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TRANSFORMATION AND PUSHING UP OF *bsh* GENE IN *S. FEACALIS* TO GALLBLADDER STONES FRAGMENTATION

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ABSTRACT
Stones in the common duct are associated with increased risks of complications and death. Consequently, they need to be removed. The risk of gallstone formation rises markedly with the use of estrogen and clofibrate. Bacterial stains and plasmid used in this work was *S. salivarius* and *S. feacalis.* The bile salt tolerance to test the bacterial growth tolerance. Also, antibiotic tolerance was made to test bacterial growth tolerance. The extraction of plasmid PMG36bsh vector uses ethanol 96%. Total RNA extraction was made then cDNA synthesis with PCR machine. The fragmentation of gallbladder stones in *S. feacalis* broth media. The using of PMG36bsh vector carry bsh gene to get duplicate bsh gene expression in *S. feacalis* probiotic strain and became suitable drug to remove gallbladder stones. Tolerance to bile salt genes allows probiotic bacteria to survive in the small intestine. Total RNA expression levels of bacteria *S. feacalis* which show in control of *S. feacalis* total RNA conc. was 35.3ng/µl and cDNA conc.77.6 ng/µl. In wild type 97.7 ng/µl and 197.6ng/µl in *S. feacalis* (transformer type) 140.6 ng/µl and 237 ng/µl. Also the gall bladder reducing concentration and reducing ratio, which show in wild type bacterial with stone, bile salt and free cholesterol, the gallstone conc. 0.814 g/l in transformer type bacterial with stone, bile salt 0.748g/l. The bacterial modification can become principal noninvasive non-surgical treatment for cholesterol gallstones before and after treatment.

KEYWORDS: Bacterial stains, plasmid, RNA extraction, cholesterol.

INTRODUCTION

Gall stones are abnormal masses of a solid mixture of cholesterol crystals, calcium carbonate, phosphate, bilirubinate and palmitate, phoshplipids, glucoproteins and mucopolysaccharides, which were first described in 1507 by Antonio Benivenius [1]. Gall stone disease is one of the most prevalent and costly gastrointestinal tract disorders in the world. The gall stone formed within gallbladder out of bile component which called cholelithiasis as refer to the presence of stones in the gallbladder or the diseases caused by gall stones[2]. Streptococcus or Enterococcus, particularly *Enterococcus faecalis* are involved in the reduction or prevention of gastrointestinal tract infections [3]. Enterococci belong to group of LAB, which is known to produce lactic acid as the end product of sugar fermentation and antimicrobials which are active against pathogens including *Staphylococcus aureus* and listeria monocytogenes[4]. Further, many stains of *E. faecalis* produce bacteriocins named enterocins a family of safe and ribosomally synthesized antimicrobial peptide[4]. *Enterococcus faecalis* is the main probiotic found in doctor Ohhiras probiotic. Doctor Ohhairas probiotics stain of *Enterococcus faecalis* is a specific strain of Enterococcus Known as TH10. *Enterococcus faecalis* TH10 may be a safer strain of *Enterococcus faecalis* [5,6]. Bile salt hydrolase (bsh) plays an essential role in the cholesterol removing effects which hydrolyze conjugated bile salts to amino acid and deconjucated bile salts. Streptococcus faecalis lacks the bsh gene. Thus this enzyme plays a vital role in the colonization and survival of commensal bacteria in mammalian intestine and determination of their probiotic potential. The bile deconjugation also leads to lower of cholesterol and alterations in energy homeostasis and clinically making bsh an important enzyme [7].

MATERIALS & METHODS

Bacterial strain and plasmid

S. salivarius and *S. feacalis* cells bacteria was growing in nutrient broth medium at 37ºC for 18h , for activated bacteria was streaked and were propagated media contain 100 µg/ ml con. of Erythromycin antibiotic for plasmid detection and for bacterial antibiotic sensitively. The bacterial strain and plasmid used in this work (table 1).

