DIPLOMA THESIS

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Influence of sodium deoxycholate in biofilm production by *Salmonella enterica* serovar. Typhi

Diploma thesis

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Vliv deoxycholátu sodného na produkci biofilmu u *Salmonella enterica* serovar. Typhi

Diplomová práce

Vedoucí práce: Prof. Carlos Gamazo
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CONTENT

Salmonellae are facultative anaerobic intracellular parasites that ferment glucose, reduce nitrate to nitrite, and synthesize peritrichous flagella when motile. Over 2000 serotypes of *Salmonella* infect humans and virtually all known wild and domestic animals, including birds, reptiles and insects. These infections result in significant morbidity and mortality. Human gastroenteritis, caused by nontyphoidal *Salmonellae*, is globally increasing because of zoonotic contamination of food. Typhoid fever caused by the exclusively human pathogens *S.* Typhi and *S.* Paratyphi was a major cause of death throughout the world in nineteenth and early twentieth centuries. Due to improvement in sanitation, the incidence of typhoid fever has dropped dramatically in developed nations; however, it remains a significant problem in the developing world. Antibiotic resistance of both typhoidal and nontyphoidal serotypes is increasing and has magnified the public health problem of *Salmonella* infections (Groisman E., 2001).

Salmonellae can get from the liver into the human gallbladder. Bacteria cause an active infection (cholecystitis) or a chronic infection (carrier state) can develop. The carrier state occurs in about 3 to 5% of the people infected and is frequently associated with gallbladder abnormalities, such as gallstones. *Salmonella* Typhi form biofilm on the surfaces of gallstone. A biofilm is defined as a population of one or more organisms attached to each other on a surface by means of bacterium-initiated matrix. This matrix provides a very stable environment and results in high levels of resistance to antimicrobial agents (such as antibiotic treatment or high concentration of bile). But on the other hand, efficient biofilm formation by *Salmonella* Typhi on gallstones is dependent upon the presence of bile, as the biofilm is not formed on gallstones alone. Therefore, bile appears to be an important environmental signal for *Salmonella* pathogenesis (Prouty A. M., 2002).

This thesis is a part of project of the Department of Microbiology, University of Navarra, Spain. This project tries to find out why bile is so essential for biofilm formation of *Salmonella spp.* on gallstones. And which particular substance of bile compounds is the most active one, and also, how this substance modulates the bacteria behaviour. In fact, the initial hypothesis is based on the possibility that the bacteria by itself may be considered as a nucleation factor during gallstone generation

1. INTRODUCTION

1.1 Salmonella and Salmonellosis

1.1.1 Organism

The genus *Salmonella* is a member of the family Enterobacteriaceae (kingdom Bacteria, phylum Proteobacteria, class Gamma Proteobacteria, order Enterobacteriales). The *Salmonellae* are Gram-negative, flagellated, nonsporulating and nonencapsulated bacilli, approximately 2 – 3 x 0.4 – 0.6 μm in size (Groisman E., 2001). Colonies of *Salmonella enterica* are on LB agar circular, convex, entire, grey colour.

The molecular characterization divides the genus into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into 6 subspecies (groups) that are designated by names or Roman numerals (I – *enterica*, II – *salamae*, IIIa – *arizonae*, IIIb – *diarizonae*, IV – *houtenae*, VI – *indica*). *Salmonella bongori* was originally designated *S. enterica* subspecies V. (US Department of Health and Human Services, CDC, 2004). Group I (*enterica*) includes many of serotypes pathogenic for humans, including *S.* Typhi and *S.* Typhimirium. As the correct taxonomic classification for *Salmonella* subspecies is not widely used, the common species name that prevailed before reclassification of the species is still being used. Thus *Salmonella enterica* ssp. *enterica* (or group I), serovar Typhi, is referred to by its common name, *S.* Typhi (Fig. 1) and this name will be used in this thesis.

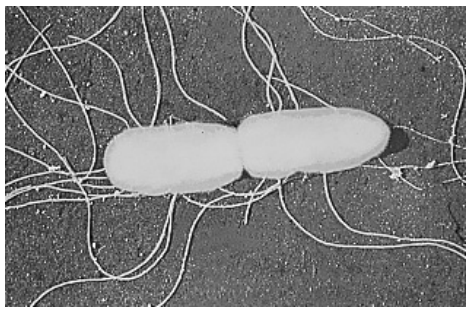


Figure 1: *Salmonella* Typhi (www.vacationsalacarte.co.uk)

Salmonella has been grouped into over 2200 serotypes according to three major antigenic determinants: the flagellar H antigen, the somatic O antigen, and the Vi antigen. The Vi antigen, a homopolymer of *N*-acetylgalactosaminouronic acid, is predominantly found on *S.* Typhi, and is considered an identifying feature of this serotype. Identification of nontyphoidal serogroups is based on differential agglutination by specific antibodies to O antigen. O antigen is a polysaccharide structural component of bacterial lipopolysaccharide, the major component of the outer leaflet of the bacterial membrane. Agglutination by antibodies specific for the various O antigen is used to group *Salmonellae* into 6 serogroups: A, B, C1, C2, D and E. Although these groupings can help identify pathogenic bacteria as *Salmonella*, cross reactivity between groups does not allow definitive identification of serotype. For instance, both *S.* Enteritidis and *S.* Typhi express O antigens of group D. Further classification of serotypes is based on the antigenicity of the flagellar H antigen and other more specific genetic and molecular methods (Groisman E., 2001).

1.1.2 Salmonellosis

The two major clinical syndromes that result from *Salmonella* infection are typhoid (or enteric) fever and gastroenteritis. Focal infections of the vasculature (endocarditis), bone (osteomyelitis), and joints (arthritis) as well as variety of other organs can occur but are much less common and are often associated with specific immune defects.

Before nineteenth century, human typhoid fever was often confused with typhus, a rickettsial disease. The typhoid bacillus was first isolated in 1884, when the German microbiologist Gaffkey obtained *S.* Typhi from human spleens. In 1885, the veterinary pathologist Daniel Salmon isolated *S.* Choleraesuis from the intestines of pigs infected with hog cholera and according to him are *Salmonella*e named. First typhoid vaccine was introduced in 1896 and the modern era of antibiotic treatment was initiated in 1948 with chloramphenicol (Groisman E., 2001).

1.1.2.1 Gastroenteritis

Human gastroenteritis is caused by many serotypes of *Salmonella*, the most common are *S.* Enteritidis and *S.* Typhimirium (Groisman E., 2001). There is a large animal reservoir of infection which is transmitted to man via contaminated food (especially poultry and dairy products). Waterborne infection is less frequent. *Salmonella* infection is also transmitted from person to person and thus secondary spread may occur. Most common manifestation of this infection is diarrhoea. Diarrhoea is produced as a result of invasion by the *Salmonella*e of epithelial cells in the terminal portion of the small intestine (Fig. 2).

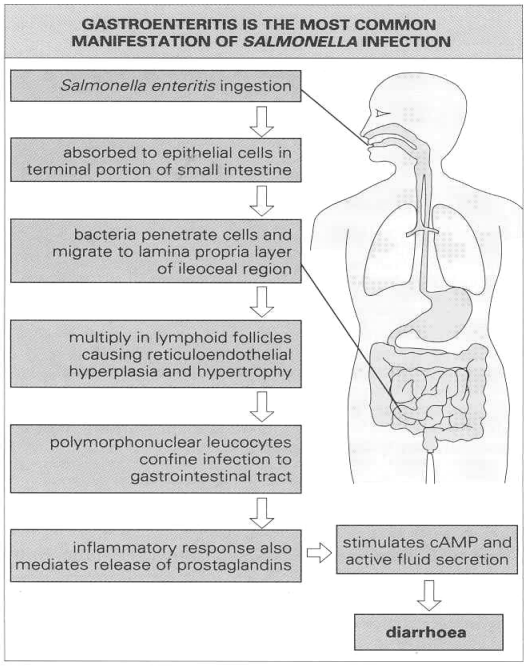


Figure 2: The passage of *Salmonellae* trough the body to the gut (Groisman E., 2001).

The bacteria migrate to the lamia propria layer of the ileocaecal region, where their multiplication stimulates an inflammatory response which both confines the infection to the gastrointestinal tract and mediates the release of prostaglandins. These in turn activate cyclic AMP and fluid secretion, resulting in diarrhoea. *Salmonella* do not appear to produce enterotoxins. The diarrhoea, that *Salmonella* spp. cause is in the vast majority of cases acute but self-limiting. But in the young and the eldery symptoms may be more severe. Fever is usually a sign of invasive disease. Other symptoms are nausea, abdominal pain and rarely either vomiting (Mims C. A., 1993).

Incidence of nontyphoidal salmonellosis for year 2002 was in The Czech Republic 272.3 per 100.000 inhabitants, in Spain 19.8 per 100.000 inhabitants. (www.czso.cz).

Disease onset approximately 8-48 hours after ingestion, and usually resolve within 5 to 7 days without treatment, although some infections may result in bacteraemia or other complications and require treatment. Except of rare cases, antibiotic treatment is not advised, as it has been observed to prolong the presence of bacteria in the stool. Fluid and electrolyte replacement may be required. *Salmonella* gastroenteritis is life threatening in a small percentage (1%) of total cases, particularly infants, the eldery, and imunocompromised individuals.

Nontyphoidal serotypes on average persist in the gastrointestinal tract, depending on serotype, from 6 weeks to 3 months. However, persistence beyond 6 months is rare. Only about 0.1% of nontyphoidal *Salmonella* cases are shed in stool samples for period exceeding 1 year, the clinical definition of chronic carriage (Groisman E., 2001).

1.1.2.2 Typhoid fever (enteric fever, Eberth disease)

Typhoid fever is a serve multisystemic illness. *S.* Typhi and *S.* Paratyphi are the causative agents of the human enteric fever, although *S.* Paratyphi generally produces a milder form of the disease. Both serovars are solely human pathogens, and do not exist in animal reservoirs. Disease is characterized by high fever, gastrointestinal symptoms, including diarrhoea and constipation, sometimes a characteristic rash (rose spots) from which bacteria can be cultured, headache, malaise, chills, and myalgia. Confusion, delirium, intestinal perforation, and death may occur in several cases (Groisman E., 2001).

Since ancient times, these bacteria have thrived during wartime and during the breakdown of basic sanitation. Archaeologists have found *S.* Typhi in Athenian mass graves from the era of the Peloponnesian Wars, implicating it as the cause of the Great Plague of Athens. *S.* Typhi and *S.* Paratyphi infections occur worldwide but primarily in developing nations where sanitary conditions are poor. Typhoid and paratyphoid fevers are endemic (Fig. 3) in Asia, Africa, Latin America, the Caribbean, and Oceania (Evans A. S., 1982). Typhoid fever affects 13-17 million people yearly and kills an estimated 600 thousand people. Although sporadic outbreaks occur in developed nations, most

individuals with typhoid fever in such areas have recently returned from travel to an endemic region.

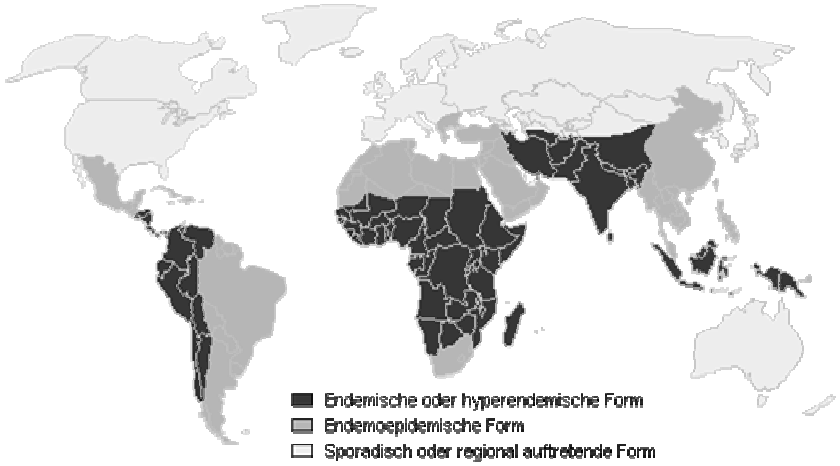


Figure 3: Geographic occurrence of typhoid fever. (www.med1.de)

Way of infection: People are typically infected with *S.* Typhi and *S.* Paratyphi through food and beverages contaminated by a chronic stool carrier. Less commonly, carriers may shed the bacteria in urine. Individuals may also be infected by drinking sewage-contaminated water or by eating contaminated shellfish or faulty canned meat. Large epidemics are most often related to faecal contamination of water supplies or street vended foods.

In studies of healthy, previously unvaccinated men, ingestion of 10^7 *S.* Typhi bacilli caused disease in 50% of volunteers (the minimum infectious dose ID_{50}). Investigations of outbreaks seem to indicate that an inoculum of as few as 200 organisms may lead to the disease. Perhaps such a discrepancy exists because many who ingest *S.* Typhi are not healthy and may have any number of risk factors (e.g. children aged 1-5 years and eldery are at the highest risk of infection) (www.emedicine.com). Conditions that increase susceptibility to lower inoculum include decreased stomach acidity, chronic gastrointestinal disease such as inflammatory bowel disease, gastrointestinal surgery, and alternation of the intestinal flora by antibiotics administration. Immunocompetency also affects the $ID_{50,}$ as those within immune disorders, such as AIDS. As the number of ingested bacilli increases, the incubation period decreases.

Physiopathology: After ingestion by the host, successfully passing through the stomach *S.* Typhi invades through the gut and multiplies within the mononuclear phagocytic cells in the liver, spleen, lymph nodes, and Peyer´s patches of the ileum. Peyer´s patches are grossly visible aggregates of 5-100 lymphoid follicles in the small bowel submucosa; these patches are larger and more numerous distally. They are the primary mechanism for sampling antigens in the gut and initiating response. Passage of *Salmonella* trough the intestinal epithelial barrier most likely occurs through specialized microfold (M) enterocytes, which overlay Peyer´s patches (Fig. 4). The primary function of M cells is to sample intestinal antigens. The bacteria penetrate the Peyer´s patches probably in the jejunum or the distal ileum (Groisman E., 2001).

