RESEARCH ARTICLE

Biotherapy Exploration for Gallbladder Stones Removing *In vitro*

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ABSTRACT

Latterly, non-surgical pharmaceutic have been based on dissolving gallstones and saturated bile salt, which is contained 70% in near cholesterol. Therefore, the article takes care of the transforming agent *Streptococcus sp*. Such as a model for inducing plasmid p*MG36bsh* for processing of gallstones removing *in vitro*.

Whole transcripting messenger ribonucleic acid (mRNA) of *Streptococcus sp*. was gauged in Prime script TM RT reagent Kit, to monitor the change after attending p*MG36bsh* vector and set up *Streptococcus sp*. have two copies of cholesterol-reducing genes (*cgh, bsh*) used to look like gallstone fragments in bacterial growth culture. To find data that was assumed to be released in bacterial *bsh* expression, reduce the amount of gallstones in culture when bile salt serves like inducing stiff for associated *bsh* genes. The removing ratio depended on bacterial species with their living environmental kind. Whereas, *Streptococcus mutans* has given a 56% (n = 2) signification removing ratio, and *Streptococcus parasanguinis* was 24% (n = 1), considering non-significant result when they comparted with wild type reducing ratio and *S. salivarius* used like digestive balance probiotic drugs, its get 82% (n = 1) significant removal ratio.

Keywords: *pMG36bsh* vector, Transformer bacteria, *bsh* gene, gallbladder stones, *cgh* gene.

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INTRODUCTION

The gallbladder's founding function is to save bile-like substance secreted by liver is involved in digestion activities. Sometimes gallstones are forming like crystallized bile contents. Gallstones synthesis stabilizes in the liver on excessive bile cholesterol, leading to cholesterol association and finally gallstones forming.[1]

Surgical eradication of gallstones requires removing an entire organ of the gallbladder, which led to the growth of non-surgical treatments that help prevent cholesterol-raising in body.[2]

Patients preferred surgery, neither old nor ill,[3] therefore, it was found several kinds of non-surgical therapies. One of the new non-surgical treatment options is employing transformer-friendly bacteria (probiotic, microflora bacteria) after changing the genetic expression and gene cloning methods to create biotherapy add to other non-surgical ways included thinning bile with acid pills). It can dissolve gallstones, shock waves, MTBE injection, endoscopic drainage, percutaneous cholecystectomy, transmural drainage decompresses, swollen gallbladder, and ultrasound-guided drainage procedure.[4,5]

The best option is to create modified biotherapies of one of the orally applied microorganisms (probiotics, microflora) as the main objective of the metabolic concept.[6,7]

Laterally, there has been a lot of interesting articles *in vitro* because of healthy bacterial advantage effects, which include anti-hypercholesterolemia, anti-pathogenic, anti-carcinogenic properties, antidiabetic bacteria and stimulation agents of another metabolic diseases or the immune system effect to gallstone forming,[8,9] various of *Bifidobacterium* species, *Lactobacillus s*pecies, *Xthomonas maltophilia* and *Streptococcus* species like probable bacteria have manifestation of bile salt hydrolase (BSH) activity[10,11] were extended to reduce cholesterol associated problems,this article catches up some transformer *Streptococcus* species were isolated from different areas in human body, which they have contained genotype (*bsh or cgh* genes) were coding to bile salt hydrolase liberated cholylglycine and cholyltaurine re-editing the steroid nucleus and excretion of cholesterol,[12] it will certainly continue to play a role in the management of gallbladder stones like non-surgically treatment.

The patients treated with ursodeoxycholic acid have hold 50% reduction in gallstone (volume, number, or both).[13]

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In vitro instigations, including biliary ursdeoxycholic acid, help induce the *bsh* genes of gut probiotics[14] and seems to be a secure and functional commute to surgery in patients with gallstones.[15] Subsequently, this study takes care to select *S. mutans* and *Streptococcus parasanguinis* were isolated from oral cavity and respiratory tract ad they have compared with *Streptococcus salivarius* like an intestinal probiotic in genetic expression which responsible on bile salt-resistance and cholesterol-lowering properties.