TABLE 1:- bacterial strain and plasmid used in this work

Bacterial strains and plasmid	Properties
S. salivarius	Probiotic for human treatment, (Ery^R), with pMG 36*bsh* plasmid
S. feacalis	Plasmid free probiotic strain used for human treatment, Ery^S
pMG36*bsh* vector carried in *S. salivarius*	Cloning and expression plasmid, Replicon (rep), 7promoter, Ery^R

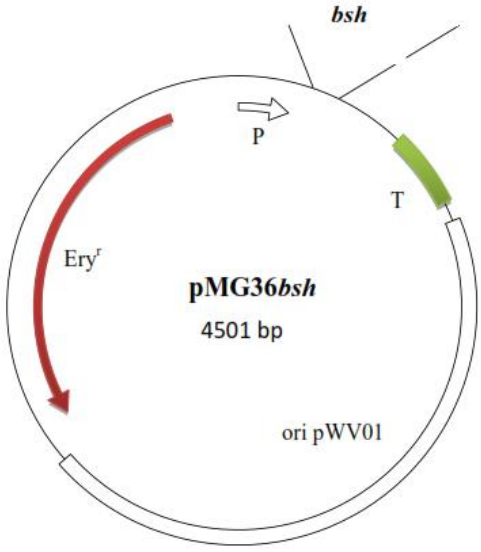

FIGURE 1 :- Physical map of pMG36*bsh* vector

Bile salt tolerance

The *S. feacalis* strain was treated with (sodium salt of urodeoxycholic acid) (SCA) using 0.5% (wt/vol) concentration and incubated at 37ºC aerobically for 18h. To test bacterial growth tolerance the experiments were performed in duplicates. In both cases, the survival percentage of LAB was calculated by the following formula [7]:

% Survival = Final (cfu/ml) /control (cfu/ml) x 100

Antibiotic tolerance

The *S. feacalis* strain was treated with 100 µg/ml erythromycin and incubated at 37ºC aerobically for 18hr. to test bacterial growth tolerance.

Plasmid pMG36*bsh* vector **extraction uses ethanol 96% method**

S. salivarius was picked up a single colony from a plate with 100 µg/ml Erythromycin and was incubated overnight at 37^{o}C in nutrient broth media. Wishing cell from overnight culture centrifuged at high speed for 15 min. take cells pellet .

suspend the pellet in 1ml solution (25 mM Tris.Cl [pH 8.0], 10 mM EDTA [pH 8.0]). Repeated the centrifugation twice . add 150 ul solution I (3 M sodium acetate) for last cell pellet for cell lysis process used sonication with a Soniprep 150 (USA) in ice-cold condition. The sonicator was set to 16 micron amplitude for 5 minutes (1\2 min turn on and 1\2 min turn off). Cells suspension was centrifuged at 8,000 x g for 30 min , centrifuge at high speed for 5 min. Carefully remove the supernatants and put them into new eppendorf tubes. Discard the old tubes and pellets. Add 2.5 volumes of ice cold 95% ethanol and mix well to precipitate the DNA. Centrifuge on high speed for 5 min ,remove the liquid y wash the pellet with 400 ul of ice cold 70% ethanol. Spin for 3 min. took the pellet back into the centrifuge ,allow the pellet to air dry for 5-10 min. Resuspend the pellet in 20 ul of TE (10 mM Tris.Cl [pH 7.5], 1 mM EDTA [pH 7.5],) .extracted plasmid was measurement in Nano drop spectronic device and Store in the freezer at -20^{o}C for later use.(1)

Natural transformation assay

In routine assays, aliquots of a growing culture were mixed with 500 µl extracted pMG36*bsh* vector and held at 37°C for 18 hr with 250 µl *S. feacalis* growth culture in eppendorf tubes complete to 2 ml from nutrient broth contain (100 µg/ml) erythromycin . The competent *S. feacalis* cells plated in selective media from (100 µg/ml) erythromycin 18hr overnight.(2)

***Bsh* gene expression assay**

Total RNA extraction

Wild type and transformer type of *S. feacalis* cells was growthing up in triple extragene centergugation tubes at 37°C for 18 hr with 0.5% (sodium salt of urodeoxycholic acid) (SCA) and without bile salt (control)to total RNA extraction used easy-spinTM [DNA free]Total RNA Extraction Kit.., prepare 1-10x106 cell in 1.5ml tube. Centrifuge it to remove culture media (13,000rpm, 10sec), and add 1ml of Lysis buffer(easy-BLUETM reagent).