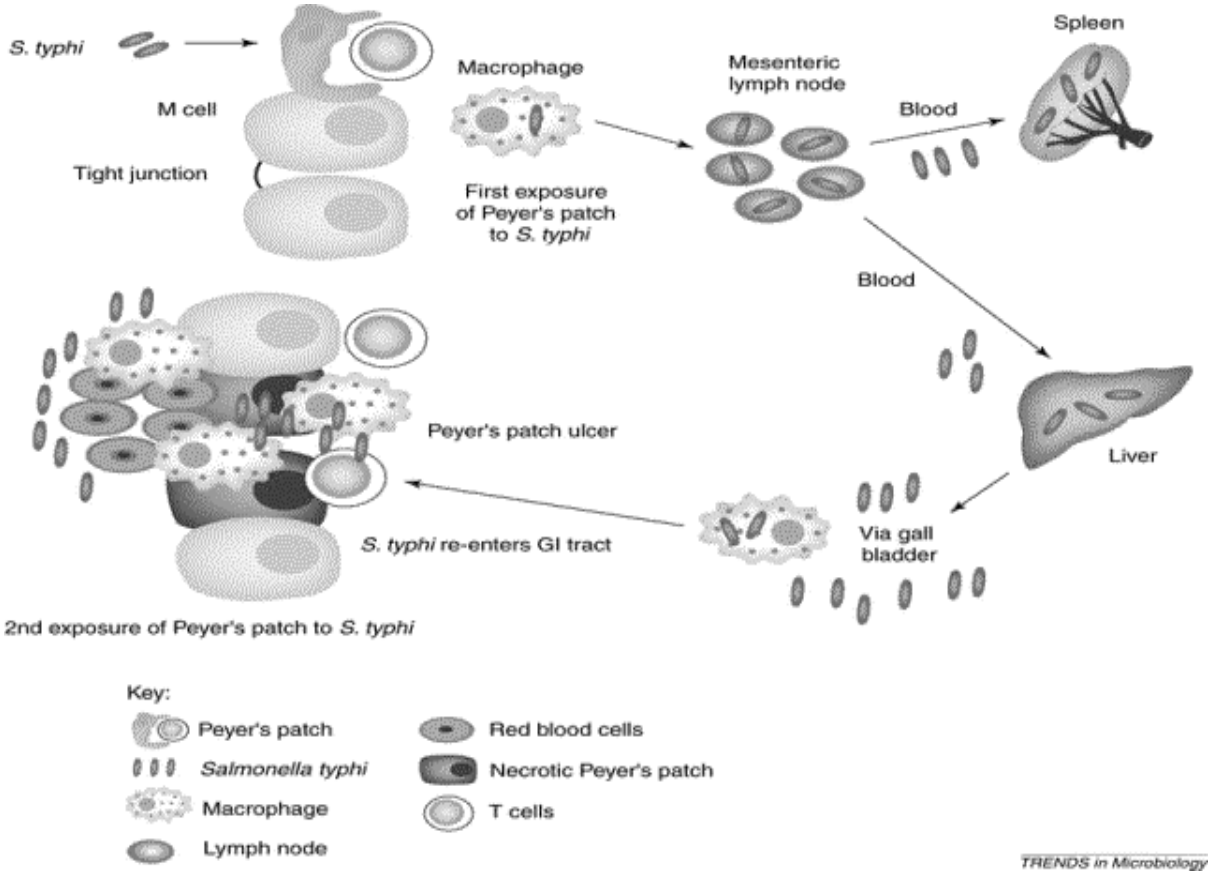


Figure 4: *Salmonella* Typhi infecting the body via the Peyer's patches of the small intestine. The bacteria migrate to mesenteric lymph nodes and arrive via the blood in the liver and spleen during the first exposure. After multiple replications in the above locations, the bacteria migrates back into the Peyer's patches of the small intestine for the secondary exposure and consequently the clinical symptoms are seen. Inflammation in the small intestine leads to ulcers and necrosis. (www.bio.davidson.edu)

Once trough the mucosal barrier, the bacteria reach the intestinal lymph nodes, where they survive and multiply within macrophages. They are transported in these cells to the mesenteric lymph nodes, then to the thoracic duct and eventually discharged to the blood stream. Circulating in the blood, the organisms can seed many organs, most importantly in areas where cells of the reticuloendothelial system are concentrated (i.e. the spleen, bone marrow, liver) and contribute to the multisystem disease characteristic of typhoid fever. In the liver they multiply in Kupffer cells. From the reticuloendothelial system there is reinvasion of the blood, to reach other organs (e.g. kidney). The gallbladder is infected either from the blood or from the liver via the biliary tract, the bacteria being particularly resistant to bile. As a result *S.* Typhi enter the intestine for the second time, in much larger numbers than in the primary encounter, and in Peyer´s patches cause a strong inflammatory response leading to ulceration, with the danger of intestinal perforation (Mims C. A., 1993).

Clinical features and complications: Untreated typhoid fever lasts at least 4 weeks. Most of the classic signs and symptoms of typhoid fever are prevented with prompt treatment. Clinical response begins about 2 days after starting antibiotics, and the patient's condition markedly improves within 4-5 days. The incubation period of typhoid fever varies with the size of the infecting dose and averages 7-14 (range 3-60) days. In paratyphoid infection, the incubation period ranges from 1-10 days. During the incubation period, 10-20% of patients have transient diarrhoea (enterocolitis) that usually resolves before the onset of the full-fledged disease. As bacteraemia develops, the incubation period ends. Patients often experience chills, diaphoresis, anorexia, dry cough, a dull frontal headache, and myalgias before the onset of a high fever. About 20-40% of patients present with abdominal pain. In immunocompetent adults, constipation is common and is most likely due to hypertrophy of Peyer´s patches. Young children and individuals with AIDS are more likely to have diarrhoea that is probably due to blunted secondary immunity. The incidence of constipation versus diarrhoea varies geographically, perhaps because of local differences in diet or *S.* Typhi strains or genetic variation.

Unusual modes of onset include isolated severe headaches that may result in mimic meningitis. *S.* Typhi infection may cause an acute lobar pneumonia. In the early stages of the disease, rigors are rare unless the person also has malaria. This is not an unusual pairing of diseases. Patients may present with arthritis only, urinary symptoms, severe jaundice, or fever. Some patients, especially in India and Africa, may present

with confusion and delirium or report parkinsonian symptoms or spastic rigidity. This regional variety in neuropsychiatric presentation may be due to the same factors that cause the variation in gastrointestinal symptoms.

Fever occurs in 75-85% of patients in the first week and is often initially remittent but becomes steady. The individual's temperature often rises to as high as 39-40°C by the beginning of week 2. At approximately the end of the first week of illness, about a third of patients develop bacterial emboli to the skin known as rose spots. These are considered a classic symptom in typhoid fever, but they occasionally appear in shigellosis and nontyphoidal salmonellosis. Rose spots constitute a subtle, extremely sparse (often $\leq$5 spots), salmon-coloured, blanching, truncal, maculopapular rash with 1- to 4-cm lesions that generally resolve within 2-5 days. During the second week of illness, the patient is toxic-appearing and apathetic with sustained fever. The abdomen is slightly distended, and soft splenomegaly is common. In the third week, the patient grows more toxic and anorexic with significant weight loss. The patient may have a thread pulse, tachypnea, conjunctivitis, and crackles over the lung bases. Pyrexia persists. The patient may enter into a typhoid state of apathy, confusion, and even psychosis. Patients may develop polyneuropathy. Abnormal cerebrospinal fluid should prompt a search for a different cause. During the fourth week, the fever, mental state, and abdominal distension slowly improve over a few days, but intestinal complications may still occur in surviving untreated individuals. Weight loss and debilitating weakness last months (www.emedicine.com).

Relapses occur in 10% of patients, mostly during the first 2-3 weeks of convalescence. Chronic carrier state occurs in about 3 to 5% of the infected people. This state is defined as individuals who excrete *Salmonella* for more than 1 year. Some individuals may continue to excrete the bacterium for decades. Stool carriage is more frequent in people with pre-existing biliary abnormalities. Chronic carriers have a greater risk for carcinoma of the gallbladder and other gastrointestinal malignancies; chronic carriers have a 6-fold increase in the risk of death due to hepatobiliary cancer. This may be due to chronic inflammation caused by the bacterium. (www.emedicine.com).

Treatment: In case of typhoid fever is necessary to find a medical care, because the disease can be fatal. Antibiotics, such as ampicillin, trimethoprim-sulfamethoxazole, and ciprofloxacin, have been commonly used to treat typhoid fever in developed countries. Usage of ofloxacin along with *Lactobacillus acidophilus* is also

recommended. Prompt treatment of the disease with antibiotics reduces the case-fatality rate to approximately 1%. When untreated, typhoid fever persists for three weeks to a month. Death occurs in between 10% and 30% of untreated cases. Vaccines for typhoid fever are available and are advised for persons travelling in regions where the disease is common. There are two types of vaccination: Typhim Vi is an intramuscular killed-bacteria vaccination and Vivotif is an oral live bacteria vaccination (www.wikipedia.org). Total time needed to set aside for vaccination is 2 weeks. In the case of oral vaccine is necessary to take 4 capsules, 2 days is the time between doses and minimum age for vaccination is 6 years. In the case of injection, 1 dose is essential and minimum age for vaccination is 2 years.

Prevention: Defence of spread infection depends on good personal hygiene, adequate sewage disposal and clean water supply. For tourists is as a first recommended to get vaccinated before travelling. Drink only bottled water, or boil water at least one minute before drinking. In restaurants is recommended to ask for drinks without ice and not to buy food from street vendors. Avoid raw vegetables and fruits that cannot be peeled. In USA is used "Boil it, cook it, peel it, or forget it" as a rule for tourists (www.cdc.gov).

1.2 Biofilm

Biofilm is a type of microbial aggregate. It is a community at a phase boundary generally, but not always, at liquid: solid interface. Spatially and temporally heterogeneous. In may have specific mechanisms for attachment to surface. It generates EPS (extracellular polymeric substance) for adhesion, protection and to facilitate community interactions (Allison D. G., 2000).

Biofilms are often characterized by surface attachment, structural heterogenity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substance. Single-celled organisms generally exhibit distinct modes of behavior. The planctonic form is the familiar free floating form in which single cells float or swim independently in some liquid medium. The biofilm is an attached state in which cells are closely packed and firmly attached to each other and surface. The change in behavior is triggered by many factors, including quorum sensing (number of

bacteria needed to form biofilm), as well as other mechanisms that vary between species (www.wikipedia.org).

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion molecules such as pili. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together (Fig. 5). Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment.

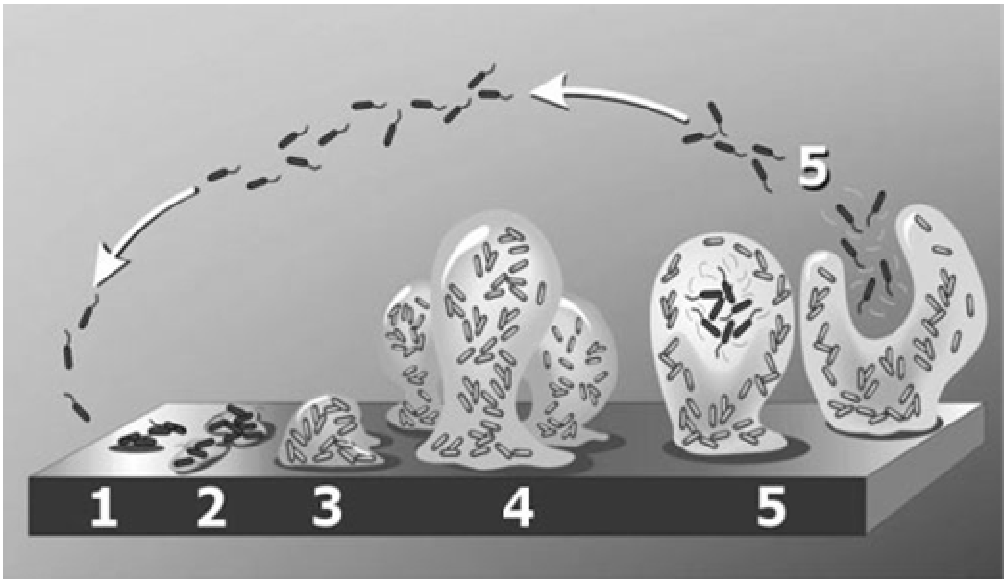


Figure 5: Formation of biofilm (www.biology.binghamton.edu)

Given sufficient resources for growth, a biofilm will quickly grow to be macroscopic. The biofilm is held together and protected by a matrix of exopolysaccharides. This matrix protects the cells within it and facilitates communication among them through biochemical signals. Some biofilms have been found to contain water channels that help distribute nutrients and signaling molecules. Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, as the dense and protected environment of the film

allows them to cooperate and interact in various ways. One benefit of this environment is increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. In some cases antibiotic resistance can be increased 1000 fold (www.wikipedia.org).

1.3 Gallbladder, cholelithiasis

The gallbladder (cholecyst, gall bladder) is a pear-shaped organ, about 7-10 cm long on the underside of the liver on the right side of the abdomen; it is connected to the liver and the duodenum by the biliary tract (Fig. 6). The function of the gallbladder is to store bile that is produced in the liver before the bile (about 50 ml). Bile is secreted into the intestines to help the body digest fats. It is released when food containing fat enters the digestive tract, stimulating the secretion of cholecystokinin (www.wikipedia.org).

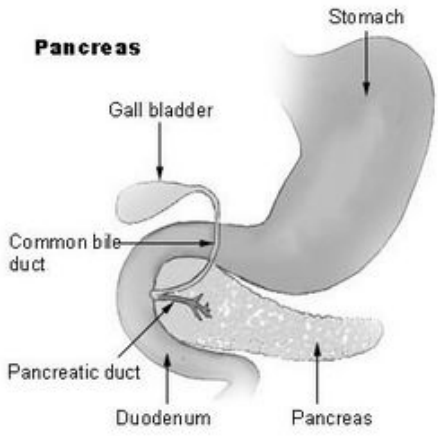


Figure 6: Position of gallbladder in the human abdomen. (www.wikipedia.org)

When the gallbladder is not functioning properly, the components of the bile become out of balance leading to the formation of solid crystals called gallstones (disease cholelithiasis). The majority of stones (80%) are composed of cholesterol; the remainders are pigmented stones consisting of bilirubin (Tab. 1.) (www.markfuscomd.com).

