The Scientific Journal of King Faisal University

Basic and Applied Sciences

A Novel Design of Continuous Culture for In Vitro Formation of Gallstones by *Salmonella Typhi*

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LINK	RECEIVED	ACCEPTED	PUBLISHED ONLINE	ASSIGNED TO AN ISSUE
https://doi.org/10.37575/b/sci/210055	26/09/2021	26/11/2021	26/11/2021	01/12/2021

NO. OF WORDS	NO. OF PAGES	YEAR	VOLUME	ISSUE
4431	5	2021	22	2

ABSTRACT

The current research focused on detecting the role of *Salmonella typhi* (*S. typhi*) in the formation of gallbladder stones in the laboratory following isolation and diagnosis of *S. typhi* from bile samples of patients suffering from gallstone. Locally and for the first time, a novel continuous culture was designed, and Brilliant Green Bile Broth (BGBB) was used by adding 60% cholesterol and 20% Calcium Carbonate ($CaCO_3$) to form the gallstone nucleation. The continuous culture was inoculated with 1% *S. typhi* then incubated in optimal conditions for 20 days. After 14 days of incubation, results revealed the formation of spherical aggregations with various sizes in the test flask compared to the control flask. Moreover, an increase in the size of the stone formed was observed after 20 days of incubation. The morphology of cholesterol and Calcium Carbonate crystals were studied using light, fluorescent and scanning electron microscopes, and the functional groups were diagnosed using the Fourier transform infrared spectrometry (FTIR) technique.

KEYWORDS

Biliary microbiota, model Bile, polysaccharide production

CITATION

Al-Sanjary, S.I.H. and Al-Rawi, A.M.M. (2021). A novel design of continuous culture for in vitro formation of gallstones by *Salmonella typhi*. *The Scientific Journal of King Faisal University: Basic and Applied Sciences*, **22**(2), 81–5. DOI: 10.37575/b/sci/210055

1. Introduction

Salmonella enterica subspecies *enterica* serovar *typhi* is described as part of the intestinal family; *S. typhi* is characterised by rod-shaped, flagellated, aerobic, Gram-negative bacilli. This bacterium invades the mucus surface of the intestine, causing an infection that spreads to infect the liver, pancreas, and bone marrow, and their ability to survive in bile and intestine with the presence of bile salts causes a chronic infection without any symptoms. Epidemic studies on *S. typhi* showed that most chronic infections are accompanied by gallstones (Di Ciaula *et al.*, 2019; Di Domenico *et al.*, 2017).

Gallstones are considered one of the primary digestive system diseases affecting humans (Kadhim, 2020; Al- Amedy *et al.*, 2020). The bile is a homogenous yellow-greenish liquid that is sterile under normal circumstances and consists of bile acids, salts, lecithin, and cholesterol (Rudling *et al.*, 2019). The supersaturation of the bile is regarded as the first step of gallstone formation compared to the bile acids, salts, and lecithin. The cholesterol determines the physical state of the bile and other factors that decrease the solubility of the cholesterol in the bile, and the presence of Mg^{++} and Ca^{++} are the critical factors in the formation of the nucleation of the cholesterol stone (Murphy *et al.*, 2020). Moreover, the increase of bile concentration, the slow discharge of the bile, the change in the bile contraction, the excessive secretion of the mucin or infection of bile ducts with some bacteria or diseases like sickle-cell disease increases the red blood corpuscles breaking and results in vast amounts of bilirubin (Grigor'eva and Romanova, 2020).