Vigorously vortex in room temperature for 10sec. Add 200ml of Chloroform and apply vortex. After centrifuging the solution at 13,000 rpm (4) for 10 min, transfer 400 ml of the upper fluid to an empty 1.5ml tube. Add 400 ml of Binding Buffer and mix it well by pipetting or gently inverting the 2-3 times. Do not centrifuge and leave it for 1min at room temperature.6. Load the upper solution to the column, but do not load the whole upper solution because the maximum volume of the column reservoirs is 800 ml. After loading the optimum of the upper solution to the column, and centrifuge at 13,000rpm for 30sec. Discard the low through after centrifuging and place the spin column back in the same 2ml collection tube. Then repeat this step, the maximum volume of the column reservoirs is 800 ml. For same volume or larger volume, reload the remained sample in the column and spin again.. Add 700 ml of Washing Buffer A to the column. Close the tubes gently, and centrifuge for 30 sec at 13,000rpm to wash the column. Discard the flow-through and place the spin column back in the same 2ml collection tube. Wash by adding 700 ml of Washing Buffer B to the column and centrifuge for 30 sec at 13,000rpm. Discard the filtrates and place the spin column back in the same 2ml collection tube, washing Buffer B is supplied as a concentrate. Ensure that ethanol is added to Washing Buffer B before use. Centrifuge for 1-2 min at 13,000rpm to dry the column membrane. Place the column in a clean 1.5ml

microcentrifuge tube (not provided), and add 50 ml of Elution Buffer directly onto the membrane. Incubate at RT for 1min, and centrifuge for 1min at 13,000rpm to elute. Total RNA extraction was measured at nanodrope machine O.D 260nm , also load 1 µg samples on 1% agarose gels in TBE (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA) with 0.5 µg/ml ethidium bromide added to the gel. Add 10X native agarose gel loading buffer loading dye to the RNA samples to a final concentration of 1X to 90min ,the remaining samples saved at -70 C°(3).

cDNA synthesis

Maxime RT PreMix Kit is the product for cDNA synthesis just with PCR machine. Add 0.1-1ug template RNA and 0.1-1ug (Oligo dT or Random primer) with complete the mix distilled water to a total volume of 20ml. Dissolve the blue pellet by pipetting.. Perform the cDNA synthesis reaction cycles as follows using PCR machine:

Reaction step	Tem	Time
RTase inactivation step	95 C°	5min
cDNA Synthesis	45C°	60 min

Dilute the reactant above by adding 50-80ml sterile water into a tube containing the cDNA obtained at RT reactant. Transfer 1-5ml of RT product (synthesized cDNA) to *Maxime* PCRPreMix tube. 5-2) Perform PCR cycles according to the PCR condition, PCR product measured at nanodrope machine O.D 260nm. Generally load 1µg samples on 1% agarose gels in TBE (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA) with 0.5 µg/ml ethidium bromide added to the gel. Add 10X native agarose gel loading buffer with loading dye to the RNA samples to a final concentration of 1X to 90 min , the remaining samples saved at -20 C°[4].

Fragmentation of gallbladder stones in *S. feacalis* broth media

After cholecystectomy the gallbladder was opened to take 0.66 mg stone was dissolved at 25ml ethanol 96% and 0.33 mg free cholesterol at 50ml ethanol 96% to corporation[6] (Department the whole stone was immersed in 70% ethanol for 10 min for surface sterilization)(5), were transferred (1ml) to a culture(nutrient broth media) to make 3 tubes to each once . The control twice tube for growthing wild type bacteria empty from bile salt , gallbladder stone and free cholesterol. The other once media tubes for growthing wild type bacteria with stone once and other for growthing in free cholesterol. The last media tubes for growthing transfer type bacteria with stone once and with free cholesterol the other once, all was incubated aerobically environment for 24 hours at 37C°. For cholesterol amount detection in all of last tubes used cholesterol liquicolor kit to fellow enzymatic colorimetric test for cholesterol estering, , unutilized cholesterol was estimated in the 300µl supernatant from Stagnant bacterial growth media and standard cholesterol(STD) with 700 µl reagent tester(RGT) after 10 min at room temperature were measured all samples in spectrophotometer absorbance at O.D (500-520 nm) , and compared to the control. Isolates having *in vitro* reduction of cholesterol on the media were selected. the values was calculated in this math equation to took cholesterol concentration for all samples(6,7):-

$$C = 200^* \frac{\Delta A \text{ sample}}{\Delta A \text{ STD}} \text{ (mg/dl)}$$