Table 1: General view of gallstones

characteristic	**cholesterol**	**black pigment**	**brown pigment**
colour	white-yellow with pigmented centre	black or brown pigmented	brown-yellow or orange
surface	solid, glazed	grey, glazed	soft, greasy
form	round, with facets	hirsute, with facets	ovoid, irregular
quantity	one, various	various, numerous	one, various
location	generally in gallbladder	generally in gallbladder	generally in bile duct
composition	45-98% cholesterol	10-50% black pigment polymer	calcium salt of bilirubin
	2-20% calcium salts of bilirubin	calcium and phosphate salts of bilirubin	10-60% calcium palmitate
	with coat or pigment of $CaCO_3$	$CaCO_3$	20-45% cholesterol
etiology	increase of cholesterol, lower secretion of bile salts in the bile	higher secretion of bilirubin, calcium in the bile, increase of bile pH	bacteriological infection which cause hydrolysis of bilirubin, lecithin conjugations

Although solubility of cholesterol in aqueous solutions is extremely limited, in gall-bladder bile a relatively large amount (20 mM) of the sterol can be kept in solution. This significant increase in solubility is explained by incorporation of cholesterol in mixed micelles, together with bile salts and phospholipids (mainly phosphatidylcholine). Supersaturation occurs when either too much cholesterol or not enough solubilising bile salt and phosphatidylcholine molecules are secreted to allow complete micellar solubilisation of all cholesterol. Excessive cholesterol may be kept in vesicles (i.e. spherical bilayers of cholesterol and phospholipids, without bile salts), provided that enough phospholipids is available. When relatively low amounts of phospholipids are present, cholesterol crystal formation occurs in supersaturated bile, which is the beginning of gallstone formation. Cholesterol crystallization is promoted by hydrophobic bile salts (chenodeoxycholate, deoxycholate) and by phospholipids with unsaturated acyl chains. Biliary bile salt, rather than phospholipids, composition may affect human gallstone formation with potential therapeutic implications (van Erpecum K. J., 2005).

To find out possibilities of gallstone formation due to the presence of bacteria, many researches were done. And DNA of various bacteria was already found in

gallstones (Manabu, K. et al., 2002; Frashad, Sh. et al., 2004; Dong, K. L. et al., 1999) This supports the hypothesis of this thesis .

Risk factors for gallbladder disease are: age over 40, obesity, rapid weigh loss, more common in women, estrogen intake, diabetics (www.markfuscomd.com).

In most situations gallbladder problems require surgical treatment. Since gallstones are often just a sign of the problem, treatment is directed towards the gallbladder rather than the gallstones. The treatment involves removing the gallbladder. This is done in most cases using laparoscopic surgery. Non-surgical treatments such as dissolution therapy and lithotripsy have a poor success rate and high recurrence rates so are used only in very limited circumstances (www.markfuscomd.com).

1.4 Bile, bile salts

1.4.1 Bile

Bile is a complex fluid containing water, electrolytes and a battery of organic molecules including bile acids, cholesterol, phospholipids and bilirubin that flows through the biliary tract into the small intestine. Initially, hepatocytes secrete bile into the canaliculi, from which it flows into the bile ducts. This hepatic bile contains large quantities of bile salts, cholesterol and other organic molecules. As bile flows through the bile ducts it is modified by addition of a watery, bicarbonate-rich secretion from ductal epithelial cells. Bile is critical for digestion and absorption of fats and fat-soluble vitamins in the small intestine. Many waste products, including bilirubin, are eliminated from the body by secretion into bile and elimination in faeces. Adult humans produce 400 ml to 800 ml of bile daily. Large amounts of bile acids are secreted into the intestine every day, but only relatively small quantities are lost from the body. This is because approximately 95% of the bile acids delivered to the duodenum are absorbed back into blood within the ileum. The net effect of enterohepatic recirculation is that each bile salt molecule is reused about 20 times, often two or three times during a single digestive phase (www.vivo.colostate.edu).

1.4.2 Bile salts (bile acids)

Bile salts are derivatives of cholesterol synthesized in the hepatocytes, liver. Cholesterol, ingested as part of the diet or derived from hepatic synthesis is converted into the bile acids cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (glycine or taurine) to yield the conjugated form that is actively secreted into cannaliculi (see the synthesis on Fig. 7). Bile acids are facial amphipathic, that is, they contain both hydrophobic (lipid soluble) and polar (hydrophilic) faces. The cholesterol-derived portion of a bile acid has one face that is hydrophobic (methyl groups) and one that is hydrophilic (hydroxyl groups); the amino acid conjugate is polar and hydrophilic.

Their amphipathic nature enables bile acids to carry out two important functions:
Emulsification of lipid aggregates: Bile acids have detergent action on particles of dietary fat which causes fat globules to break down or be emulsified into minute, microscopic droplets. Emulsification is not digestion per se, but is of importance because it greatly increases the surface area of fat, making it available for digestion by lipases, which cannot access the inside of lipid droplets (www.vivo.colostate.edu).
Solubilisation and transport of lipids in an aqueous environment: Bile acids are steroidal detergents, which together with lipids/fats/cholesterol form mixed micelles in the intestine to enable fat digestion and absorption trough the intestinal wall (Mukhopadhyay, S. et al., 2004). Bile acids are also critical for transport and absorption of the fat-soluble vitamins, such as vitamin E (www.vivo.colostate.edu).

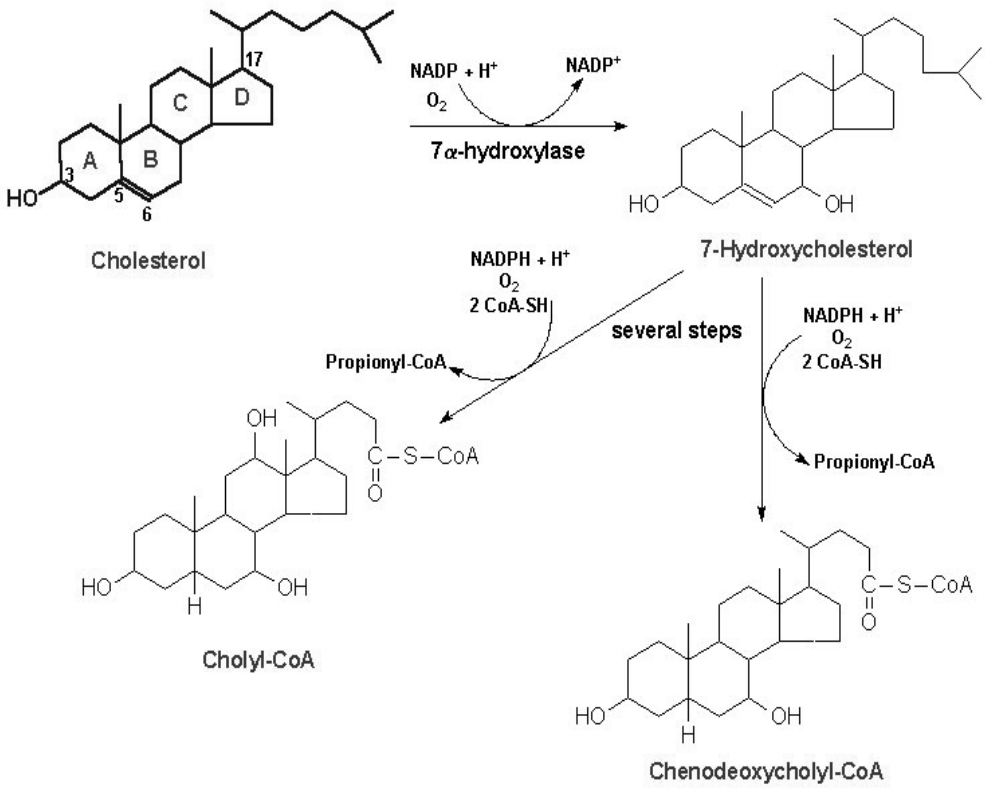


Figure 7: Bile acids synthesis and utilization (www.dentistry.leeds.ac.uk)

The most abundant bile salts in human are cholate, chenodeoxycholate and deoxycholate, and they are normally conjugated with either glycine (75%) or taurine (25%). Conjugation increases the aqueous solubility of bile salts under physiological conditions. These conjugates are membrane-impermeable, water-soluble, amphiphilic molecules having a powerful ability to transform lamellar arrays of lipids into mixed micelles (Mukhopadhyay, S. et al., 2004).

Also there are studies about a series of cholanic-acid derivates, and showed that the cationic bile salts are potent in accelerating bacterial swelling (Mukhopadhyay, S. et al., 2004).

1.4.2.1 Micelle and gel formation in aqueous solutions.

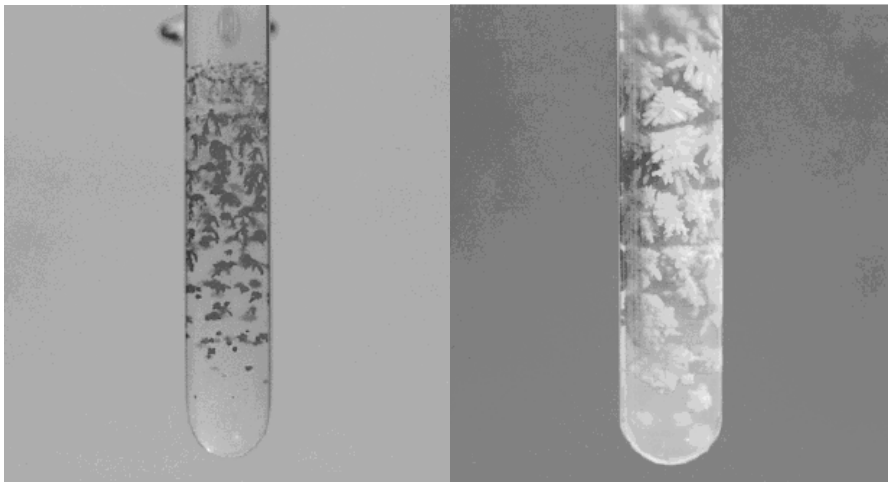
In an aqueous environment, bile salts aggregate to form micelles. These micelles under physiological conditions are transformed into mixed-micelles with lecithin and glycerides, which are responsible for fat/cholesterol solubilisation in the small intestine. This process is called emulsification and water-solubility increase rapidly. For example the aqueous solubility of cholesterol (1 nM) can increase more than million fold in the

presence of bile-salts micelles. The cholesterol solubilisation ability is far better with dihydroxy bile-salts than with trihydroxy bile-salts.

Some of the bile acids and their analogues act as potent gelators (gel-forming agents) in both organic and aqueous media. This unusual behaviour is known for a long time. Sodium cholate, sodium deoxycholate and sodium lithocholate were shown to form gels in water. The gel is pH-dependent and gels are thixotropic (the gel becomes more fluid (less rigid) as an unstable net and transparent (Mukhopadhyay, S. et al., 2004).

The detergent action of bile salts is mainly due to their micelle-forming capacity and these are known as „bio-surfactants“. Fundamental parameter in the evaluation of their biological activity is critical micelle concentration (CMC). This concentration is largely determined by its hydrophilic: hydrophobic balance. But also increasing temperature leads to the formation of fluffier micelles with less rigid interior (Subuddhi, U. et al., 2007).

Bile salts have been found in pigment gallstones in concentration ten times greater that found in cholesterol stones, the presence of metal ions as Cu, Co, Ni, Zn and Mn have been repeatly identified. Deoxycholic acids the one of physiologic bile acids, that could form periodic and/or fractal precipitates with metal ions (Fig. 8). This phenomenon was considered to be related to ring formation in gallstones (Peng Q. et al., 1995).



(a) **(b)**

Figure 8: (a) - Fractal pattern formed in $CuCl_2$–NaDC agar gel system. $CuCl_2$ = 10 mM*;* NaDC = 5 mM; (b) - Fractal pattern of $CoCl_2$–NaDC gel system with periodic precipitation. $CoCl_2$ = 0.5M*;* NaDC = 5 mM. (Peng Q. et al, 1995)

1.4.2.2 Deoxycholic acid

Chemical name is 3α,5β,12α)-3,12-Dihydroxycholan-24-oic acid. Molecular mass is 392.58 g/mol and melting point 174 - 176 °C (Fig. 9). When pure, it comes in a white to off-white crystalline powder form. Deoxycholic acid is one of the 4 main acids produced by the liver. It is soluble in alcohol and acetic acid. In bile occurs as a sodium deoxycholate which is a sodium salt of deoxycholic acid. Formula is $C_{24}H_{39}NaO_4$ and molecular weight: 414.55. It is soluble in water (www.wikipedia.org)

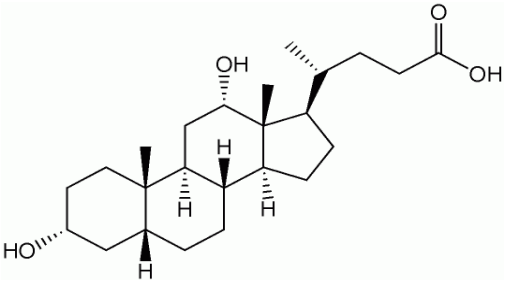


Figure 9: Deoxycholic acid (www.wikipedia.org)

1.5 Brief information about work with virulent microorganisms.

To work with bacteria in the laboratory of microbiology require high level of security and responsibility. Microbiological laboratory has strict rules, which are necessary to be followed. Exact register of these rules must be attendant at workplace. Variety among the rules depends on the types of microorganisms with which is working. Generally there are 4 classes of microorganisms (or biological agents) according to their dangerousness.

1. class includes biological agents with very low probability to cause a human disease.
2. class includes biological agents that can cause a human disease and are dangerous for workers, but there is a low possibility of expanding disease collectively. General prophylaxis or treatment exist and is efficacious (e.g. *Salmonella* Enteritidis, *Pseudomonas aerugionosa*, *and Listeria monocytogenes*).
3. class includes biological agents that can cause serious human disease, are dangerous for workers of laboratory; there is a high risk of expanding disease collectively. General prophylaxis or treatment exist and is efficacious (*Salmonella* Typhi, *Rubella mellitus's*).

4. class includes biological agents that can cause serious human disease, are very dangerous for workers of laboratory; there is a very high risk of expanding disease collectively. General prophylaxis or treatment does not exist (e.g. *Virus Ebola*, *Areola virus*).

Class 3 is the highest class of microorganisms with which is worked in a Department of Microbiology, University of Navarra. To work with these bacteria means (inclusive of basic security rules valid for class 1 and 2) also to work always in box of streamline flow in a zone P3. Zone P3 is a room equipped with HEPA filters, UV lamps and uderpressure protection of microorganisms outflow while door's open
(Gamazo C., 2006).

2. PLANS AND OBJECTIVES

Salmonella Enteritidis is able to produce biofilms under *in vitro* conditions. In order to have some more light on the pathogenesis of *S.* Typhi, we have investigated the *in vitro* conditions required for this serovar to produce biofilms.