Non-surgical biotherapies for gallbladder stones dissolving has held to take advantage of transgenic of utilizing *pMG36ebsh* vector to increase the number of bile salt hydrolase gene copies and enhance bile salt hydrolase *bsh* and (3.5.1.24 choloylglycine hydrolase *cgh*) genes (www.brenda-enzymes.org › enzyme) in an extraordinary expression of genetic modification of (*bsh,cgh*) phenotype pretending through on the issue of the quantity of cDNA generated by oligo(dT) priming. These genes have caught up genetic containing of *Streptococcus* species which using in report to gallbladder stones removing.

MATERIAL METHOD

S.mutans, S. parasanguinis was detected as dental strains in College of Dentistry, University of Baghdad, *S. salivarius* (probiotic bacteria) were taken, and nutrient broth medium were used for inoculation and cultured at 37ºC for 18 hours, the activating bacteria were growing on media consisted of 100 µg/mL Erythromycin antibiotic[16] was used for bacterial antibiotic sensitivity checking (Table 1).

Bile salt tolerance capacity

The sodium salt of ursodeoxycholic acid (SCA) 0.5% (wt/vol) concentration was prepared in nutrient media and used to inoculate S.mutans, S. parasanguinis *S. salivarius* strains then incubated aerobically for 18h at 37ºC for bacterial growth tolerance.[17] It's replicated two times. In two cases, pretending the calculated of bacterial growth at altogether by the following formula:

A600 nm control = A600 nm control-A600 nm bile salt)

Then: A600 nm control - optical density of the broth culture without bile salt.

A600 nm bile salt-optical density of the broth containing bile salt, the number of live bacteria (cfu/ml) was determined.[18]

Transformation Assay

The modeling for transformation was carried out according to Bigas with modifications. *S.mutans, S. parasanguinis and S. salivarius* strains as sensitive recipient bacteria were grown in Luria-Bertani medium (LB) to OD_{600} = 1.6 (about 2.4×10^7 cfu/mL), a late-stationary phases. The bacteria were resuspended in 350 µL aliquot LB to supplement with 500 µL of p*MG36bsh* vector. The mixture was incubated for 18 hours at 37°C with shaking incubator then cultured on solid LB again with 100µg/mL erythromycin for 18 hours to induce antibiotic resistance expression.[18,19]

pMG36*bsh* Vector Extraction

A single colony of transformer *S.mutans, S. parasanguinis and S. salivarius* strains were picked up to incubate overnight at 37°C in LB media with erythromycin, p*MG36bsh* vector was extracted to get through using the Presto™ Mini Plasmid Kit, which it belonged the harvesting step that transfers 1.5 mL of cultured bacterial cells (1-2 x 109 bacteria strains grown in LB medium) to a 1.5 mL microcentrifuge tube. Plasmid p*MG36bsh* vector served as a vector for cloning. p*MG36bsh* vector was extracted above from transformer *S.mutans, S. parasanguinis and S. salivarius* strains; it was migrated at 0.7% gel agarose electrophoresis to insure its presentation in transformer strains.[20]

Bsh Gene Expression Test

Total RNA Extraction

The Wild and transformer *S.mutans, S. parasanguinis, and S. salivarius* strains used for total RNA extraction used ZR RNA MiniPrep kit (DNA free).

Bacterial cells were suspended by adding 48 mL 100% ethanol to the 12 mL RNA Wash Buffer concentrate. Afresh or frozen cell pellet in 800 µL RNA Lysis Buffer was Resuspended and transferred the mixture to a tube of ZR BashingBead™ Lysis. Centrifuged at 12,000 x g for 1 minute. then 400 µL supernatant to a Zymo-Spin™ IIIC Column tube was Transferred and centrifuged at 8,000 x g for 30 seconds. 0.8 volume ethanol (95–100%) to the flow-through in the Collection Tube was added to centrifuge at 12,000 x g for 30 seconds.[5] A 400 µL of Prep Buffer was added to the column. Centrifuged at12,000 x g for 1 minute. Then 800 µL of Wash Buffer was added to the column. Centrifuged at12,000 x g for 30 seconds. the wash step was repeated, then carefully removed into tube of 25 µL DNase/RNase-Free Water directly was added and Centrifuged at 10,000 × g for 30 seconds to elute the RNA from the column. Yield and integrity of RNA were assessed by electrophoresis using a 1% non-denaturing agarose gel.[21]