And calculated cholesterol reducing ratio from this math equation(6):-

$$\text{Cholesterol reducing r} = \frac{\text{Absorbance of cholesterol in media after growthing bacterial cells}}{\text{Absorbance of Cholesterol in media before growthing bacteria cells}} \times 100$$

RESULTS

Bacterial strain characterization

S. feacalis probiotic strain was isolated from tablet treatment for get diseases, Its has ability to remove cholesterol because have *bsh1* gene responsible convert LDL cholesterol to healthy case and sensitive for erythromycin, these gene do not have enough expression for fragmentation of gallbladder stones cholesterol, therefore its was transformed by using pMG36*bsh* vector carry *bsh* gene to get duplicate *bsh* gene expression in the bacteria (Fig 1) and become suitable drug to removal gallbladder stones .

Bile salt tolerance

Tolerance to bile salt genes allows probiotic bacteria to survive in the small intestine. At the beginning of the performed experiments this study strains cultured with 0.5% (sodium salt of urodeoxycholic acid) (SCA) resulted to increasing of live cells number from ($O.D_{600}$= 1.25 to 1.85) cfu\ml to ensure gene *bsh* stimulation and the survival percentage was 98% can thus be classified as resistant isolates. Since they are tolerant to high bile salts concentration, they are beneficial to the body because according to[8]. high bile acid will co-precipitation in low pH environment with cholesterol rich food and facilitate the uptake of cholesterol (Cholesterol assimilation, incorporation to cell membrane or attachment to the bacterial cell surfaces Bile salt resistance is recommended as a suitable parameter for selecting probiotic strain[9].

Plasmid extraction and *S. feacalis* transformation

Getting from ethanol 96% protocol suitable for DNA purity that was 80% (1.7 nm) gave 7-10 cell\ 100µl competent single colonies, to each dish in selective media with 100 µl Erythromycin when *S. feacalis* transformation overnight and instead bacteria form sensitive to resistance Erythromycin because pMG36*bsh* vector presenting.

Total RNA expression levels and cDNA synthesis assay

The addition of 0.5% (sodium salt of urodeoxycholic acid) (SCA) play a predominant role in shaping expression levels of individual genes in growthing bacteria *S. feacalis* (wild type and transformer type). The results indicated an increase in the expression and the existence of a difference between bacterial types, the change occurred in total RNA concentrations. The cDNA synthesis reinforced the validity of these results that it will be easy to fragment bacteria gallbladder stones process in bacterial media (Table: 2), the gel will have sharp 23S and 16S rRNA bands of bacteria. (23S:16S) (800bp, 1000bp) are a good indication that the RNA is intact (Fig: 3). Equally

important that appearance bright band in gel agarose of cDNA migration give proximally 4500bp[10]. Considering that equal amounts of RNA were used for cDNA synthesis, copy number calculations demonstrated similar 16S and 23S rRNA[11] (Fig. 4).

TABLE 2: Total RNA expression levels of bacteria *S. feacalis*

Bacterial types	Total RNA con.	cDNA con.
S. feacalis (control)	35.3 ng\µl	77.6 ng\ µl
S. feacalis (wild type) cultured in 0.5% bile salt	97.7 ng\µl	197.6 ng\ µl
S. feacalis (treansformer type) cultured in 0.5% bile salt	140.6 ng\µl	237 ng\ µl

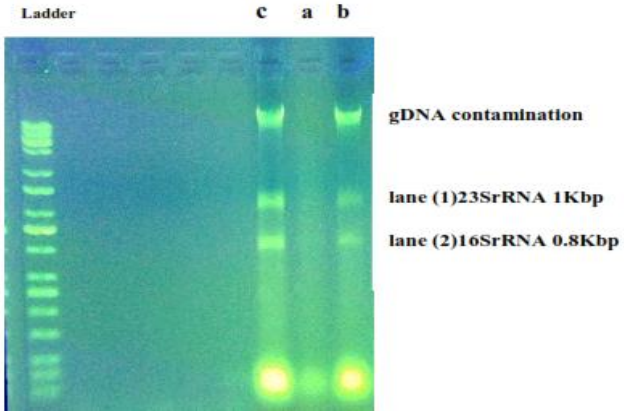

FIGURE 3: 1% gel agarose of total RNA expression levels lane 1,2 (23S:16S) of bacteria *S. feacalis* (a): *S. feacalis* (control)
(b) *S. feacalis* (wild type) cultured in 0.5% bile salt.(c): *S. feacalis* (transformer type) cultured in 0.5% bile salt.(Ladder) 100bp