- To confirm and define conditions for biofilm production of S. Enteritidis and *S.* Typhi.

During the course of this research we found a significant and original result: *S.* Typhi is able to gelificate a medium containing deoxycholic salts. As a consequence, new objectives were proposed:

- To characterize gelification of medium caused by presence of sodium deoxycholate, to prove the influence of *S.* Typhi on the gelification and to describe the nature of gel.

- To investigate influence of mutant bacterial strains on gelification process.

- To investigate effects of various physico-chemical factors on gelification.

- To study possibility of *S.* Typhi and other bacteria strains resistance to NaDC, *in vitro*. With the help of Bioscreen C method determine growth curves of bacteria strains in various incubation conditions.

- To observe conceivable possibility of changes in gene/protein expression of *S.* Typhi under various incubation conditions.

- To discuss the hypothesis of gallstone generation caused by *S.* Typhi.

3. MATERIAL AND METHODS

3.1 Biological material

The bacterial strains that were used in this thesis are listed in Tab. 2:

Table 2: Used strains of bacteria.

Strain	Species	Phenotype
MIC-567	*Salmonella* Typhi	E2 Biofilm ATM: -
3934	*Salmonella* Enteritidis	clinico
25922	*Escherichia coli*	ATCC
MIC-591	*Salmonella* Typhi	Mutant ΔsiiE :: Km of *Salmonella* Typhi 567
MIC-592	*Salmonella* Typhi	Mutant ΔcsgD :: Km of *Salmonella* Typhi 567
MIC-593	*Salmonella* Typhi	Mutant ΔyhsjL :: Km of *Salmonella* Typhi 567
MIC-594	*Salmonella* Typhi	Mutant ΔyhjS :: Km of *Salmonella* Typhi 567
MIC-595	*Salmonella* Typhi	Mutant REXBAD :: bapA of *Salmonella* Typhi 567
MIC-596	*Salmonella* Typhi	Mutant ΔbapA :: Km of *Salmonella* Typhi 567
	Yersenia enterocolitica	
9177	*Shigella sonnei*	
O:111	*Escherichia coli*	

3.2 Chemical material:

Sodium deoxycholate (Sigma)

Glucose (Panreac)

LB broth (Lennox)

American Bacteriological Agar (Conda)

Other chemical substances are mentioned in single experiments.

3.3 Methods – experimental part

3.3.1 Culture of bacteria for general purposes. Preparation of media (broth and agar)

Bacteria were cultivated in Luria-Bretani broth medium – LB (Lennox) which is a nutritionally rich medium. For cultivation on Petri plates, agar (American Bacteriological Agar; Conda) was added and plates prepared. Usual cultivation conditions on plates were 24h after inoculation in 37°C, than restored in 4°C. Cultivation conditions in broth were varied.

Formula LB broth: Tryptone 10 g, Yeast Extract 5 g, Sodium Chloride 5 g

Preparation of broth:
LB 20 g
Distilled water a 1000 ml
Sterilize in the autoclave at 121°C for 15 min.

Preparation of plates:
LB 20 g
Agar 15 g
Distilled water 1000 ml
Sterilize in the autoclave at 121°C for 15 min. In sterile environment distribute in sterile Petri plates while hot and leave to become cold (at least 24 h).

3.3.2 Determination of cfu, plating, counting of colonies

For some experiments with bacteria it is necessary to use same initial inoculum in all samples. Therefore it is important to know how many bacteria are included in the initial inoculum. This value is characterized by cfu/ml (colony forming unit per millilitre). Each colony on agar plate is formed by unit. One forming unit is one bacteria cell. According to number of colonies on plate it is possible to determine primary

amount of bacteria (Fig. 10). Countable number of colonies on 1 plate is 20 – 200, that is why it is necessary to make a range of dilutions before. Dilution is usually done as a logarithmic line (10^{-1}, 10^{-2}, 10^{-3}, 10^{-6}, ...). Then the plate is inoculated by 100 µl of inoculum, this inoculum is spread on the plate with sterile glass beads (5-7); plates incubated 24 h in 37°C.

This is also the way how to estimate number of bacteria by absorbance measurement. Measure absorbance samples before dilution. As a blank sample, broth medium is used and wave-length is set on 600 nm.

From previous experiments was determined that an absorbance of 0.125 (A=0.125; wave-length 600) is equal to 10^8 cfu/ml.

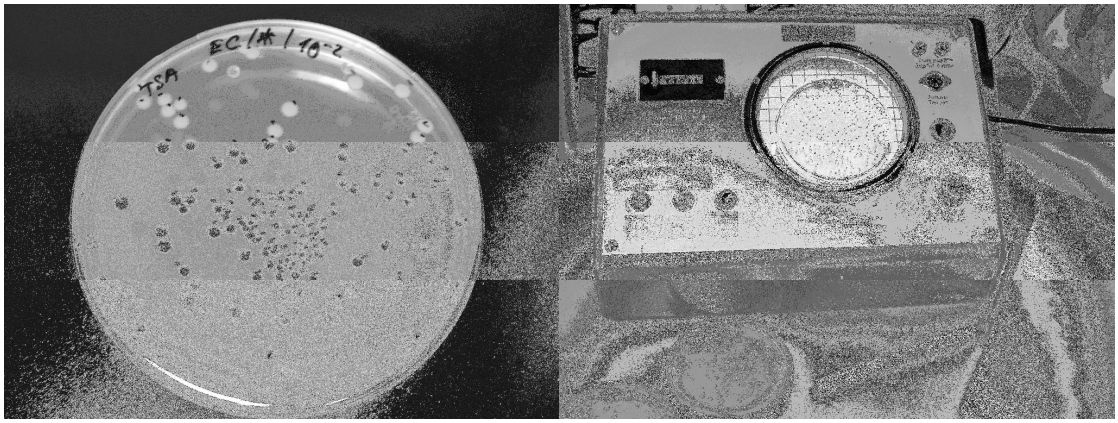


Figure 10: Agar plate with colonies to determine cfu/m1; machine for colonies counting.

Instrumental equipment: test tubes, microbiological loop, micro-pipettes, agar plates, Spectronic® (20 Genesys™), machine for counting colonies on plates (Kolonienzahlgerat), vortex (Heidolph reax 2000), sterile glass beads, gloves.

3.3.3 Gram staining

It is a kind of differential staining that helps define purity of culture and also identify microorganisms. *Salmonellae* are gram negative (G-) bacillococci and thus can be seen as small pink particles (gram positive bacteria are dark blue).

Instrumental equipment: microscope slides, microbiological loop, Bunsen burner, microscope axioscope with integrated photo camera MC 80 (Zeiss)

Protocol: Fix a small amount of media from tube (or a small amount of bacteria suspended in a drop of physiological saline) on a microscope glass by heat (optional way on fixing is by methanol). Then do the staining by using the following procedure:

1. colour with crystal violet (40 seconds)
2. wash with water
3. apply solution of Lugol (1 minute)
4. wash with water
5. wash with alcohol-acetone (1:1)
6. wash with water
7. colour with saphranine (2 minutes)
8. wash with water
9. dry by heat (Bunsen burner)

The cells were observed under the microscope.

3.3.4 Alcian blue staining

This staining is used for colouring both cells and acidic-polysaccharide surrounding (e.g. the matrix in a biofilm).

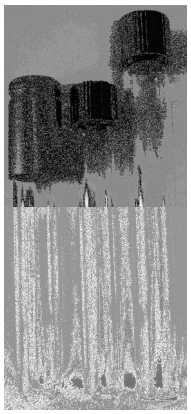
Instrumental equipment: microscope slides, microbiological loop, Bunsen burner, microscope axioscope integrated photo camera MC 80 (Zeiss).

Protocol: Fix a small amount of biofilm coat on a microscope glass by heat. Then do the staining by using the following procedure:

1. colour 2 minutes with Alcian blue (1 g/100ml of methanol)
2. wash with water
3. colour 5 minutes with carbophuscine or saphranine (saphranine was used in this thesis)
4. wash with water
5. dry by heat (Bunsen burner)

The cells (pink) and polysaccharide surrounding (bluish) can be observed under the microscope.

3.3.5 *In vitro*, Incubation of bacteria in glass tubes



Experiments for this thesis were done in glass tubes (Fig. 11) mostly with a screw cap or metal lid (air may go through in contrast to the screw capped one).

Volume of broth medium was 4 ml, environment 37°C (exception noted). Tubes must be sterile (autoclaved, 121°C, 15 min.) and work with tubes, medium and bacteria must be prepared in sterile environment (e.g. box of streamline flow).

Figure 11: from left: metal cap tube, spectronic screw cap tube, screw cap tube (length 12 cm, diameter 1 cm)

Other instrumental equipment: test-tube racks, microbiological loop (plastic or metal), gloves, Bunsen burner, micro-pipettes, vortex (Heidolph reax 2000), sterile plastic tubes, sterile eppendorffs, incubator shaker (C24, New Brunswick Scientific Co.), incubator (Heraus electronic), box of streamline flow (Faster BH 2004), autoclave Auster-E (P Selecta)

Reactives: LB broth, sodium deoxycholate, saline (physiological solution), (special reactives mentioned in single experiments)

Protocol:

1. Prepare sterile medium and sterile tubes.
2. Put 4 ml of medium in each tube.
3. Inoculate tubes by microbiological loop, 1 bacterial colony from LB agar plate in each tube (exceptions noted).
4. Vortex (shake) each tube for 5 seconds.
5. Incubate in 37°C, without agitation (exception noted, if used: 150 rpm).
6. Visual observation.

In case another substance is present in the medium (e.g. sodium dexycholate – can not be autoclaved), first put appropriate amount straight in the medium and shake properly to dissolve then inoculate with bacteria. Never forget to prepare control tubes

either without substance or bacteria. Always prepare more than 1 tube from each sample (usually triplicates).

3.3.6 Bioscreen C

Bioscreen C is a highly developed instrument designed to study the bacterial growth curves (Fig. 12). It is a well known principle that as microorganisms grow, they increase the turbidity of their growth medium. By measuring the turbidity of this medium over time, an optical density (OD) curve can be generated. The curve reflects the growth increase of the organism.

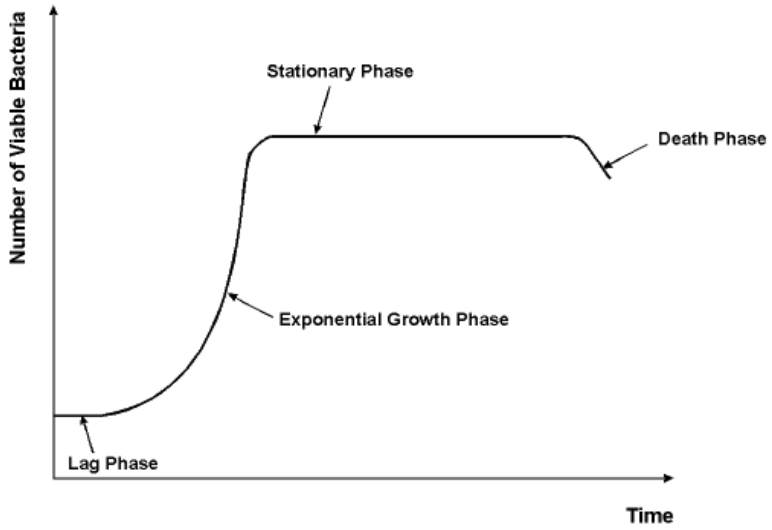


Figure 12: Typical bacterial growth curve. In graphs produced by Bioscreen C, the number of viable bacteria is demonstrated by absorbance.

Bioscreen C system (Fig. 13) provides a platform for these measurements to be made. Turbidity is measured by a wide band filter which is rather insensitive to colour change in the sample. For colour measurement Bioscreen C has 7 other filters with special wavelengths. There are special Honeycomb Microplates, the 10 by 10 well plate (400µl per well). For one experiment, two plates can be used, which means that 200 wells can be filled with the sample. Machine can be set on various experiment lengths, temperature, measuring interval and agitation conditions. Data can be interfaced via MS Excel.

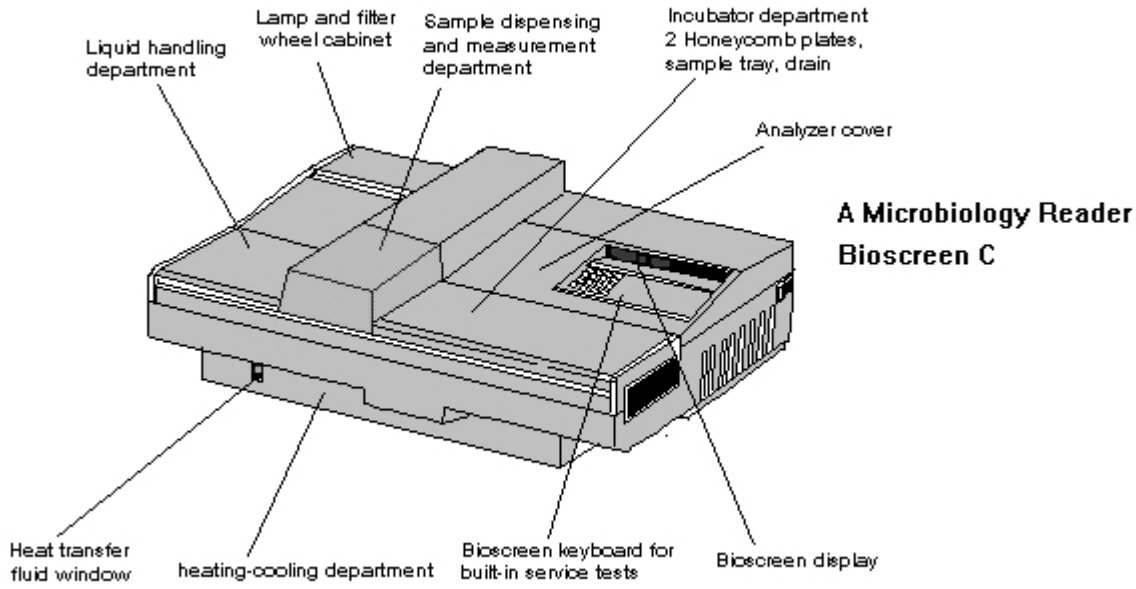


Figure 13: View of Bioscreen C machine, this machine is connected with PC.

