate source	Total isolates	Isolate degrading cholesterol	percentage of cholesterol degrading isolates
1	Fresh raw cow's milk	14	8	57.1%
2	Fresh raw buffalo milk	11	6	54.5%
3	Rayeb (local)	15	9	60.0%
4	Yogurt (local)	8	3	37.5%
5	Mesh (local)	8	4	50.0%
6	White cheese	5	2	40.0%
7	Cow butter	9	5	55.6%
8	Buffalo butter	8	4	50.0%
9	Buffalo ghee	6	3	50.0%
10	Cow ghee	9	3	33.3%
11	Lamb meat	12	5	41.7%
12	Brain	8	3	37.5%
13	Liver	11	4	36.4%
14	Fresh salmon fish	11	3	27.3%
15	Egg yolk	4	1	25.0%
16	Soil	17	3	17.6%
17	Serum sample of hypercholesterolemia patients	6	2	33.3%
Total		162	68	42.0%

Table (2): Effect of pH and Cholesterol Concentration on cholesterol degradation *by Lactobacillus sp.* determined as optical density.

Cholesterol conc. (g/L)	pH 2			pH 3			pH 6			pH 7			pH 8		
	12h.	24h.	48h.	12h.	24h.	48h.	12h.	24h.	48h.	12h.	24h.	48h.	12h.	24h.	48h.
0.5	0.15	0.15	0.15	0.16	0.16	0.17	0.17	0.22	0.28	0.24	0.29	0.36	0.20	0.25	0.33
1	0.17	0.17	0.17	0.19	0.21	0.22	0.20	0.25	0.35	0.27	0.33	0.41	0.23	0.29	0.36
1.5	0.18	0.18	0.18	0.20	0.22	0.23	0.25	0.29	0.38	0.31	0.38	0.46	0.27	0.33	0.39
2	0.16	0.16	0.16	0.18	0.19	0.20	0.19	0.23	0.30	0.25	0.31	0.38	0.22	0.27	0.34

It can be arranged in descending order as to the suitable concentration of cholesterol the medium as followed: O.D. reading was 1.5, 1, 2 and 0.5 g/L, Respectively. Irrespective to the most suitable pH with cholesterol concentration as shown in the table, it was found that pH 7.0 gave the highest value reached 0.46 O.D. at 1.5 g/L cholesterol concentration of followed by pH 6 and 8. Other pH gave to somewhat less value. It is worth to mention that the optimum incubation period existed at 48 h. At all tested pH. It was also noted that at pH 2.0 bacterial activity was almost stopped as the O.D. value after 12 h. incubation period was the same and continue to 24 and 48 h. At pH 3.0 the growth was very weak which indicated by relatively low O.D. recorded. It may be said that Lactobacillus sp. was able to adapt, to certain degree, and proliferate at relatively low pH. **(Soliman et al., 2013)** who recorded that, the viability of Lactobacillus sp. did not loss it viability over 3 h of exposure to pH 3.0, indicating a naturally high level of acid resistance in Lactobacillus sp. to acidity.

In contrast, exposure to pH 2 eliminated more than 11.2 of Lactobacillus sp. during an incubation period of 2 h and then after 3 h elimination was more than 22.0 % at the same pH, acidic pH range (1.5, 2.0, 3.0 and 3.5) and **(Shivram and Vishwanath., 2012)** reported that, the isolated Lactobacillus strains were tolerable to pH 2 and 3, These results disagree with the current results because this step was testing the bacteria's ability to tolerate low pH for a period of 2-3 hours, while my study was testing bacteria's tolerance to low pH for 12, 24 and 48 hours. While in case of pH6.

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تكسير الكوليستيرول الغير مرغوب فية بواسطة اللاكتوباسيلس

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الملخص العربي

الكوليسترول مركب غير متجانس في تركيبه الكيميائي، مهم جدا سواء على المستوى الطبي أو الصحه الغذائية. البروتين الدهني منخفض الكثافة (L.D.L) يشكل بعض الخطورة على حياة الإنسان ويستحق العديد من الأبحاث عليه لتوضيح فكرة سلوكه المحدد أو الآمن في حياة الإنسان. تم اختيار مجموعة من العزلات ١٦٢ عزلة على أساس الإختلاف المورفولوجي للمستعمرات ، ووجد أن ٦٨ منها تؤدي إلى تكسير الكوليستيرول الموجود في بيئة أجار الكوليسترول الغذائية للميكروبات . Basel medium بلغت نسبة البكتيريا التي تستطيع تكسير الكوليستيرول ٤٢٪ من إجمالي العينات التي تم العزل منها. تم العثور على البكتيريا السائدة لتكون عصيات موجبة الجرام. من أصل ١٦٢ عزلة ، ٩ كانت من اللبن الرايب المحلي ، ٨ من الحليب البقري الطازج ، ٦ من الحليب الجاموسي الطازج ، ٥ من الزبدة البقري ولحم الضأن ، ٤ من المش، والكبد ، ٣ من الزبادي ، السمن البقري الجاموسي والمخ وأسماك السالمون الطازجة والتربة الزراعية . أشارت النتائج عزلات اللبن الرايب (محلي) كانت تحتوي على أعلى العزلات في تكسير الكوليستيرول . بكتيريا حمض اللاكتيك كانت نشطة في هذا المجال. تم العثور على الظروف البيئية المثلى لتكسير الكوليسترول بشكل عام عند درجة الحموضة ٧ ودرجة الحرارة ٣٧ وتركيز الكوليسترول ١.٥ جرام في اللتر وفترة التحضين ٤٨ ساعة.