Bacteria play an essential role in the formation of the gallstone, and this motivated the researchers to study the role of bacteria and their relationship with gallstone formation (Di Ciaula *et al.*, 2018). Various types of bacteria were isolated from the patients suffering from gallstone using different microbiological and molecular techniques, including *E. coli*, *Salmonella spp*, *Enterococcus spp*, *Klebsiella spp*, *Enterobacter spp*, *Citrobacter spp*, *Staphylococcus spp*, *Pseudomonas spp*, and *Acinetobacter spp*. (Grigor'ova and Romanova, 2020; Hazrah *et al.*, 2004). Researchers pointed to the role of microbes that produce urease enzyme in terms of gallstone crystals precipitation, and they confirmed that urease production changes pH values, which in turn causes the precipitation of calcium crystals (Belzer *et al.*, 2006). Wang and his colleagues (2020) demonstrated intestinal microbes in gallstone formation, although the epidemic and the mechanism of the gallstone formation are not yet understood.

Until now, there has been no local study on the critical role of *S. typhi* in forming gallstones because all the previous – local or international – studies have focused on the bacterium's ability to precipitate the crystals. The current study aimed at investigating the role played by *S. typhi* in the stages of gallstone formation using a new continuous culture system designed for *in vitro* conditions with a suitable medium as a model for bile.

2. Materials and Methods

2.1. Isolation and Identification of *S. Typhi*:

Bile samples were obtained from patients subjected to gallbladder cholecystectomy at Alzahrawi Teaching Hospital and Alzahrawi Private Hospital in Mosul City. A sterilised syringe was used to take 2 –5 ml of bile and was immediately inoculated on MacConkey agar, SS agar and XLD agar. The plates were incubated at 37ºC for 24 hours. Several diagnostic biochemical tests were conducted, as mentioned in (Willey *et al.*, 2017). These tests included indole production, methyl red test, Voges–Proskauer, citrate utilisation test, urease enzymes production, cytochrome oxidase, catalase, and TSI test.

VITEK2 compact system was employed using the diagnostic kit of Enterobacteriaceae to confirm the diagnosis, and the work method followed according to the instructions of the manufacturing company, Bio Merieux, for diagnosis (Renaud *et al.*, 2005).

2.2. Slime Production Test:

The pure isolates were inoculated on Congo red agar and incubated at 37ºC for 24 hours. Positive isolates were investigated by the appearance of black colonies, while the negative isolates appeared

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in reddish pink (Freeman *et al.,* 1989).

2.3. *In Vitro* Formation of Cholesterol Gallstones:

2.3.1. Design of the continuous culture system

This system was designed to be used in preparing a continuous culture for *S.typhi* and using Brilliant Green Bile Broth (BGBB) as a model for the bile. The system consists of the continuous culture flask with its accessories as follows:

A glass flask with a side glass arm, an air pump to provide oxygen, a collecting tank with a volume of (250 cm^3) with a tap to control the addition of (25 cm^3 / hour) of the sterilised bile to get a dilution average of (0.05 / hour). Steel holders with clamp bottles were used to collect the excess solution from the culture flask, which was changed every 24 hours; a water bath with a thermometer was used to keep the temperature at 37ºC. All the accessories of the system were sterilised by autoclaving at (121 ºC) for 15 minutes.

2.3.2. Preparing the Culture

After confirming the diagnosis of *S.typhi* and testing its ability for slime production, a culture was prepared by inoculating the bacteria in a Brain Heart Infusion (BHI) medium and incubating at 37ºC for 18 hours.

2.3.3. A Model for Continuous Culture of Gallstone Nucleation *In Vitro*

A BGBB medium was prepared as a model of the bile; it contained 2% ox bile as a suitable nutritious media for *S.typhi*. Cholesterol was gradually added to the culture, and saturation was reached at a concentration of 60%. Calcium carbonate ($CaCo_3$) was added at a concentration of 20% with continuous stirring to form the nucleation of the stone. Then the pH was set to 7.2 using a pH-meter and sterilised with the autoclave at 121ºC for 15 minutes.

A 500 cm^3 sample of artificial bile was placed in the continuous culture flask and inoculated with 1% (5 cm^3) of *S.typhi* culture growing on BHI (18 hours old), which equals (5×10^5 CFU / cm^3). The flask was incubated under optimum temperature and pH. Excess liquid was discharged from the culture through a lateral discharge tube with the continuous addition of the artificial bile as drops (an average of 25 cm^3 / hour). A second sample was prepared that contained the same components but without *S.typhi* as a control sample.