Instrumental equipment: Bioscreen C (Labsystems), Honeycomb Microplates (Thermo electron corporations), incubator shaker (C24 New Brunswick Scientific Co.), Spectronic® (20 Genesys™), gloves, micro-pipettes, vortex (Heidolph reax 2000), sterile plastic tubes

Reactives: LB broth medium, sodium deoxycholate, glucose

Protocol of the common procedure:

1. Incubate one colony of bacteria in 3 ml of LB broth medium in spectronic glass tubes (3 h, 37°C, continuous agitation 150 rpm)
2. Measure absorbance (wave-length 600 nm, LB broth medium as a blank), dilute the sample with medium to the absorbance 0.125 (=10^8 cfu/ml)
3. Dilute this sample again 1/100 (=10^6 cfu/ml) and put 20 µl (=$2x10^4$ ml/well as a final concentration) in each well, use special honeycomb microplate with cover
4. Prepare medium (varied in single experiments) and put 180 µl per well (final volume of well is 200 µl)
5. Always make at least triplicates of all various media and bacterial strains.
6. Set the Bioscreen C machine, temperature 37°C, OD wideband, continuous low agitation, experiment length 24 h, measuring interval 30 min.
7. Create growth curves in MS Excel.

3.3.7 SDS – PAGE

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used electrophoretic technique for the analysis of proteins. This is due to the ability of strong anionic detergent SDS to solubilise, denature and dissociate most of proteins to produce single polypeptide chains, when used in the presence of disulphide bond cleaving reagents. The resulting SDS-proteins complexes can be separated according to a molecular size by electrophoresis in gels containing SDS. The effective separation range of SDS-PAGE is determined by the concentration of polyacrylamide gel that was used. In experiments for this thesis the commercial gels were used.

Though the SDS-PAGE method does not provide any direct information on the identity or functional properties of the separated proteins, the comparison of proteins or lipopolysachcarides (LPS) expression between single samples is allowed, silver staining technique was used in this thesis.

Instrumental equipment: Vertical slab electrophoresis apparatus (Criterion™ Cell, Bio-Rad), commercial gel (Criterion™ XT Precast Gel, Bio-Rad), micro-pipettes, gloves, eppendorffs.

Reactives:

Upper buffer (4x) 0.5 M Tris-HCl, pH 6.8

Tris (Sigma) 6.0 g

Distilled water 60.0 ml

Adjust pH with HCl 2N

Distilled water up to 100.0 ml

Buffer was filtered and the acidity (pH) had to be exactly the same after the dilution 1:3 with water.

Sample buffer

Distilled water 12.0 ml

Glycerol (Panreac) 2.4 ml

Upper buffer (4x) 3.0 ml

SDS 10% 4.8 ml

2-mercaptoethanol 1.2 ml

Bromophenol blue 0.05% 0.6 ml

Running buffer

XT MOPS, Running buffer, 20X (Bio-Rad) 50.0 ml

Deionised water 950.0 ml

Marker: Rainbow high-range (RPN 756, Amersham)

3.3.7.1 Electrophoresis

Protocol:

1. Take 200 µl of samples with bacteria for detection, centrifuge 20 minutes (10000 rpm).
2. Separate out the supernatant, add sample buffer (1 ml) and boil for 10 minutes.
3. Centrifuge again (20 minutes, 10000 rpm), further work with the supernatant (=samples). Samples can be used repeatedly, but before each electrophoresis they must be boiled for 5 minutes again.
4. Dilute the rainbow marker (1:5) and prepare SDS-PAGE apparatus.
5. Insert in the commercial gel and running buffer.
6. Introduce 2 µl of marker and 20 µl of samples in a gel, same samples and markers twice in a gel (if is not enough space, run 2 gels at once).
7. Switch on the apparatus and let the sample run down through the gel (about 1 hour).
8. Detect the gel by silver staining.

3.3.8 Silver staining

Silver stain is the most sensitive method for permanent visible staining of proteins and lipopolysaccharides (LPS) in polyacrylamide gels. The sensitivity, however, comes at the expense of high susceptibility to interfere with a number of factors.

In the silver stain, the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions forming an insoluble brown precipitation of metallic silver. This reduction is promoted by protein.

Instrumental equipment and material: orbital shaker (Bibby Stuart), slab gel dryer LKB 2003 (Bromma), bowls, flasks, pipettes, tinfoil.

Reagents:

Methanol (Sigma)
Acetic acid (Panreac)
Glutaraldehyde (Panreac)
Sodium hydroxide (Panreac)
Silver nitrate (Panreac)
Citric acid (Panreac)
Formaldehyde (Panreac)
Ammonium hydroxide (Sigma)
Paraperiodic acid (Panreac)

Protocol:

If are samples twice in one gel, cut the gel appropriately, and one half stain for proteins and the second half for lipopolysaccharides (LPS).
From the 5th step is the method same both for proteins and LPS.

Method for protein (double fixation):

1. Fix (30 minutes, agitation) with 100 ml of solution 1 (methanol 50%, acetic acid 10%, H_2O d).
2. Fix (over night, or at least 60 min., agitation) with 100 ml of solution 2 (methanol 7,5%, acetic acid 5%, H_2O d).
3. Wash twice with H_2O d, 5 minutes each washing.
4. Fix (30 minutes, agitation) with 100 ml of glutaraldehyde 10%.
5. Remove glutaraldehyde and wash 4 times (2 minutes) with 100 ml H_2O d and 4 times (5 minutes) with 100 ml H_2O d.

Method for LPS (fixation and oxidation):

1. Fix (30 minutes, agitation) with 100 ml of solution 1 (methanol 50%, acetic acid 10%, H_2O d).
2. Fix (over night, or at least 60 min., agitation) with 100 ml of solution 2 (methanol 7.5%, acetic acid 5%, H_2O d).

3. Wash twice with H_2O d, 5 minutes each washing.
4. Oxidate with peroxide: 10 min in paraperiodic acid 0.7 % (H_5IO_6) in solution 2.
5. Remove glutaraldehyde and wash 4 times (2 minutes) with 100 ml H_2O d and 4 times (5 minutes) with 100 ml H_2O d.

Following steps are common for both proteins and LPS:
6. Add 27 ml of H_2O ultra pure and diamine solution (described later) to the gel.
7. Wash 3 times with H_2O d (5 min each).
8. Revelation: solution for revelation (described later), with agitation (5 – 30 min, as necessary), keep in dark.
9. Stop the reaction with acetic acid 1%, with agitation.
10. Dry gels (2 h).

Diammine solution

NH_4OH 1.05 ml
NaOH 3.6% 1.6 ml (prepared freshly)
H_2O ad 42.0 ml

Mix all reagents and add drop by drop under strong agitation to 3 ml 20% $AgNO_3$ (prepared freshly), keep the flask in dark (e.g. by tinfoil) and agitation until use.

Solution for revelation

Citric acid 10.0 mg
Formaldehyde 37% 100.0 µl
H_2O ad 100.0 ml

3.3.9 Immunoblotting – western blotting

For identification and characterization of proteins separated by gel electrophoresis, the ligands of a high specifity and affinity such as antibodies are used. The transferation of separated proteins from gel onto the surface of the thin membrane is done by method of semi-dry blotting. As a result, the proteins are immobilised on the matrix and are readily accessible for interaction with antibodies. After the proteins have been transferred to the membrane and before probing with a specific ligand, all

unoccapied binding sites on the support must be locked. For this purpose a solution of non-fat dried milk is used. The most important step in the blotting procedure is the reaction with a specific antibody and visualisation of the proteins in the sample with which it reacts. It is not common to derivate directly primary antibodies to visualize the blot. This derivation should adversely affect its specifity or affinity. Therefore, usually an indirect or sandwich approach utilising labelled (enzyme-conjugated) secondary antibody reagents is used. Detection is achieved using appropriate substrates that form insoluble, stable coloured reaction products at the sites where the enzyme conjugated secondary antibodies is bound (in our case 4-chloro-1-naphtol).

Instrumental equipment and material: semi-dry transfer cell: Trans blot ® SD (Bio-Rad), power supply: LKB Bromma 2197, membrane: Westran ® PVDF Protein transfer and sequenting membrane (Schleicher&Schnell), paper Whatman ® 3, orbital shaker (Bibby Stuart), bowls, flasks, pipettes, tinfoil.

Reactives:

Buffer transblotting (pH=8.3):

- Glycerol (Panreac) 43.5 g
- Tris (Sigma) 9.0 g
- Methanol (Merck) 300.0 ml
- H_2O d ad 3000.0 ml

Methanol (Merck)

H_2O_2 39%

Non-fat dried milk (Sveltesse, Nestle)

4-chloro-1-naphtol for detection of peroxidase (Merck)

Serum – primary antibodies: anti-bapA (rabbit, 1:1000, Universidad Publica de Navarra), anti-groel (rabbit, 1:100, Universidad de Navarra), anti-humano (human, 1:50, Universidad de Navarra)

Serum – secondary antibodies: anti-conejo-PO (rabbit, 1:1000, GantiIgG(Fc)/PO, Nordic), anti-humano-PO (human, 1:500, GAHu/IgG(H+L)/PO, Nordic)

PBS-STOCK:

- Na_2HPO_4 (Panreac) 54.8 g

$Na_2HPO_4.2\ H_2O$ (Panreac) 20.47 g

H_2O d a 1000.0 ml

PBS-TWEEN 20 (pH=7.4)

PBS-stock 80.0 ml

NaCl (Panreac) 17.0 g

Tween 20 (Sigma) 3.0 ml

H_2O d 1900.0 ml

TRIS BUFFER SALINE (TBS)

50 mM Tris 6.05 g

200 mM NaCl 11.69 g

H_2O d a 1000.0 ml

pH = 7.5 was adjusted by HCl

Protocol:

3.3.9.1 Electro-blot (semi-dry method)

1. Run SDS-PAGE electrophoresis according to the protocol.
2. Wash the membrane and 6 pieces of Whatman paper in Buffer transblotting (5 min). Never touch the membrane with fingers (use tweezers, gloves).
3. Place the gel on the membrane and sandwich between two stacks of Withman papers (3+3), wet with Buffer transblotting and remove bubbles (helping with glass rod).
4. Close and switch on the Trans-blot machine (8 V, 200 mA).

3.3.9.2 Immuno-blot

1. Remove membrane and put it in the bowl with 100,0 ml of solution of non-fat dried milk (3% w/v in PBS-TWEEN 20), 1 hour or over the night.
2. Wash in PBS-TWEEN 20 (4 times, 5 min).
3. Add serum with primary antibodies in appropriate dilution (see particular experiments) (1 h or over the night).
4. Wash in PBS-TWEEN 20 (4 times, 5 min).
5. Add serum with secondary antibodies in appropriate dilution (see particular experiments), 1 hour or over the night, keep in dark.
6. Wash in PBS-TWEEN 20 (4 times, 5 min).

7. Prepare reactives, keep them in dark. Reactive A: 60.0 mg of 4-chloro-1-naphtol + 20.0 ml of methanol. Reactive B: 60.0 ml of H_2O_2 39% + 100.0 ml of TBS

8. Mix the reactives together and add them to the bowl with membrane, incubate 5 – 30 minutes, till the bands get visible and dark enough.

9. Document membrane well by scanner or camera, save the membrane in dark and freeze.

4. RESULTS

4.1 Biofilm production of *S.* Enteritidis and *S.* Typhi in LB broth

- ***S.* Enteritidis 3934 is able to produce a biofilm in LB broth.**

Glass tubes with loosely attached metal cap and with a screw cap were used. Tubes with metal cap allow air circulation, glass tubes with screw cap do not.

After inoculation according to the protocol, tubes were incubated at room temperature, without agitation.

In tubes with metal cap, the thin layer of biofilm appears on interface liquid: air after 2 - 3 days (at higher initial inoculum, faster biofilm production; Fig. 13, 14). In tubes with screw cap biofilm is not produced. Air circulation is for biofilm production by *S.* Enteritidis 3934 essential, by contrast to *S.* Typhi as will be shown later.

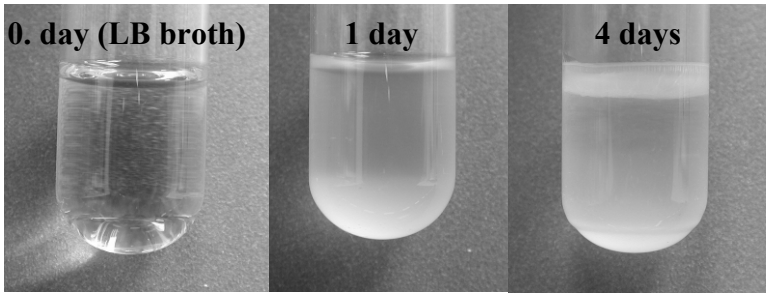


Figure 13: Biofilm production by *S.* Enteritidis 3934, biofilm appears after 2-3 days (initial inoculum one colony from LB agar plate, RT, no agitation).

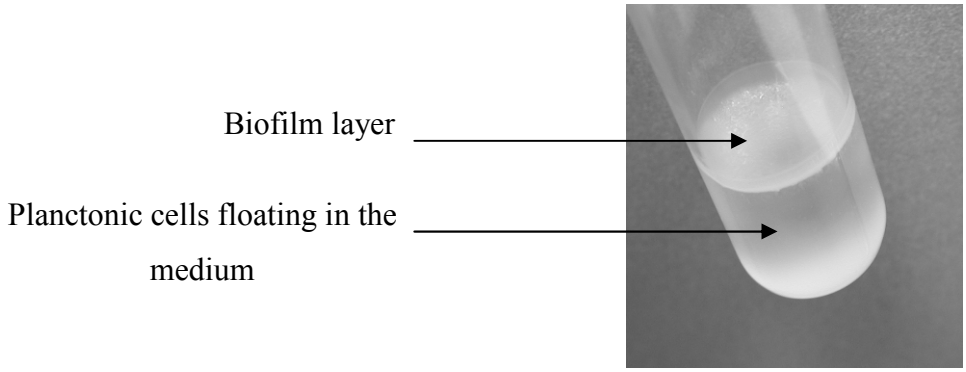


Figure 14: Tube with typical biofilm produced by *Salmonella* Enteritidis 3934 in LB broth (initial inoculum one colony from LB agar plate, RT, no agitation, 2-3 days).

- ***S.* Typhi is not able to produce a biofilm in LB broth.**

Similar conditions were used as in the experiment with *S.* Enteritidis 3934 (initial inoculum one colony of *S.* Typhi 567 from LB agar plate, metal cap tube, screw cap tube, no agitation, 2-3 days). Tubes were incubated at 37°C.