Complementary Deoxyribonucleic Acid (cDNA) Synthesis

Last total RNA and detected liberated gene expression changing in bile salt exposition through on quantity of cDNA generated. The cDNA essay has used the PrimeScriptTM kit to includ Random 6 mers and Oligo dT Primer for use as reverse transcription primers. Reaction mixture on ice from

Table 1: Stander strains of bacteria and plasmid used in the present study

Bacterial strains and plasmid	*Features*
S. mutans	Microflora in human saliva (oral cavity) (Ery^S)
S. parasanguinis	Microflora in human Respiratory tract (Ery^S)
S. salivarius	a free probiotic plasmid treatment for human digestive balance, (Ery^S)
p*MG36bsh* artificial vector	Artificial expression vector, Replicon (rep), *T7*promoter, *EryR* with *bsh* gene

2 µL 5 × PrimeScript TM was added with total RNA and 10 µ l RNase Free dH_2O. The reaction mixture was Incubated under 37°C, 15 minutes*3 (Reverse transcription) and 85°C, 5 seconds (Inactivation of reverse transcriptase with heat treatment).[22]

Total RNA and cDNA Assessing

The quantitation of extracted total RNA and cDNA were occurred by using the Quantus™ Fluorometer prior to using dye systems highly sensitive, easy-to-use, fluorescent dye for RNA and dsDNA Quantitation was determined from working solution with 1X TE buffer add to 1X with nuclease-free water, the nucleic acid standard in a 0.5 mL PCR tube was Prepared and the blank sample for the QuantiFluor®. The centrifuge was prepared at 2,000 × g for 5–10 seconds; tubes were incubated at room temperature for 5 minutes, protected from light for taking quantity measuring (ng/µL).[23]

Bacterial Broth Media for Gallbladder Stones Removing Assay

Cholecystectomy remnants of stones after gallbladder enucleation, by dissolved 0.66 mg stone in 25 mL ethanol 96% then calibrated with 0.33 mg free cholesterol **(**70% ethanol was prepared for immersing the whole stone for 10 minutes to sterilize the stone surface).[14,23]

In the first step, 1-mL of gallbladder stone was dissolved once and 1-mL dissolved free cholesterol other once, but they empty from bile salt for each bacteria species tested at the study. The second step is to conclude tubes contain on the media with 0.5% bile salt for the growth of wild-type bacteria once in stone and others for growth in free cholesterol.[25] The final step is involved**,** tubes contain 0.5% bile salt for growth transform type bacteria with stone once and with free cholesterol the other once, all tubes were incubated aerobic conditions for 18 hours at 37°C. To detect the amount of cholesterol in each tube liquicolor kit to follow enzymatic colorimetric test for cholesterol esterification, unutilized cholesterol was assessed in the 300 µL supernatant from remaining bacterial growth media and standard cholesterol (STD) with 700 µL reagent tester (RGT) after 10 minutes at room temperature were measured all samples in spectrophotometer absorbance at O.D 500–520 nm and compared to the control[26] (Tables 3 and 4).

Isolates which have abled to cholesterol dissolving *in vitro* were selected from growth media. A well as know samples reducing attitude for cholesterol concentration, the values were calculated by the following math equation.[27]

$$C = 200 \times [\Delta A \text{ sample}/\Delta A \text{ STD}] \text{ (mg/dL)}$$

Cholesterol reducing ratio was calculated from this math equation

The cholesterol assimilated by bacterial strains was determined as follows:

$$\text{cholesterol assimilated } (\mu g/mL) = [\text{cholesterol } (\mu g/mL)] \text{ 0 h} - [\text{cholesterol } (\mu g/mL)] \text{ 24 h} \quad (1)$$

Cholesterol assimilated by each bacterial strain was also calculated in terms of percent cholesterol assimilation:

$$\%\text{ cholesterol assimilated} = [\text{cholesterol assimilated } (\mu g/mL)/\text{cholesterol } (\mu g/mL) \text{ 0 h}] \times 100 \quad (2)$$

Cholesterol assimilated by each bacterial strain was calculated considering a dose of 10^{10} cells:

$$\text{Cholesterol assimilated} = [\text{cholesterol assimilated (mg/mL)}/\text{Bacterial cell viability (cfu/mL)} \times 10^{10}] \quad (3)$$

Samples and standards were tested in duplicate to ensure accuracy and reproducibility.