After seven days of inoculating the continuous culture, a new *S.typhi* culture was added (18 hours old) and a size of 5 cm^3 growing on BHI to ensure the continuity of the metabolic activity with continuous incubation under optimum temperature and pH. Continuous culture persisted for 20 days from the beginning of inoculation.

2.4. Detection of the Crystals Forming the Stones with the Presence of *S.typhi*:

2.4.1. Examination under the Light Microscope

A light microscope was used to detect the stones' formation and observe *S.typhi* in the precipitate. From day one to day 10 of continuous culture, a precipitate drop was sampled daily and placed on a clean glass slide. Safranin stain was added, and a cover slide was fixed on top. It was examined using (100 X) magnification power (Sharma *et al*., 2020) and then photographed using the digital camera of the microscope (CH3 ORF 200, Olympus optical Co., Ltd., Japan).

2.4.2. Examination under the Fluorescent Microscope

A fluorescent microscope was used to detect *S.typhi* and its ability to produce polysaccharides and to detect crystals forming the stones by following the steps:

- Acridine orange dye was prepared by dissolving 0.1 gram of the dye in 100 ml of distilled water.
- Thin smears of the precipitate formed every day from the first day of continuous culture to 10 days were prepared and left to dry at room temperature. Smears were fixed using ether mixed with methanol in a ratio of (1:1) for ten minutes and then washed with distilled water.
- The slides were immersed in ethanol 50% and 70% for two minutes and stained with acridine orange for five minutes.
- The slides were washed with distilled water for one minute, then rinsed in phosphate buffer solution for 1-2 minutes, then dried, examined by the fluorescent microscope, and photographed (MacCarthy and Senne, 1980).

2.4.3. Examination under the Scanning Electron Microscope

The Scanning Electron Microscope (SEM) Inspect S50/FE/ Company- Netherland, at the College of Sciences, Kufa University was used. First, the specimen was prepared (the stone formed after 20 days of continuous culture), the stone was crushed and left to dry at room temperature for 15 minutes. Then carbon adhesive tape was fixed on the specimen holder surface (Aluminum stubs), and then the specimen was placed on the stubs. Then, the specimen was coated with gold using Sputter Coater (Quorum- 15 ORES- England) for 10 minutes, then the specimen on the stubs was moved to its place inside the microscope chamber, examined and photographed with the electron microscope camera that is connected to the computer of the microscope (Echlin, 2009).

2.4.4. Measurement with the Fourier Transform Infrared Spectroscope

The FTIR (Alpha-Bruker-Germany) at the College of Sciences, Kufa University, was used. The stones were crushed into fine powder; 2 mg of the powdered stone sample was used to make potassium bromide (KBr) discs. Then the specimen was analysed and measured using the Fourier transform infrared spectrometer (FTIR) at a frequency of 500-4000 cm^{-1} and a resolution of 4 cm^{-1} (Ha and Park, 2018).

3. Results

3.1. Isolation and Identification

Each isolate was initially identified using the Gram stain, and the culture characteristics of *S. typhi* on the selective media included MacConkey, SS, and XLD agar after they were incubated aerobically at 37ºC for 24 hours. The colonies of *S. typhi* appeared pale on MacConkey agar and paled with a black centre on SS agar. While they appeared pink with black centres on XLD agar. Moreover, the biochemical tests showed their ability to produce the catalase and positive methyl red test, and the VITEK2 compact system 99% match was given. Isolates of *S. typhi* showed a positive result in producing the polysaccharides as black colonies appeared on Congo red medium.