Biofilm was not observed. *Salmonella* Typhi 567 is not able to produce biofilm in LB broth in any kind of tubes.

4.2 Induction of bacterial aggregation of S. Typhi in LB broth and presence of sodium deoxycholate (NaDC)

For this experiment glass tubes with screw cap were used, both for the security reasons and resemblance to the airless environment in gallbladder.
A variety of experiment conditions was tried, see single experiments. Environment of 37°C was used (exceptions noted) to model conformable environment to human gallbladder. Without exceptions, manipulation with *S.* Typhi must be done in the box of streamline flow!

4.2.1 *S.* Typhi is able to produce a biofilm in LB broth and presence of NaDC.

Tubes were prepared according to the protocol with gradual-range concentrations (2%, 1%, 0.5%, 0.25%, 0.12% and 0%) of NaDC. Three initial inoculums were tested: one colony in each tube, one colony in 10 ml of saline and its 1/10 dilution (100 μl in each tube).

Positive result was possible to be observed in a few days (Tab. 3, Fig. 15, 16).

Table 3: Results of incubation *S.* Typhi 567 in presence of NaDC (NaDC), three different concentration of initial inoculum: 1 colony (one colony from LB agar plate in each tube), 1/10 (1colony in 10 ml of saline), 1/100 (1/10 dilution of previous inoculum); 37°C, no agitation, ++ very rigid gel and clear biofilm layer, + rigid gel and biofilm layer, +/- soft gel, thin biofilm layer, - liquid, no biofilm.

amount of NaDC	triplicates - different initial inoculum								
	1 colony			1colony/10ml			1/100		
2%	++	++	+	++	++	++	++	++	+
1%	++	++	++	++	++	++	++	++	+
0.5%	++	++	++	++	++	++	++	++	++
0,25%	+	+	+	+	+	+	+	+	+
0,12%	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
0%	-	-	-	-	-	-	-	-	-

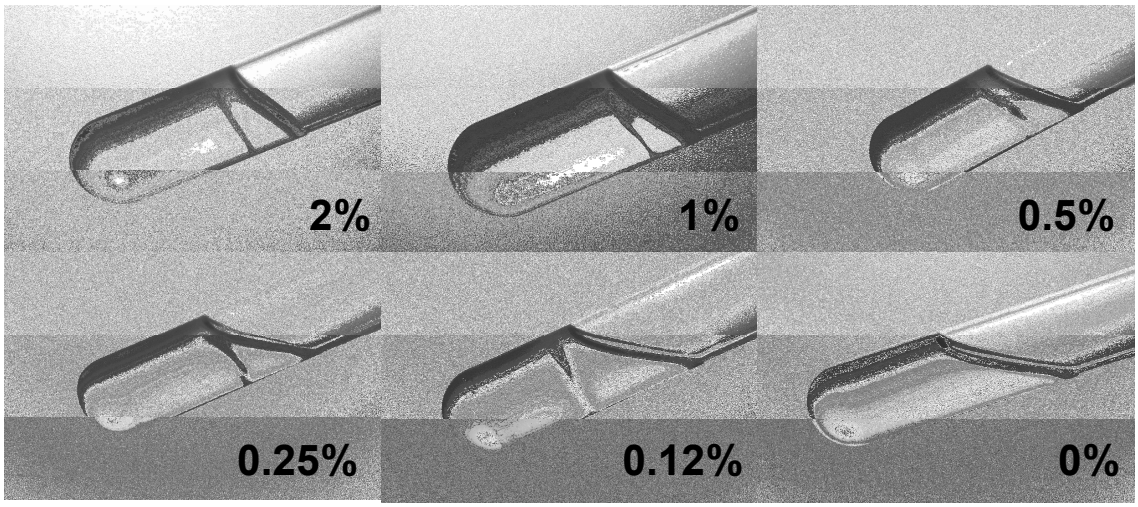


Figure 15: Tubes with concentration line and *S.* Typhi 567, initial inoculum 1/100, 37°C, no agitation.

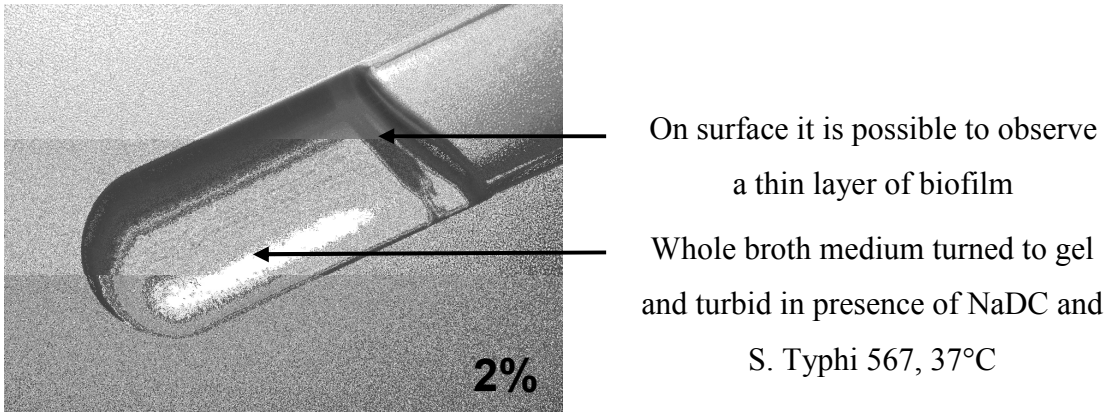


Figure 16: Typical positive result of incubation of *S.* Typhi 567 in presence of NaDC, 37°C, initial inoculum – 1 colony, no agitation.

NaDC provides positive results. *S.* Typhi 567 is multiplying enough, because the turbidity that occurs in tubes column with NaDC is same as in tubes without NaDC. Control tubes of LB broth with and NaDC without bacteria are transparent. The thin layer of biofilm is possible to be observed only in tubes containing NaDC, this phenomenon does not depend on concentration level of NaDC. Gelification appears in tubes, higher rigidity of gel is in tubes with higher concentration of NaDC.

Concentration of bacteria in the initial inoculum has no significant interest, only that the higher concentrated initial inoculum provides the phenomenon faster. Generally, the gel and turbidity are possible to be observed in a few hours (3-6 h), visible and certain biofilm layer is surely possible to be observed after 24 hours.

For following experiments 0.5 % concentration of NaDC was selected as a representative concentration. This concentration provides a sure positive result (gel and biofilm) and is low enough to avoid conceivable toxicity for bacteria by presence of bile salt (see the following results from Bioscreen C method).

4.2.2 Gel produced by *S.* Typhi is stable in 37°C in the comparison with control without bacteria.

With the respect of protocol, control tubes with LB broth and 0.5% NaDC were prepared.

Tubes were placed in room temperature. In 1 hour gelification was possible to be observed in all of them.

Tubes were shaken (Vortex) properly, till liquid appeared again and were placed in 37°C environment. It was not possible to observe the gel again, medium remained liquid (Fig. 17). Tubes containing *S.* Typhi 567 produced rigid gel and biofilm layer at 37°C.

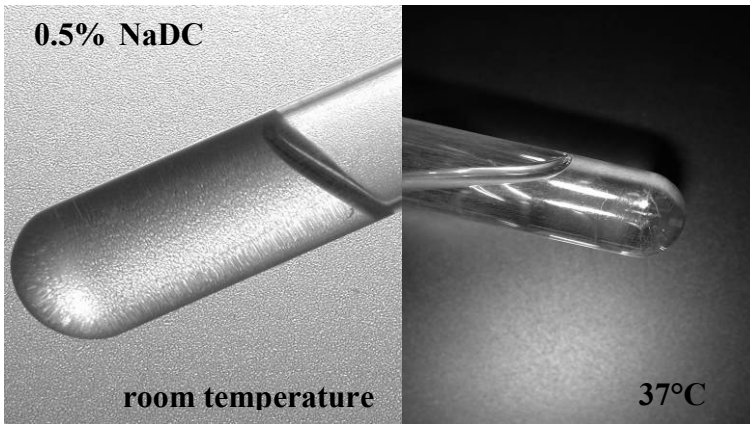


Figure 17: Control tubes without bacteria are solid (gel) at RT and liquid at 37°C.

4.2.3 The air atmosphere affects to the gelification process.

In this experiment, influence of changes in air condition was tested. Tubes with loosely attached metal cap and with screw cap were used.

1. Metal cap tubes access the circulation of air. 4 ml of LB and 0.5% NaDC and 1 colony was put in and incubated according to the protocol.
2. Screw cap tubes do not allow access of air. Tubes were prepared according to the protocol (4 ml, LB, 0.5% NaDC, 1 colony of *S.* Typhi 567).
3. Other screw cap tubes were prepared with an only difference - 12 ml of media was put in to stimulate environment with a lack of air, inoculated and incubated as usual.

Table 4: Influence of air condition on gelification. ++ typical positive result, +/- gel is disappearing, - liquid

	1 day	2 days	5 days
metal cap	++	+/-	-
screw cap, lack of air	++	++	+/-
screw cap, standard	++	++	++

After 24h, positive results as gel and biofilm were observed. But after 48h (2 days) the gel from the metal cap tubes started disappearing (turbidity remained). After 5 days, there was no gel in the metal cap tube and the medium was liquid and turbid). The screw cap tube with grater volume started loosing the gel from the lower part to the upper part. Gel and biofilm in standardly prepared tube maintained (Tab. 4, Fig. 18).

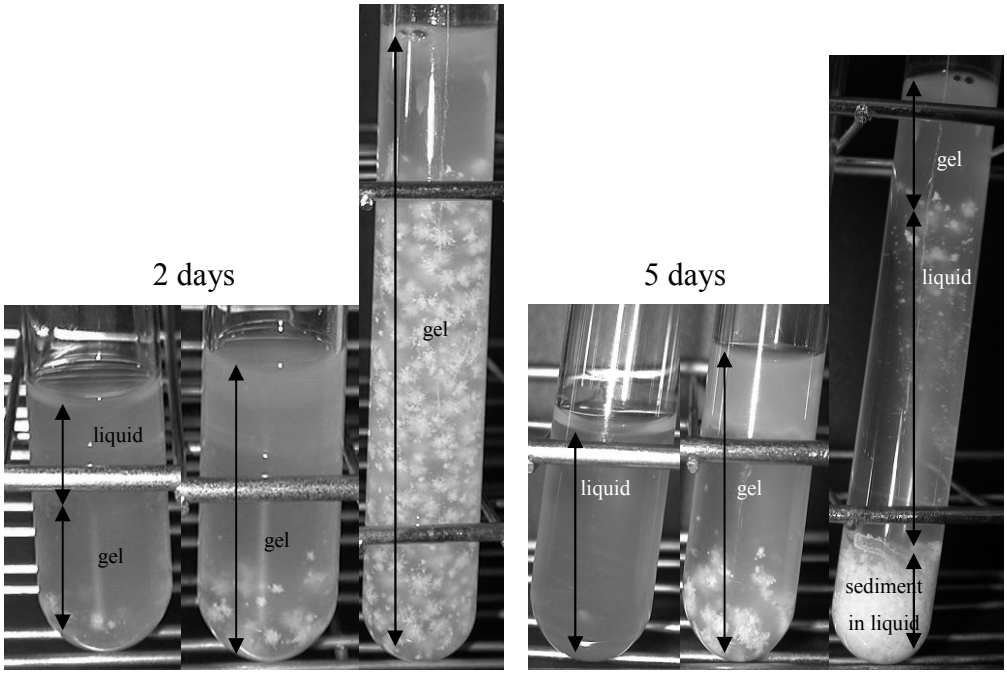


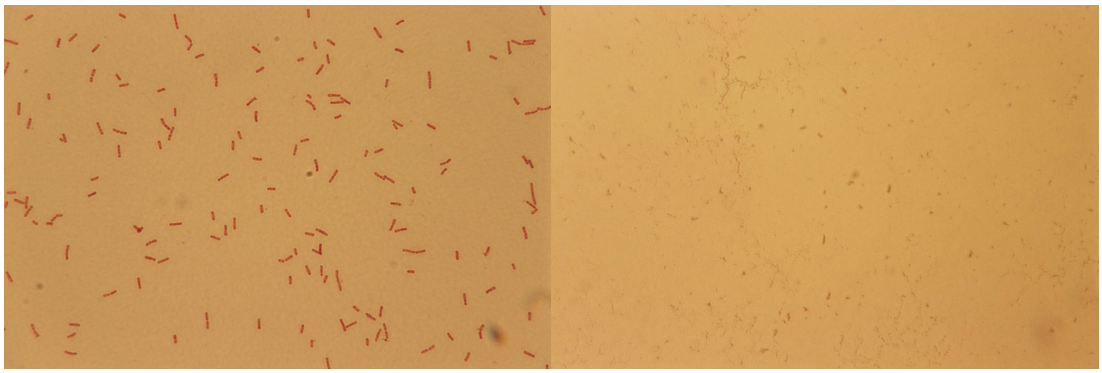
Figure 18: Influence of air access on the gel degradability (2 and 5 days: from left - metal cap tube, which allows access of air, standardly prepared screw cap tube, screw cap tube with higher amount of media and lack of air), *S.* Typhi 567 in presence of NaDC, 37°C, no agitation. .

4.2.4 *S.* Typhi 567 growing in presence of glucose and NaDC needs longer time period for adaptation to the environment, there are also significant changes in cells morphology

Tubes of LB broth and 0.5% NaDC and other tubes of LB broth and 0.5% NaDC and 1% glucose were prepared, inoculated by *S.* Typhi 567 and incubated at 37°C, agitation (150 rpm).

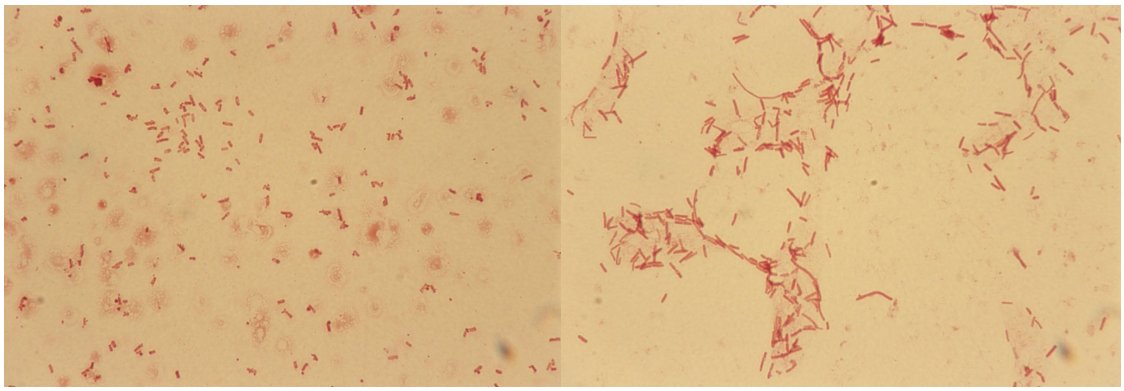
They were observed visually and every hour (from 5^{th} to 10^{th}) a drop was taken from and Gram stained.