Table 2: Levels of Total RNA expression from bacteria

Types of Bacterial growing media	*S. parasanguinis*		*S. mutans*		*S. salivarius*	
	RNA con. ng/µL	*cDNA ng/µL*	*RNA con. ng/µL*	*cDNA ng/µL*	*RNA con. ng/µL*	*cDNA ng/µL*
Wild type bacteria without stone, bile salt and free cholesterol	27.6	12	47	13	19	11
Wild type bacteria with stone, bile salt and free cholesterol	33.9	16	54	29	55	22
Transformer type bacteria with stone, bile salt and free	41	20	378	245	500	360

Table 3: Explain the gallbladder stone reducing concentrations

	S. parasanguinis		*S. mutans*		*S. salivarius*	
Bacterial growing media types	*Gall stones ratio (%)*	*Free cholesterol ratio (%)*	*Gall stones ratio (%)*	*Free cholesterol ratio (%)*	*Gall stones ratio (%)*	*Free cholesterol ratio (%)*
Wild type bacteria without stone, bile salt and free cholesterol	1	1	1	1	1	1
Wild type bacteria with stone, bile salt, and free cholesterol	16	74	47	45	78	61
Transformer type bacteria with stone, bile salt, and free	24	92	56	46	82	63

RESULT AND DISCUSSION

Bacterial Strain Characterization

Strains of *S.mutans*, *S. parasanguinis,* and *S. salivarius* were isolated to diagnose species through biochemical tests provided and *16S* rRNA, and samples have the ability to remove by the cholesterol-reducing gene that uses blood cholesterol (https://www. Brenda-enzymes.org Enzyme). php = Streptococcus + mutans) (https://www.brenda-enzymes.org/enzyme.php=Streptococcus) responsible for converting LDL cholesterol into a healthy state, all of which were sensitive to erythromycin, and the *cgh* gene does not contain adequate expression of gallstones fragmentation. Consequently, cholesterol was converted using p*MG36 bsh* vector, and it was transferred to the *bsh* gene for all bacteria carrying an expression of the dual *bsh* genes (Table 1), and biotherapy be more convenient for removing gallstones.

Detection and Quantification of *bsh* Activity

The activity of *bsh* was detected in previous studies 0.5% sodium salt of urodeoxycholic acid[27] in media. Positive bacterial growth was survival passages through the stomach and tolerant to bile salts concentrations in the small intestine. The comparison of various bacterial growing for bile salts tolerance throughout many studies showed that. All strains exhibited significant differences with growth in control broth after 24 hours. The optical densities of all strains approach 1.85×10^{10} besides the concentration of 0.5% bile salts, had the highest enhancement for a growth rate of bacterial strains.[28]

Bacterial Transformation and Plasmid Extraction

Transformation of p*MG36bsh* vector was done in *S.mutas, S. parasanguinis, and S. salivarius* like sensitive bacteria for erythromycin, for each dish in selective media the competent cells selective by natural transformed protocol gave 7-15cell\100µL competent single colonies, with 100 µL Erythromycin device for p*MG36bsh* vector presenting. The pMG36bsh vector renewal was extracted from all transformer strains to ensure bacterial transformation was achieved successfully.[29] (Figure 1).

Total RNA Expression Levels and cDNA Synthesis Test

Additionally, the 0.5% (sodium salt of urodeoxycholic acid) play a great role in the levels of expression to (*cgh,bsh*) gene copies while *S.mutans S. parasanguinis and S. salivarius* were growing. The results demonstrated an increasing in expression for these genes and the existence of a difference between bacterial types; the change in total RNA expression concentrations that occurred lead to cDNA synthesis reinforced the validity of these rates that its will be easy to fragment gallbladder stones process in bacterial media (Table 2, Figure 3), The migrated samples will have sharp *23S* and *16S rRNA* bands of transformer bacteria,they have (*23S:16S*) (850bp,1000bp) are a good evidence for RNA indicted[30] (Figure 2). Given equal amounts of RNA were used for cDNA synthesis, calculations showed a similar number of copies *16S* and *23S* rRNA [31] .