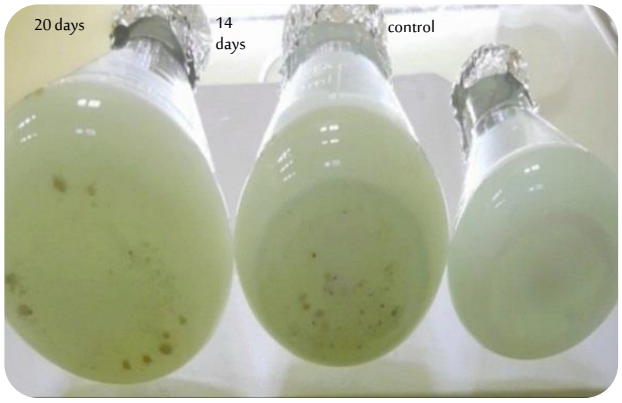
3.1.1. Results of *In Vitro* Nucleation of Gallstones Using the Continuous Culture System

Following the addition of cholesterol to BGBB, cholesterol crystals were dissolved at concentrations less than (6 mg / 10 cm^3). The gradual increase of cholesterol concentration resulted in the precipitation of crystals at the bottom of the continuous culture flask until it reached the concentration of 60%. In order to provide optimum conditions for gallstone formation, calcium carbonate was added to the flask. The gallstone was formed *in vitro*. Two models were prepared; the first represents the experiment flask, and the second is the control flask that contains the same components but without *S. typhi*.

The crystallisation process started seven days after inoculating the continuous culture with a new culture of the same bacteria, revealing the evident effect of the supersaturation of cholesterol

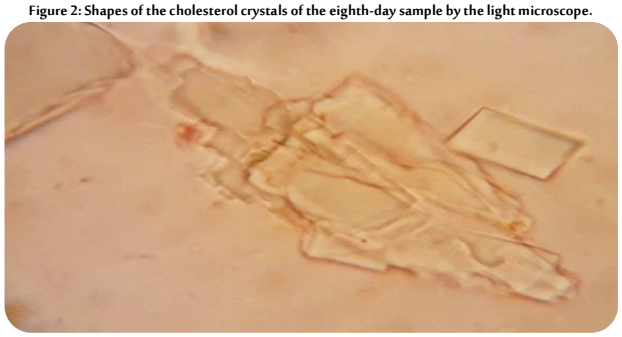
crystals. After 14 days of incubation at optimum conditions, tiny spherical aggregations with various sizes were observed in the flask compared to the control flask without *S. typhi* in which only a white precipitate was noticed. After 20 days of incubation, a continual increase was observed in the sizes of the stones in the experiment flask. Figure (1).

Figure 1: Formation of stones in the experiment flask compared to the control flask.



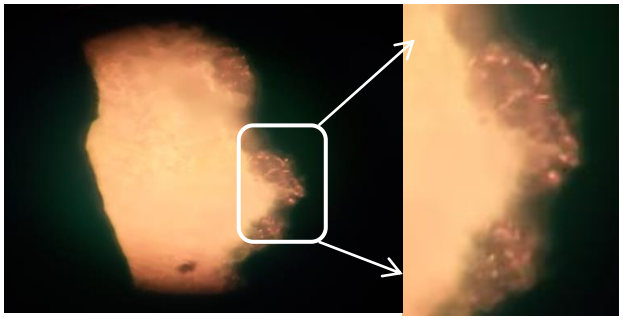
The precipitate was examined using the light microscope to investigate the shapes of the crystals from the fifth-day sample. Results showed the individual cholesterol crystals with their typical plate-like shape with *S .typhi* that were clear by microscope. The eighth-day sample showed that the cholesterol crystals plates are assembled, while the tenth-day sample showed the cholesterol crystals as compacted agglomerations, as shown in Figure 2.

Figure 2: Shapes of the cholesterol crystals of the eighth-day sample by the light microscope.