Raising turbidity was first observed (after 4 hours) in tubes without glucose. In sample containing glucose it was observed after 9 hours of incubation (same as in Bioscreen C experiment, see further). Morphology of *Salmonella*s cell is changing as consistent with exponential (multiplying) phase. In this phase *Salmonella* cells are lengthy. In presence of both glucose and NaDC this length is rather conspicuous (Fig. 19, 20).



(a) **(b)**

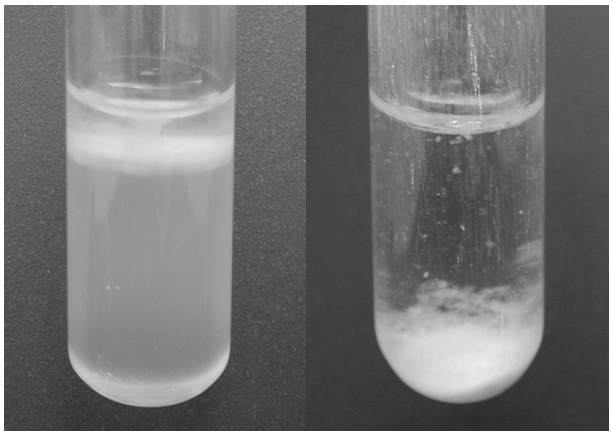
Figure 19: Gram staining, after 5th hours of incubation. (a) – *S.* Typhi 567 in presence of NaDC, (b) – *S.* Typhi 567 in presence of NaDC and glucose



(a) **(b)**

Figure 20: Gram staining, after 10th hours of incubation. (a) – *S.* Typhi 567 in presence of NaDC, (b) – *S.* Typhi 567 in presence of NaDC and glucose

After 10 hours, the tubes were put into standard incubation conditions (no agitation), after 24 hours, in the tube with NaDC, gel was observed as usually. In the tube with NaDC and glucose, the medium was liquid and transparent, whitish sediment of cells lay/floated in the lower part of the tube. Its consistence was curd/soft (Fig. 21)



(a) **(b)**

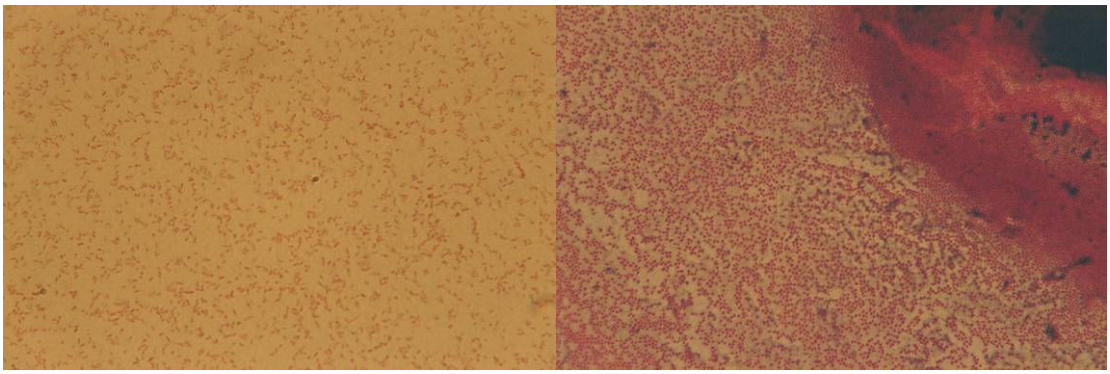
Figure 21: Tubes with *S.* Typhi 567, LB, 0.5% NaDC, 37C, no agitation
(a) – without glucose; (b) - with glucose (1%)

4.3 Study of phenomenon of aggregation and gelification.

4.3.1 Microscopy observation of biofilm

- **Biofilm of S. Enteritidis 3934**

A drop of media with bacteria was taken from the lower part of the tube (not from the biofilm part) and stained according to the Gram. Part of the biofilm layer was taken and stained according to Alcian blue (Fig. 22). Microscope glass was observed with an immersion lens of microscope (1000x).



(a) **(b)**

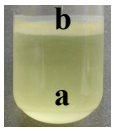
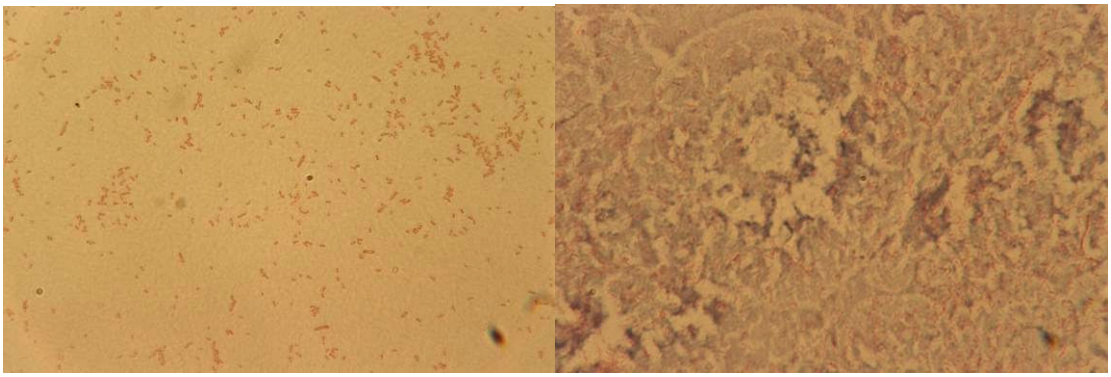


Figure 22: Cells of *S.* Enteritidis 3934, (a) – Gram staining, pink cells are observed, (b) – Alcian blue staining, pink cells and bluish polysaccharide (cellulose mostly) surrounding of biofilm is possible to observe.

- **Biofilm of S. Typhi 567**

Drop of media with bacteria was taken from lower part of tube (not from biofilm part) and stained according to the Gram. Part of the biofilm layer was taken and stained according to Alcian blue (Fig. 23). Microscope glass was observed with an immersion lens of microscope (1000x).



(a) **(b)**

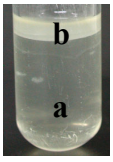


Figure 23: Cells of *S.* Typhi 567, (a) – Gram staining, pink cells are observed, (b) – Alcian blue staining, pink cells and bluish polysaccharide (cellulose mostly) surrounding of the biofilm can be observed.

4.3.2 Inductive factors of the aggregation and gelification process

- **S. Typhi 567 must be alive to produce the biofilm and the gel.**

Two initial inoculums were used. First inoculum was very high concentrated (huge amount from agar plate (comparable to 100 colonies) was added to 10 ml of saline (signed as D)), and second inoculum was very low concentrated (1 colony in 10 ml of saline and than diluted 1/100 (signed as 10^{-3})). A part of inoculum was put into a water bath, 65°C, for 1 hour to kill bacteria.

Four groups of tubes with LB broth and 0.5% of NaDC were prepared and inoculated with 100 μl of inoculum, each group with different inoculum. Tubes were incubated according to the standard protocol (37°C, no agitation).

After 24 h, gelification was observed only in tubes with living *S.* Typhi 567, the rest of tubes were liquid (Fig. 24, 25).

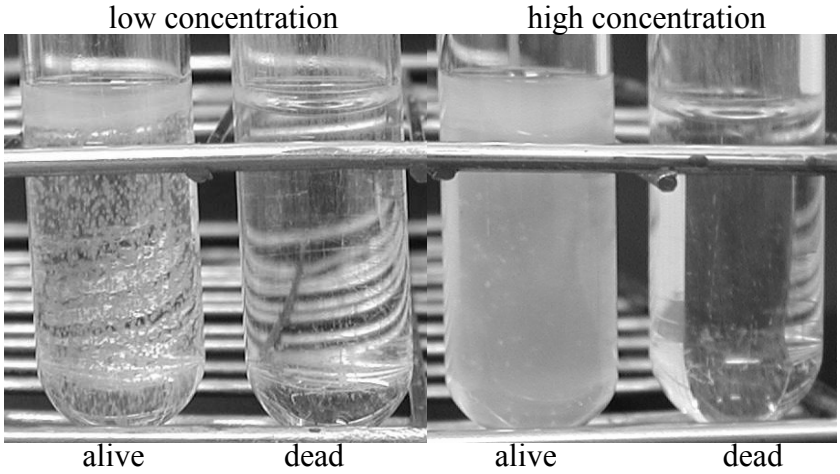


Figure 24: Living *S.* Typhi 567 and dead cells, 37°C, no agitation. Gelification is observed only in the tubes containing bacteria which are alive.

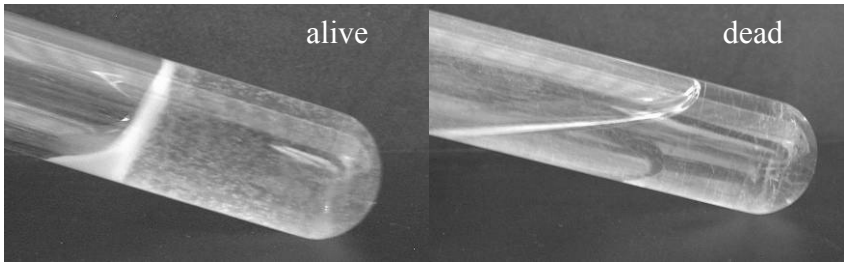


Figure 25: *S.* Typhi 567 alive and dead cells, 37°C, no agitation, lower concentration.

- **Mutant strains of S. Typhi 567 also provide efficient conditions for biofilm production and gelification in LB broth with 0.5% of NaDC.**

Table 5: Mutant strains of *S.* Typhi 567.

Strain	Species	Phenotype
591	*Salmonella* Typhi	Mutant ΔsiiE :: Km of *Salmonella* Typhi 567
592	*Salmonella* Typhi	Mutant ΔcsgD :: Km of *Salmonella* Typhi 567
593	*Salmonella* Typhi	Mutant ΔyhsjL :: Km of *Salmonella* Typhi 567
594	*Salmonella* Typhi	Mutant ΔyhjS :: Km of *Salmonella* Typhi 567
595	*Salmonella* Typhi	Mutant REXBAD :: bapA of *Salmonella* Typhi 567
596	*Salmonella* Typhi	Mutant ΔbapA :: Km of *Salmonella* Typhi 567

Tubes of LB broth and 0.5% NaDC were prepared and incubated with mutant strains (Tab. 5) of *S.* Typhi 567, according to the protocol.

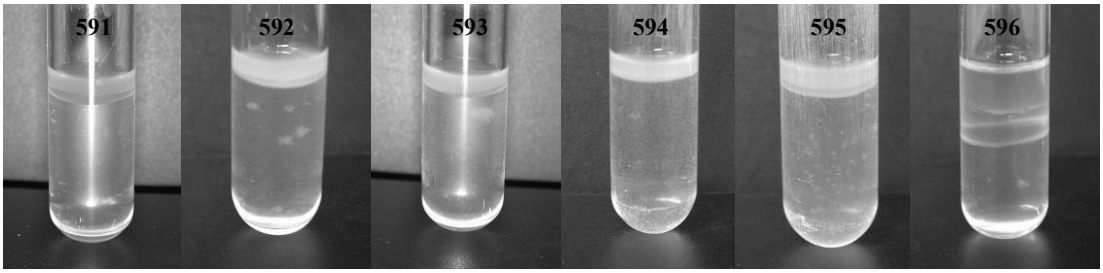


Figure 26: Mutant strains of *S.* Typhi 567, LB, 0.5% NaDC, 37°C, no agitation, one week old.

During one week of visual observation of gelification and biofilm was produced (Fig. 26, 27), both the gel and the biofilm layer were analogous to non-mutant strain *S.* Typhi 567.

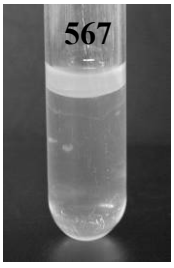


Figure 27: *S.* Typhi 567, LB, 0.5% NaDC, 37°C, no agitation, one week old.

In the case of Mutant ΔbapA :: Km of *Salmonella* Typhi 567 (596), the gel was rigid for 3 days and than started to disappear slowly, which is apparent in figure 26. It appears this mutant strain is defective in the ability to form gel.

Control tubes without NaDC prove that bacteria were multiplying (turbidity) but did not create any biofilm or gelification, they were liquid.

- **Study of the factors involved in gelification, filtration. Soluble factors released by the bacteria induce the gelification.**

Tubes were prepared according to the protocol with *S.* Typhi 567 with 0.5% NaDC. The same number of control tubes without NaDC was also prepared. Tubes were incubated for 4 hours (enough for gelification).

Then the culture was filtrated trough microbiological filters (0.22 μm, Millex™ GP) and put in other sterile tubes, in the same volume. In the control tubes, NaDC was added to the concentration 0.5% and the tubes were vortexed.

Incubation was done according to the protocol (37°C, no agitation).

After 4 hours, all tubes got positive result. Both the media that already contained NaDC and the control media, where NaDC was added later, were rigid gels. Media were transparent (did not contain cells).

Note: Centrifugation was also used to get the supernatant free of cells. But this separation was not effective. Especially in samples which already contained NaDC, the gel-like culture was not centrifuged well.

4.4 Study of the nature of the soluble(s) factor(s) induction gelification

4.4.1 Neither in *S.* Typhi 567 nor in Mutant ΔbapA :: Km of *S.* Typhi 567 (596) treatment of proteinase K and boiling temperature affect regelification after filtration.

Tubes were prepared according to the protocol with *S.* Typhi 567 and Mutant ΔbapA :: Km of *S.* Typhi 567 (596), 0.5% NaDC. The same number of control tubes without NaDC was also prepared. Tubes were incubated for 4 hours (enough for gelification). Then the culture was filtrated trough microbiological filter (0.22 μm, Millex™ GP) and put into other sterile tubes, in the same volume. In the control tubes, NaDC was added up to the concentration of 0.5%. Incubation was done according to the protocol (37°C, no agitation).