Ration of Gallbladder Stone Reducing

The diffraction of (*cgh* and *bsh*) genes expression in transformer *S.mutans, S. parasanguinis and S. salivarius* uncured when

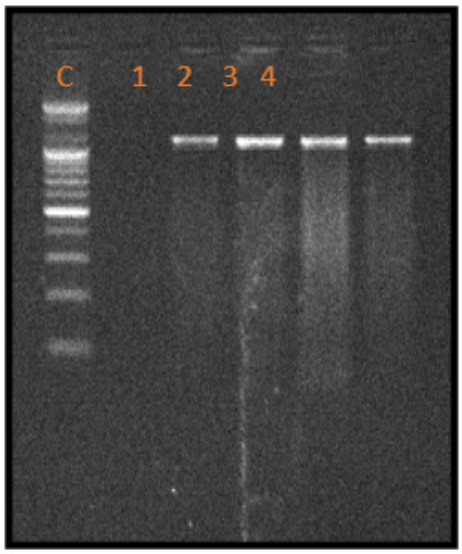

Figure 1: Gel electrophoresis 0.7% to extracted pMG36bsh vector from all transformer strains,C: Ladder 100bp, 1 : p*MG36bsh* vector (control), 2: *S.mutans* pMG36*bsh* vector *S. parasanguinis* p*MG36bsh* vector *and* 3: *S. salivarius* p*MG36bsh* vector

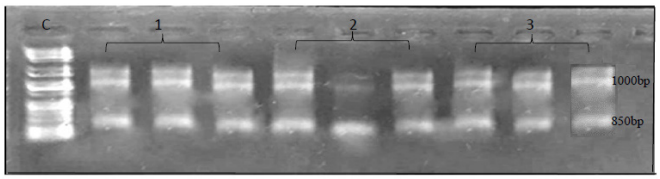

Figure 2: Total RNA expression (*23S:16S*) (85bp, 1000bp) for all strains (wild type and transformer type), C: Ladder 100 bp 1: *S. mutans*, 2: *S. parasanguinis* and 3: *S. salivarius*

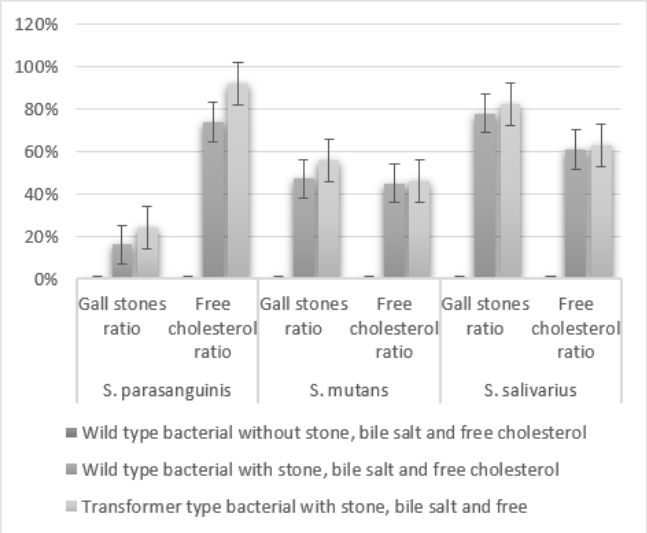

Figure 3: Total RNA expression for The comparative reducing of series (2) of gallstones cholesterol and series (1) free cholesterol concentrations in media with bile salt for growthing wild type and transformer type of *S. mutans, S. parasanguinis,* and *S. salivarius* strains.