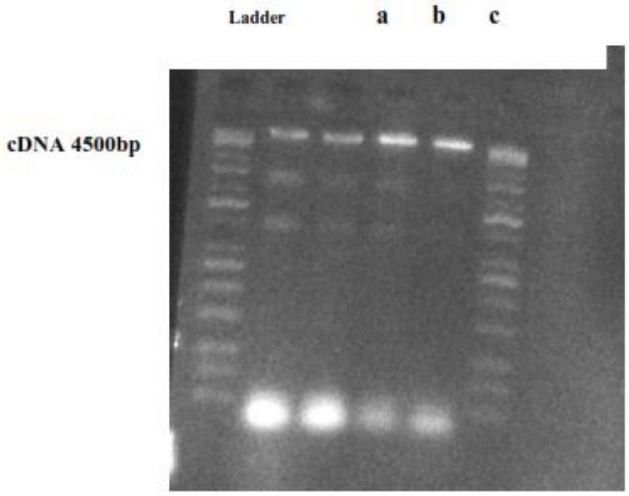

FIGURE 3: 1% gel agarose of cDNA synthesis band (a,b,c,) of bacteria *S. feacalis* (a): *S. feacalis* (control) .(b) *S. feacalis* (wild type) cultured in 0.5% bile salt.(c): *S. feacalis* (transformer type) cultured in 0.5% bile salt.(Ladder) 100bp

Gallbladder stone reducing ratio assay

The diffraction of (*bsh1*and *bsh*) genes expression in transformer *S. feacalis* was uncured when its used in cholesterol liguicolor kit by detection reagent and cholesterol reducing ratio in media before and after growth of bacteria. Recent data in rodents have demonstrated that concentrations of stone in the media reduced comparing with control a proximally (1.3to 0.748) g/L and free cholesterol reducing concentration from (1.35 to 0.873) g/L (12) relatively low, ranging 37.43% reducing ratio in bacterial media comparted with free cholesterol (table 3), to explain the decreasing ratio occur because dissolution of cholesterol gallstones .The bacterial modification can become principal non-invasive non-surgical medical treatment for cholesterol gallstones before and after cholecystectomy [6,7].

TABLE 3: Explain the gallbladder stone reducing concentrations and reducing ratio

Bacterial types	Gallbladder stone con.	The reducing ratio	Free cholesterol con.	The reducing ratio
Bacterial media empty from stone, bile salt and free cholesterol	1.3 g/L	-	1.35 g/L	-
Wild type bacterial without stone ,bile salt and free cholesterol	1.3 g/L	-	1.35 g/L	-
Wild type bacterial with stone ,bile salt and free cholesterol	0.814 g/L	40.71%	0.896 g/L	44.82%
Transformer type bacterial with stone ,bile salt and free cholesterol	0.74.8 g/L	37.43%	87.3 g/L	43.64%