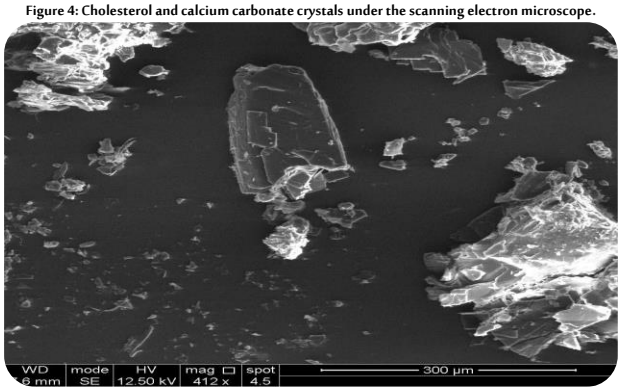
Using the fluorescent microscope appearance of *S. typhi* with the stone-forming crystals surrounded by a viscous material (polysaccharide) was evident in the seventh-day sample Figure 3.

Figure 3: *S. typhi* with crystals surrounded by polysaccharides using the fluorescent microscope.



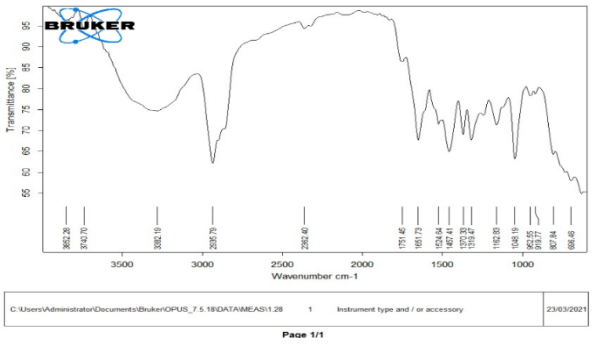
The scanning electron microscope images of the cholesterol stones formed *in vitro* showed that they appeared as regular plate-like or as lamellar-like crystals or as irregular aggregations with different sizes and three-dimensional crystals using various magnification powers. While calcium carbonate crystals appeared as cubic three-dimensional shapes and spherical, as shown in Figure 4.

Figure 4: Cholesterol and calcium carbonate crystals under the scanning electron microscope.



The results of FTIR various peaks for cholesterol and $CaCO_3$ revealed the absorption peak at 3382 cm^{-1} and 2935 cm^{-1} due to CH asymmetric stretching of CH_2 and CH_3, respectively. The peak at 1162 cm^{-1} and 1048 cm^{-1} is due to the functional groups C-C of the cholesterol. The absorption peak at 1457 cm^{-1} shows the presence of the C–O group in the calcium carbonate, and an absorption peak appeared at 1379 cm^{-1} due to CH bending of CH_3 of the cholesterol figure 5.

Figure 5: Infrared spectrum of cholesterol stones formed *in vitro*.



4. Discussion

The results of *S. typhi* identification were identical to the diagnostic tests of *S. typhi*, as mentioned by Willey *et al.*, 2017. The *S. typhi* showed the inability of producing the urease enzyme that hydrolyses urea into ammonia and carbon dioxide, the resulting ammonia raises the pH of the medium, making it alkaline, and therefore the colour changed from yellow to pink. In addition, the isolate showed the ability to produce polysaccharides. When the diagnosis of *S. typhi* was confirmed by using the VITEK2 compact technique, it showed a 99% matching, so this technique is considered quick and accurate in diagnosing bacteria.

In this study, the BGBB medium was used due to its suitability as an alternative medium, providing appropriate conditions for the growth of *S. typhi*, which are similar to the conditions of the bile in the human body. Additionally, the medium contains ox bile, which is chemically similar to the bile of the human. Therefore, the continuous culture provides similar conditions as in the human body; the chemical composition and the components necessary for the growth of *S.typhi*. The pH value and temperature were fixed, the surplus was removed from the continuous culture, and it was provided with the artificial model of the bile, which supports *S. typhi* to achieve stable growth for several generations.

Bile is one of the biological materials that prevent the growth of bacteria; despite this, bacteria were isolated from the bile and the gallstones. Previous studies confirm the role of the bacteria in precipitating the stone crystals. However, no previous study shows the S. typhi role in forming the gallstone and increasing its

solidification.