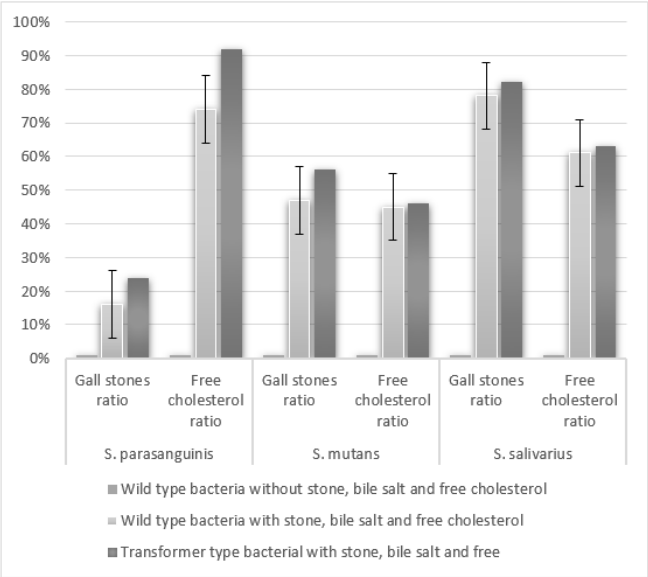

Figure 4: The comparative reducing of series (2) of gallstones cholesterol and series (1) free cholesterol concentrations in media with bile salt for growing wild type and transformer type *of S.mutans, S. parasanguinis* and *S. salivarius* strains.

its used in cholesterol liguicolor kit by detection reagent and cholesterol reducing ratio in media before and after bacterial growthing. Appeared data in rodents have demonstrated the concentrations of stone in media showed hesitated reducing comparting with control, it be a proximally 1.3–0.1 g/L and free cholesterol concentration decreased from 1.35–0.84 g/L, Bacterial reducing rate progressively have, transformer *S. salivarius* has registered higher reducing range was 82% like intestinal strain like probiotic for digestion balance, hereafter transformer *S.mutans* like dental strain has reduced range was 56% in bacterial media and their reducing ratio was compared with free cholesterol digestion was 63% respectively, the other mean its interesting strain to probiotic drug synthesis for dissolving gallbladder stones.[31] But the transformer *S. parasanguinis* like microflora branchial strains has a lower reduced ratio (26%) in comparison to other strains, even through its reducing rate (92%) differenced with free cholesterol digestion.[32] As long as last result made *S. parasanguinis* non probiotic kind and didn't have all gene expression machines to gall bladder stones destroyed, the events didn't show ribosomal compatibility with *pMG36bsh* vector expression (Table 3 and Figure 4). Multiple comparisons of reducing results also followed the genetic modification of studying affected on bacterial gene expression, it be device for bacterial reducing machines according to bacteria species in the human body. This bacterial genetic leap can be principal non-invasive or non-surgical medical treatment for cholesterol gallstones before and after cholecystectomy with other drugs.[24]

Statistical Analysis

The results are expressed as a standard error medium (SEM). Statistical analysis was performed using SPSS version 17.0, and statistical comparisons were made through multiple means comparisons using subsequent Tukey analysis. Statistical Bacterial cell viability (cfu/mL) *S.mutans, S. parasanguinis, and S. salivarius* in nutrient broth containing 130 μg/mL of gall bladder stone and other 135 μg/mL of free cholesterol, following 18 hours of incubation. Data are represented as means ± SEM, $n = 2$.

Homogeneous Tukey subgroups resulting from even comparisons are represented as A, B, and C, although A is the most important subgroup of control.

The significance of cholesterol reduction was determined at $p < 0.05$ and p values below 0.05 were considered significant for *S. salivarius* and *S. mutans*, and the chi-square statistic was 138.5199 and 57.1286 but not significant for *S. parasanguinis*.[33]

Data information attitude to environment factors of digestive tract bacteria have a singularity in genes function for cholesterol removing more than other bacterial microflora in the human body; this quality made it more convenient to synthesize bacteria like biotherapies dissolve gall bladder stones from genetic modification and bacterial changing behavior integration.