DISCUSSION

The activity of bile salt hydrolase has been observed in *lactobacillus*, *clostridium*, and *Bacteroides* spp. *lactobacillus* and *Bifidobacteria* which are used as probiotic strains[20]. *Enterococcus* spp. are also commensal of gastrointestinal tract inhabitants. Living microorganisms which upon ingestion in the certain numbers exert health benefits on the host beyond inherent basic nutrition which defined as probiotics[6]. A potential probiotic strain which is selected to be capable of performing effectively in the gastrointestinal is a significant challenge. In the present study *S. feacalis* probiotic strain was isolated from tablet treatment for get diseases, Its has ability to remove cholesterol because have *bsh1* gene responsible convert LDL cholesterol to healthy case and sensitive for erythromycin, these gene do not have enough expression for fragmentation of gallbladder stones cholesterol, therefore its was transformed by using pMG36*bsh* vector carry *bsh* gene to get duplicate *bsh* gene expression in the bacteria and become suitable drug to removal gallbladder stones . The probiotic strains which are able to hydrolyse bile salts has often been included among the selection of probiotic bacteria criteria, and a number of bile salt hydrolyses (BSHs) have been identified and characterized. However, the BSH activity in microbes has also been mooted to be potentially detrimental to the human host, and thus it is as yet not completely clear whether the activity of BSH is in fact a desirable trait in probiotic bacteria [21]. The purification and characterization of BSHs form various microorganisms and located intracellular, are oxygen insensitive, and have slightly acidic PH optima (usually between PH5 and 6)[22]. Although several reports in the literature do not correlate the bile tolerance of strains with BSH activity and possibility that these studies may have used inappropriate experimental conditions; for example, the using of tauroconjugated bile acids for detection of BSH activity, even though the majority of BSHs show a preference for glycoconjugated bile acids. Furthermore, since many different factors influence the bile tolerance of strains (*e.g.*, membrane characteristics comparing nonisogenic strains will not give a really representation of the contribution of BSH to bile tolerance [23]. Probiotic has modernized the current dietetic sense with novel therapeutic and nutritional benefits to the consumers. The presence of bile salt hydrolase (BSH) in probiotics renders them more tolerant to bile salts, which also helps to reduce the blood cholesterol level of the host [24]. BSH activity is widely distributed in many GI microbes and many bacterial groups that have been commonly used as probiotics for human as well as animal applications. However, it remains to be determined whether the BSH activity of the probiotics is beneficial or detrimental to the host. Once it is clarified, this feature could be used as one of the selection criteria for the probiotics. Future genetic analyses should concentrate on expanding the information available on the molecular mechanisms how bacteria regulate the bsh gene in the GI conditions and why the GI microbes have evolved to harbor the BSH activity[25]. This may lead to a better understanding of the interaction between animal host and microbes in their GI tracts. It is possible that information obtained from the BSH research may ultimately lead to the development of improved probiotic strains and assist in the manipulation of gut function for improving animal health. Undoubtedly, the use of probiotics has attracted lots of attention as an alternative to antibiotics in the livestock industry. Knowledge gained through bile research will provide further insight into the survival of probiotics as well as pathogens in the GI tract [26]. An understanding of how BSH active probiotics have evolved the self-defense mechanism against the toxic nature of deconjugated bile acids may also explain the antagonist action of autochthonous microorganisms of the intestinal flora such as lactobacilli against pathogens in the intestines[25]. Potential hypocholesterolemic pharmaceuticals and food products are continuously being developed in order to control serum cholesterol levels in hypercholesterolemic patients. These pharmaceuticals are mostly based on interruption of the enter hepatic circulation of bile salts. Enhanced BSH activity of probiotics may offer potential as a biological alternative to pharmaceutical interventions to prevent and treat hypercholesterolaemia [26]. The *bsh* gene could be used for the development of a new genetic marker in some bacterial groups. For example, the bsh gene is common in bifidobacteria, is present as single copies on the genome and contains conserved nucleotide signatures that are suitable targets for PCR primers. The use of bsh gene may be suitable for reliable identification and phylogenetic analysis of Bifidobacterium species. Secondary bile acids, produced solely by intestinal bacteria, can accumulate to high levels in the enterohepatic circulation of some individuals and may contribute to the pathogenesis of colon cancer, gallstones, and other gastrointestinal (GI) diseases. Bile salt hydrolysis and hydroxy group dehydrogenation reactions are carried out by a broad spectrum of intestinal anaerobic bacteria, whereas bile acid 7-dehydroxylation appears restricted to a limited number of intestinal anaerobes representing a small fraction of the total colonic flora. Microbial enzymes modifying bile salts differ between species with respect to pH optima, enzyme kinetics, substrate specificity, cellular location, and possibly physiological function. Crystallization, site-directed mutagenesis, and comparisons of protein secondary structure have provided insight into the mechanisms of several bile acid-biotransforming enzymatic reactions. Molecular cloning of genes encoding bile salt-modifying enzymes has facilitated the understanding of the genetic organization of these pathways and is a means of developing probes for the

detection of bile salt-modifying bacteria. The potential exists for altering the bile acid pool by targeting key enzymes in the 7α/β-dehydroxylation pathway through the development of pharmaceuticals or sequestering bile acids biologically in probiotic bacteria, which may result in their effective removal from the host after excretion.

CONCLUSION

The bacterial modification can become principal non-invasive non-surgical medical treatment for cholesterol gallstones before and after cholecystectomy.

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