This study is regarded as the first to highlight the role of *S.typhi* through designing a continuous culture, which provides the optimal conditions for growth and preparing an artificial model of bile to manifest the ability of *S. typhi* to form the cholesterol stones *in vitro*.

S.typhi can colonise the gallbladder, causing chronic infection, primarily due to the existence of gallstones without symptoms and its ability to form the biofilm on the gallstone and the surfaces covered with cholesterol in the laboratory. In a study by Crawford et *al.* (2010), 5% of *S. typhi* was isolated from the gallbladder containing stones, diagnosed using Multiplex PCR. Van Den Berg and his colleagues (2000) pointed out the role of calcium ions and high cholesterol concentration in forming the gallstone when they incubated a human gallstone isolated in a culture saturated with cholesterol and containing a specific calcium concentration. After incubation, they noticed an increase in stone diameter due to the precipitation of the cholesterol crystals and calcium ions on the stones.

Sharma *et al.* (2013) indicated that calcium carbonate plays a vital role in forming the nucleation of crystals. When calcium carbonate is not present, no precipitation occurs, and crystallisation nucleation is not formed. Castro-Torres *et al.* (2015) demonstrated that the presence of the cholesterols crystals alone is not sufficient to form the nucleation of gallstone crystallisation. Several factors play an essential role in forming the nucleation of gallstone, such as calcium ions Ca^{+2} and magnesium ions Mg^{+2} when the cholesterol concentration is high.

In this study, the results of the light microscope elucidate the cholesterol crystal shapes as a single regular plate or as assembled plates with the presence of *S. typhi* attached to the crystal surfaces. With the continuous incubation in suitable conditions, the culture was in progress. By the seventh day, a new culture was added with an 18-hour culture to enhance the metabolic activity of the bacteria. It was observed that a viscous matter was formed that encompasses the crystals and *S. typhi,* and crystallised materials covered a viscous material.

By using the scanning electron microscope, the results follow what has been reported by Sharma *et al.* (2020), that the cholesterol crystals and calcium carbonate can be observed clearly with the bacteria attached to the surfaces of the crystals due to its virulence factors, which help them to attach to the precipitated hard surfaces.

After the tenth day of incubation, the crystallised materials began to change gradually into small spherical masses. On the fourteenth day, an increase in the size of crystallised material was observed, and this agrees with the conclusions of Costa *et al.* (2018) that the slime provides high viscosity and consequently helps in the cohesion and attachment of crystals. Wang *et al.* (2018) demonstrated that the slime is a polymer that forms cross-linkage that aid in precipitating the crystals. They emphasised that slime production is more important than β-glucuronidase, which plays a vital role in forming the gallstone. The polysaccharide acts as additional power that increases the cohesion and adhesion of the crystals; this is a distinguishing characteristic of *S.typhi*.

Sharma *et al.* (2020) concluded that the presence of polysaccharide producing bacteria has a greater effect on forming the crystal agglomerations in a shorter time than the models inoculated with urease or β – glucuronidase producers.

Moreover, FTIR results of stones showed that they consist of cholesterol and calcium carbonate, which agree with what has been reported by Sharma *et al.* (2020), who observed the emergence of absorption peaks at 3387 cm^{-1} and 2939 cm^{-1} that were due to CH asymmetric stretching of CH_2 and CH_3 respectively. In addition, the emergence of absorption peaks at 1162 cm^{-1} and 1052 cm^{-1} belong to the C–C group, which is considered one of the functional groups of cholesterol, and the absorption peak at 1458 cm^{-1} represents the functional group C–O of calcium carbonate. Results are also consistent with Ha and Park (2018); absorption peaks at 3395 cm^{-1} and 2930 cm^{-1} belonging to the functional groups of cholesterol during the analysis of the cholesterol stones isolated from humans.

Kleiner *et al.* (2002) confirmed the presence of calcium carbonate by the emergence of an absorption peak at 1463 cm^{-1}, which indicates the presence of the functional group C–O of calcium carbonate in addition to the presence of absorption peaks at 3398 cm^{-1}, 2933 cm^{-1} and 1056 cm^{-1} that belong to the various functional groups of cholesterol.