CONCLUSION

Recent work, we have pretended a genetic determination in *S.mutans, S. parasanguinis, and S. salivarius* that contributes to bile salt resistance to examine this possibility by analyzing hydrolysis of conjugated salts in bacterial media utilize 0.5% Ursodeoxycholic acid-like dissolved therapy to the cholesterol of gallstones and *bsh* genes inducer stiff depend on. The pushing up bacterial *bsh* gene expression to extra level in *S.mutans, S. parasanguinis and S. salivarius* and help to convert like gallstones cholesterol treatment. This work adds the best explanation of the molecular kinetikes hold to change and has benefited *S.mutans, S. parasanguinis,* and *S. salivarius* products.

All studying manifestations have synthesized *S. mutans, S. parasanguinis and S. salivarius* have *cgh* gene copy and transforming *pMG36bsh* vector were carrying *bsh* gene copy to code extra quantity of bile salt hydrolase for complete bacterial physicochemical attitued for superfluity genes expression like (microflora, probiotic) therefore, become a vailed bacteria for gallstones fragmentation and synthesis therapy. Work data appeared increasing rates of bacterial whole RNA expression after transformation resulted remission gallstones amount in media when matched with control.

All most investigations have signed to variable regions of the *bsh* genes from various species that could be useful for biotherapy development for many human diseases. The bacterial genome modification in mammalian cells for implementation is considered a leap of future challenges for pharmaceutical improvement.

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REFERENCES

1. Agostino Di C. Recent advances in understanding and managing cholesterol gallstones F1000 Research. 2018;7:1529.

2. Gabriel E N .Gallstones . Niger J Surg. 2013;19(2):49–55.
3. Sara C. Non-surgical Treatments for Gallstones. Everyday health. 2013;7.
4. Joey Ho Y C. and Anthony Yuen B Y.. Current Status of Endoscopic Gallbladder Drainage. Clin Endosc. 2018;51:150-155.
5. Choi JH, Lee SS, Choi JH, *et al*. Long-term outcomes after endoscopic ultrasonography-guided gallbladder drainage for acute cholecystitis Endoscopy. 2014;46:656-661.
6. Markowiak A, Katarzyna S. Effects of Probiotics, Prebiotics, and Synbiotics on Human Health Paulina. nutrients. 2017;9:1021.
7. Marteau P, Shanahan F. Basic aspects and pharmacology of probiotics: An overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract. Res. Clin. Gastroenterol. 2003;17:725–740.
8. Goldiner I, Leikin-Frenkel A, Konikoff FM. Innovations in the medical treatment of gallstones and fatty liver: FABACs (Fatty Acid Bile Acid Conjugates). PMID. 2008;147(4):344-349.
9. Flore E, Tiepma N B, Tiencheu B, Tatsinkou A, Fossi D, Ufuan A, Marcel H S, Macaire Z W, François N. Isolation and Identification of Cho- lesterol Lowering Probiotic Bacteria from Palm Wine (*Raffia mambillensis*) J. of Micro. Res. 2016; 6(5):93-102.
10. Manoj K, Ravinder N, Rajesh K, Hemalatha R, Vinod V, Ashok K, *et al*. Cholesterol-Lowering Probiotics as Potential Biotherapeutics for Metabolic Diseases. Exp Diabetes Res. 2012: 902917.
11. Geun-Bae K, Byong H L. Iochemical and Molecular Insights into Bile Salt Hydrolase in the Gastrointestinal Microflora. Asian-Aust. J. Anim. Sci. 2005;18(10):1505-1512.
12. Iyer R, Tomar S K, Kapila S, Mani J, Singh R. Probiotic properties of folate producing *Streptococcus thermophiles* strains. Food Res. Intern. 2010;43(1):103-110
13. Tint GS, Salen G, Colalillo A, Graber D, Verga D, and Speck J, Shefer S. Ursodeoxycholic acid: a safe and effective agent for dissolving cholesterol gallstones. Ann Intern Med. 1982;97(3):351-6.
14. Atheer A M. Cloning and over expression of bile salt hydrolase gene A (*bshA*) from *Lactobacillus acidophilus Ar* in *E. coli* Bioengi. and sci. Insti., Kahraman marash sutchu imam University /Turkey; 2014.
15. Muriel C, Daniela C, Ouidad S . Evaluation of the incidence of cholelithiasis after bariatric surgery in subjects treated or not with ursodeoxycholic acid. Surgery for Obesity and Related Dis. 2016;13(4):681–685.
16. Beilei Ge, Kelly J D, Qianru Y, Shenia R Y, Crystal L R, Sonya M B, Stuart A G, *et al*. Effects of low concentrations of erythromycin, penicillin, and virginiamycin on bacterial resistance development *in vitro* . Scie. Reports .(2017); 7: 11017.
17. Atheer AM. Fragmentation of gallbladder stones using transformer *Streptoccocus salivarius* and measuring of RNA expression for cholesterol lowering gene. Pak. J. Biotechnol. 2017;14(4):745-751 .
18. Kamila G. Zbigniew C. Char- acterization of selected strains from *Lacto- bacillusacidophilus* and *Bifidobacterium bifidum.* African J. of Micro. Res. 2007;1(6):065-078 .
19. Ke D. Lvqin H. Yung-Fu C, Sanjie C, Qin Z, Xiaobo H, *et al*. Basic Characterization of Natural Transformation in a Highly Transformable *Haemophilus parasuis* Strain SC1401 . Frontiers in Cellular and Infection Microbiology. 2018;8(32):1-18.
20. Jan N, Jitka H, Miroslav P. An integron of class 1 is present on the plasmid pCG4 from Gram-positive bacterium *Corynebacterium glutamicum* . FEMS Microbiology Letters. 1998;169:391-395.
21. Alessandro B, Silvia S, Roberta P, Francesco B, Massimo A. Rapid methods to extract DNA and RNA from *Cryptococcus neoformans* . FEMS Yeast Research. 2001;1:221-224.
22. Douglas K N, Sanggyu L, Guolin Z, Xiaohong C, Clarence W, Terry C *et al*. Oligo (dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription . PNAS. 2002;99(9):6152–6156.
23. Tony Y, Elisa W, Robert L, Pfeffer, Barbara J, Ebersole S C, Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays *Nucleic Acids Research.* 2002;30(10): 48.
24. Al-Saleh AA, Metwalli AAM, and Abu- Tarboush H B. Bile Salts and Acid Tolerance and Cholesterol Removal from Media by some Lactic Acid Bacteria and Bifidobacteria. J. Saudi Soc. for Food and Nutrition. 2006;1(1).
25. Małgorzata Z. The influence of cholesterol and biomass concentration on the uptake off cholesterol by lactobacillus from MRS broth. Scientific journal. 2007;6(2):29-40.
26. Eckfeldt J M. Laboratory Procedure Manual Analyte of HDL-cholesterol in serum. Univ ersity of Minnesota Medical Center. 2008;1-21.
27. Tomaro-Duchesneau M L, Jones D, Shah P, Jain Sh. Saha, and Prakash S. Cholesterol Assimilation by Lactobacillus Probiotic Bacteria: An *In Vitro* Investigation. BioMed Research International. 2014;9.
28. Bernard B. Laboratory Methods for the Diagnosis of Meningitis. Center for diseases for control and privation. 1999; CH: 8.
29. Ludwig W, Schleifer KH. Bacterial phylogeny based on *16S* and *23S* rRNA sequence analysis . FEMS Microbiol Rev. 1994;15(2-3):155-173.
30. Sung K, Khan S A, Nawaz M S, and Khan A A, A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. FEMS Microbiol. Letter. 2003; 229(1):97-101.
31. Santichai N, Chatrudee S. and Kornchanok W. Effect of Prebiotics-Enhanced Probiotics on the Growth of *Streptococcus mutans* .International Journal of Microbiology. 2019;7.
32. Kirsten P R G, Andrew G, Cecere N P, Wasilko A L, Williams K M, Bultman M J, Mandel T M. Incompatibility of *Vibrio fischeri* strains during symbiosis establishment depends on two functionally redundant hcp genes. *Journal of Bacteriology.* 2019; 201(19):e00221-19
33. Carson D D, and Daneo-Moore L. Effects of fatty acids on lysis of *Streptococcus faecalis* Journal of Bacteriology. 1980;141(3): 1122–1126.