S. typhi can form cholesterol stones under continuous culture using an *in vitro* artificial model of the bile. The ability of *S. typhi* to produce slime as a polysaccharide provides a sufficient viscosity for the adhesion of the bacteria to the crystal surfaces, the crystals with each other, and the agglomeration and the quick growth of the crystals to become mature stone. Also, *S. typhi* possesses virulence factors that enhance the agglomeration, and also there are certain factors related to the bile components and its chemical structure, altogether leading to the formation of the gallstone.

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References

Al-Amedy, O.S., Saido, G.A. and Hussein, M.R. (2020). Assessment of the women's knowledge regarding cholelithiasis diseases in Duhok city. *Mosul Journal of Nursing*, **8**(1), 42–59. DOI: 10.33899/mjn.2020.164623.

Belzer, C., Kusters, J.G., Kuipers, E.J. and Vanvleit, A.H.M. (2006). Urease induced calcium precipitation by Helicobacter species may initiate gallstone formation. *Gut*, **55**(11), 1678–9. DOI: 10.1136/gut.2006.098319

Castro- Torres, I.G., Cardenas- Vazquez, R., Velazquez- Gonzalez, C., Ventura- Martinez, R., Delao-Arciniega, M., Naranjo- Rodriguez, E.B. and Martinez- Vazquez, M. (2015). Future therapeutic targets for the treatment and prevention of cholesterol gallstones. *Eur. J. pharmacol,* **765**(n/a), 366–74. DOI: 10.1016/j.ejphar.2015.08.045

Costa, O.Y.A., Raaijmakers, J.M. and kuramae, E.E. (2018). Microbial extracellular polymeric substances: Ecological function and impact on soil aggregation-review. *Frontiers in Microbiology*,

9(n/a), 1635. DOI: 10.3389/fmicb.2018.01636

Crawford, R.W., Rosales- Reyes, R., Ramirez- Aguilar, M.L.,Chapa- Azuela, O., Alpuche- Aranda, C. and Gunn, J.S. (2010). Gallstone play a significant role in *salmonella spp*. Gallbladder colonisation and carriage. *Proc Natl Acad Sci USA*, **107**(9), 4353–8. DOI: 10.1073/pnas.1000862107

Di Ciaula, A., Wang, D.Q. and Portincasa, P. (2018). Update on the pathogenesis of cholesterol gallstone disease. *Curr. Opin. Gastroenterol,* **34**(2), 71–80. DOI: 10.1097/MOG.0000000000000423

Di Ciaula, A., Garruti, G., Frühbeck, G., De Angelis, M., De Bari, O., Wang, D., Lammert. F. and Portincasa, P. (2019). The role of diet in the pathogenesis of cholesterol gallstones. *Curr Med Chem.*, **26**(19), 3620–38. DOI: 10.2174/0929867324666170530080636.

Di Domenico, E.G., Cavallo, I., Pontone, M., Toma, L. and Ensoli, F. (2017). Biofilm producing *Salmonella typhi*: Chronic colonisation and development of gallbladder cancer. *Int. J. Mol. Sci*, **18**(9), 1887. DOI: 10.3390/ijms18091887

Echlin, P. (2009). *Handbook of Sample Preparation for Scanning Electron Microscopy and X-Ray Microanalysis*. London: Springer Science Business Media.

Freeman, D.J., Falkiner, F.R. and Keane, C.T. (1989). New method for detecting slime production by coagulase negative Staphylococci. *J Clin Pathol.*, **42**(8), 872–4. DOI: 10.1136/jcp.42.8.872

Grigor'eva, I.N. and Romanova, T.I. (2020). Gallstone disease and microbiome. *Microorganisms,* **8**(6), 835. DOI: 10.3390/microorganisms8060835

Ha, BJ and Park, S. (2018). Classification of gallstones using Fourier-Transform Infrared spectroscopy and photography. *Bio Materials Research*, **22**(1), 2–8. DOI:10.1186/s40824-018-0128-8

Hazrah, P., Oahn, K. T.H., Tewari, M., Pandey, A.K., Kumar, K., Mohapatra, T. M. and Shukla, H.S. (2004). The frequency of live bacteria in gallstones. *HPB*, **6**(1), 28–32. DOI: 10.1080/13651820310025192

Kadhim, H.M. (2020). Review of pathogenicity and virulence determinants in *Salmonella*. *Eurasian Journal of Biosciences*, **14**(1), 377–81. DOI:10.36380/scil.2019.wvj21

Kleiner. O., Ramesh, J., Huleihel, M., Cohen, B., Kantarovich, K., Levi, C., Polyak, B., Marks, R.S., Mordechai, J., Cohen, Z. and Ordechai, S. (2002). A comparative study of gallstones from children and adults using FTIR spectroscopy and fluorescence microscopy. *BMC Gastroenterology*, **2**(1), 3. DOI:10.1186/1471-230X-2-3

MacCarthy, L.R. and Senne, J.E. (1980). Evaluation of Acridine orange for Detection of Microorganisms in Culture. *J. Clin. Microbiol*, **11**(3), 281–5. DOI: 10.1128/JCM.11.3.281-285.1980

Murphy, M.C., Gibney, B., Gillespie, C., Hynes, J. and Bolster, F. (2020). Gallstones top to toe: What the radiologist needs to know? *Insights into Imaging*, **11**(1), 13. DOI: 10.1186/s13244-019-0825-4.

Renaud, F.N., Bergeron, E., Tigaud, S., Fuhrmann, C., Gravagna, B., Freney, J. (2005). Evaluation of the new Vitek 2 GN card for the identification of gram-negative bacilli frequently encountered in clinical laboratories. *Eur. J. Clin. Microbiol. Infect. Dis,* **24**(10), 671–6.
DOI: 10.1007/s10096-005-0026-6.

Rudling, M., Laskar, A., and Straniero, S. (2019). Gallbladder bile super saturated with cholesterol in gallstone patient preferentially develops from shortage of bile acids. *J. Lipid. Res*, **60**(3), 498–505. DOI: 10.1194/jlr.S091199

Sharma, R., Sachan, S.G. and Sharma, S.R. (2020). *In vitro* analysis of gallstone formation in the presence of bacteria. *Indian journal of Gastroenterology*, **39**(5), 473–80. DOI: 10.1007/s12664-020-01055-6.

Sharma, R., Sachan, S.G. and Sharma. S.R. (2013). Urease and slime producing bacterial activity results in gallstone precipitation and solidification. *Arch. Clin Infect Dis*, **8**(4), 1–6. DOI: 10.5812/archcid.17090

Van Den Berg, A.A., Van Buul, J.D., Ostrow, J.D. and Groen, A.K. (2000). Measurement of cholesterol gallstone growth *in vitro*. *J. Lipid. Res.*, **41**(2), 189–94. DOI: 10.1016/S0022-2275(20)32051-4

Wang, Q., Hao, C., Yao, W., Zhu, D., Lu, H., Li, L., Ma, B., Sun, B., Xue, D. and Zhang, W. (2020). Intestinal flora imbalance affects bile acid metabolism and is associated with gallstone formation. *BMC Gastrenteriology,* **20**(59), 2–13. DOI: 10.1186/s12876-020-01195-1

Wang, Y., Qi, M., Qin, C. and Hong, J. (2018). Role of the biliary microbiome in gallstone disease. Expert Rev. *Gastroenterol Hepatol*, **12**(12), 1193–205. DOI: 10.1080/17474124.2018.1533812.

Willey, J.M., Sherwood, L.M. and Woolverton, C.J. (2017). *Prescott's Microbiology*. 10th edition. USA: McGraw-Hill Education, Inc.