After the gelification appeared again, the tubes were treated with:

- boiling temperature for 35 minutes
- proteinase K (Merck) (0.5 mg/ml, 37°C, with agitation)
- nothing, control group

Tubes were kept in 37°C over night. All of them were regelificated again with neither any difference between samples which contained NaDC before the filtration nor the samples to which NaDC was added after filtration.

4.4.2 Microbial filtration through filters for DNA isolation does not affect regelification after filtration.

Tubes were prepared according to the protocol with *S.* Typhi 567, in LB, 0.5% NaDC. The tubes were incubated for 4 hours (enough for gelification).

After 4 hours the culture was filtered through microbiological filter (0.22 μm, Millex™ GP) and put into Spin filters (Microbial DNA Isolation KIT, UltraClean™, Mo Bio) in the volume of 500 μl.

Filters were centrifuged (10000 rpm, 15 minutes). Supernatant was put in screw cap tubes and another 500 µl of LB were put in filters. Filters were vortexed (5 seconds) and sonicated (sonicator, Fungilab s.a.) for 10 min.; samples must be under the water surface. Culture was put in screw cap tubes; incubated in 37°C without agitation.

Gel appeared in the supernatant part. The upper part (with the rests from the filter) remained liquid (Fig. 28).

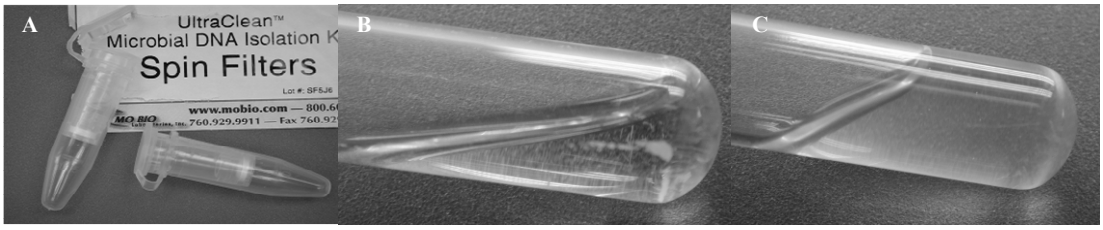


Figure 28: A – filters for DNA isolation, B – liquid media from filter after vortex and sonication, C – gel-like supernatant

4.4.3 Filtrations with 300.000 MWCO filters (Sartorius) do not allow regelification of filtrate.

Tubes were prepared according to the protocol with *S.* Typhi 567 in LB, 0.5% NaDC. The same number of inoculated control tubes without NaDC was also prepared. Tubes were incubated for 4 hours.

Later, the culture was filtered trough microbiological filter (0.22 µm, Millex™ GP) and was divided into 300.000 MWCO filter tubes (Fig. 30) and centrifuged (3000g, 2 hours – a long time was necessary, because it is really difficult for gelificated samples to pass trough the filters).

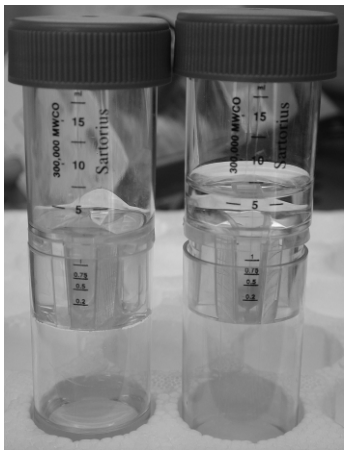


Figure 30: 300.000 MWCO filter tubes (Sartorius), separation is afforded by centrifugation.

After filtration, NaDC was added to the concentration 0.5% in the control sample. Samples were put in screw cap tubes and incubated according to the protocol (37°C, no agitation).

In 24 hours, regelification was not observed in any tube. Supernatant was liquid and transparent.

4.5 Monitoring of physico-chemical factors that may act as gelators of NaDC.

- **Gelification of NaDC in control tubes is pH dependent.**

Four tubes with 0.5% of NaDC were prepared according to the protocol. And pH was measured (CRISON pH-meter 2001) and modified to 7; 6.5; 6 and 5.5 by HCl 0,2N (exact values were 7.1; 6.45; 5.93; 5.54).

The tubes were placed in a room temperature. In 1 hour, gelification was observable. Rigidity of gel depends on pH, with increasing pH, rigidity decreases.

Tubes were shaken (vortex) properly, till liquid appeared again and were placed in 37°C environment. After few hours the gels appeared again, but with lower rigidity (Tab. 8, Fig. 31).

Table 8: Rigidity of gel depends on pH.

pH	room temperature	37°C
5.5	very rigid gel	gel
6	rigid gel	soft gel
6.5	gel	viscous liquid
7	viscous liquid	liquid

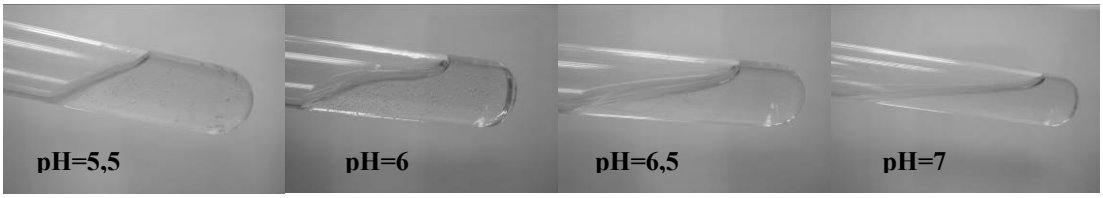


Figure 31: Rigidity of gel depends on pH, 37°C, no agitation.

In order to check whether pH affected the gel formation of *S.* Typhi, the tubes with LB, 0.5% NaDC and bacteria were prepared and incubated according to the protocol. After 24 h of incubation, pH was measured by pH-meter (CRISON pH-meter 2001; the tubes were autoclaved before measuring due to the security reasons, it was not possible to measure pH by pH-meter in a box of streamline flow). An average pH was 7.42.

This means that pH has no influence because the control sample of pH 7 was liquid in 37°C.

To confirm this, the tube containing both the liquid and the gel-like phase (lack of air, after 5 days) from experiment of air influence on gelification was used. Samples of 2 ml from each phase were taken. Filtration by microbiological filters (0.22 µl, Millex™ GP) was done (due to the security) and pH measured by pH-meter. The gel-like part pH was 6.86 and the liquid part 7.07. The very small (0.2) difference again proves that pH has no influence on this phenomenon.

- **Co^{2+}, EDTA and vitamins B do not form rigid gel at 37°C, in LB broth and 0.5% NaDC, while the yeast extract does.**

Control tubes were prepared according to protocol with various compounds: yeast extract (Difco), $CoCl_2$ (Aldershof), EDTA (Panreac), vitamin B_1 (Sigma), vitamin B_6 (Sigma), vitamin B_{12} (Sigma) (Tab. 6). The tubes were placed in 37°C.

After 4 hours, the tube with the yeast extract contained gel. The rest of tubes were liquid.

Tubes were shaken (vortex, 5 seconds) and placed in a room temperature.

After another 4 hours, gel appeared in all tubes (except tube 1, which contains only LB, Tab.6).

Table 6: Monitoring of gelification of NaDC in various conditions.

tube	after 4h, room temperature	after 4h, 37 °C
1. LB	liquid	liquid
2. LB, 0.5% NaDC	gel	liquid
3. LB, 0.5% NaDC, yeast extract (10g/l)	gel	gel
4. LB, 0.5% NaDC, $CoCl_2$ (5μM)	gel	liquid
5. LB, 0.5% NaDC, EDTA (0.1 mM)	gel	liquid
6. LB, 0.5% NaDC, vitamin B_6 (2.26 μg/ml)	gel	liquid
7. LB, 0.5% NaDC, vitamin B_6 (22.6 μg/ml)	gel	liquid
8. LB, 0.5% NaDC, vitamin B_1 (0.2 μg/ml)	gel	liquid
9. LB, 0.5% NaDC, vitamin B_1 (2 μg/ml)	gel	liquid
10. LB, 0.5% NaDC, vitamin B12 (0.2 μg/ml)	gel	liquid
11. LB, 0.5% NaDC, vitamin B12 (2 μg/ml)	gel	liquid

- **Co^{2+}, EDTA, vitamins B and yeast extract do not form gel at any temperature in sterile water and 0.5% NaDC.**

Control tubes were prepared according to protocol with various compounds: yeast extract (Difco), $CoCl_2$ (Aldershof), EDTA (Panreac), vitamin B_1 (Sigma), vitamin B_6 (Sigma), vitamin B_{12} (Sigma). The tubes were incubated in the same conditions as in the previous experiment (37°C, room temperature, Tab. 7).

Gel was not observed in any tubes at any temperature of incubation.

Table 7: Monitoring of gelification of NaDC in various conditions.

tube	after 4h, room temperature	after 4h, 37 °C
1. H_2O	liquid	liquid
2. H_2O, 0.5% NaDC	liquid	liquid
3. H_2O, 0.5% NaDC, yeast extract (10g/l)	liquid	liquid
4. H_2O, 0.5% NaDC, CoCl2 (5μM)	liquid	liquid
5. H_2O, 0.5% NaDC, EDTA (0.1 mM)	liquid	liquid
6. H_2O, 0.5% NaDC, vitamin B6 (2.26 μg/ml)	liquid	liquid
7. H_2O, 0.5% NaDC, vitamin B6 (22.6 μg/ml)	liquid	liquid
8. H_2O, 0.5% NaDC, vitamin B1 (0.2 μg/ml)	liquid	liquid
9. H_2O, 0.5% NaDC, vitamin B1 (2 μg/ml)	liquid	liquid
10. H_2O, 0.5% NaDC, vitamin B12 (0.2 μg/ml)	liquid	liquid
11. H_2O, 0.5% NaDC, vitamin B12 (2 μg/ml)	liquid	liquid

4.6 Study of bacterial sensibility in presence of NaDC, *in vitro*

4.6.1 Sensitivity of *S.* Typhi 567, *S.* Enteritidis 3934 and *E. coli* ATCC 25922 to presence of NaDC in medium related to time, CFU determination

Tubes with 0.5% NaDC and control tube without NaDC were prepared, inoculated with 3 strains of bacteria: *S.* Typhi 567, *S.* Enteritidis 3934, *E. coli* ATCC 25922 and incubated at 37°C with agitation (150 rpm). In time: 0 h (initial), 4 h, 8 h and 24 h, samples were withdrew and inoculated (volume 100 μl) on agar plates in four various dilutions: directly from the tube, 10^{-4}, 10^{-6} and 10^{-8}.

CFU were counted and the calculus was done (Tab. 9).

Table 9: t – time; ST - *S.* Typhi 567, SE - *S.* Enteritidis 3934, EC - E. coli ATCC 25922, * (star) – bacteria treated with NaDC, 0 – control

t = 0h (initial inoculum)			
bacteria	**log**	**bacteria**	**log**
ST *	7,6	ST 0	7,48
SE *	7,5	SE 0	7,5
EC *	5,11	EC 0	7,48

t = 4h			
bacteria	**log**	**bacteria**	**log**
ST *	8,44	ST 0	8,51
SE *	8,53	SE 0	11,26
EC *	4,1	EC 0	8,77

t = 8h			
bacteria	**log**	**bacteria**	**log**
ST *	11,8	ST 0	11,7
SE *	11,74	SE 0	11,78
EC *	5,48	EC 0	11,2

t = 24h			
bacteria	**log**	**bacteria**	**log**
ST *	**13,36**	ST 0	**12,88**
SE *	13,15	SE 0	13,11
EC *	4,29	EC 0	13,34

E. coli was not growing in presence of NaDC, *S.* Enteritidis was growing on the same level both in presence of NaDC and the control after 24h, *S.* Typhi 567 was stimulated by presence of low concentrated NaDC after 24h and multiplied more in the sample containing NaDC.

4.6.2 Monitoring of bacterial growth with Bioscreen C machine

- **Growth curves of Salmonella Typhi 567 in presence of NaDC.**

Special honeycomb microplate with cover was prepared with *S.* Typhi 567 and the media according to the protocol.

Concentration of NaDC: 100 mg/ml, 20 mg/ml, 5 mg/ml and 0 mg/ml.

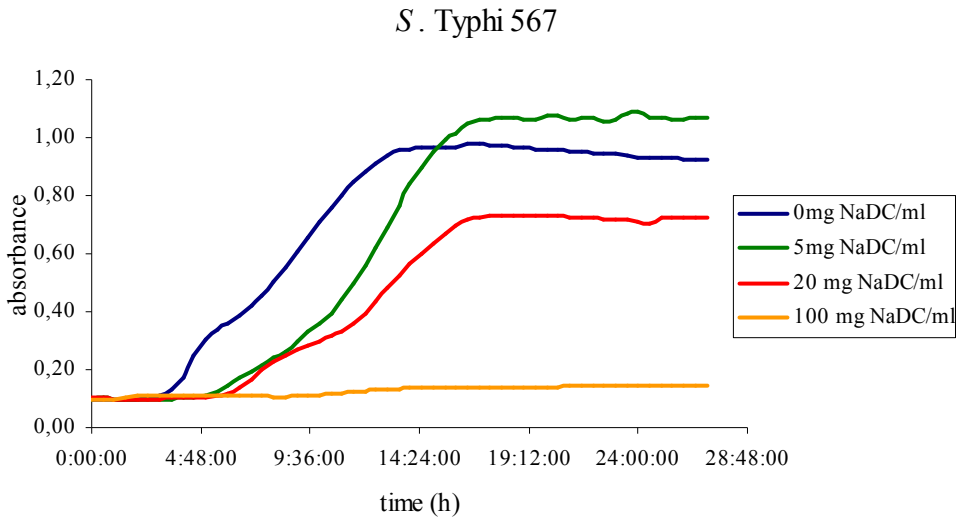


Figure 32: Growth curve of S. Typhi 567 in presence of various concentrations of NaDC.

S. Typhi 567 growth is stimulated by presence of low concentrated (5 mg/ml) NaDC, the lag phase is longer (adapting of bacteria) and in exponential phase overgrow bacteria, which are not treated. Higher concentration (20 mg/ml, 100 mg/ml) of NaDC inhibited bacteria growth and multiplication (Fig. 32).

- **Growth curves of S. Enteritidis 3934 and E. coli 25922 in presence of NaDC.**

Special honeycomb microplate with cover for Bioscreen C was prepared with *S.* Enteritidis 3934 and *E. coli* 25922 and media according to the protocol.
Concentration of NaDC: 20 mg/ml, 5 mg/ml and 0 mg/ml.

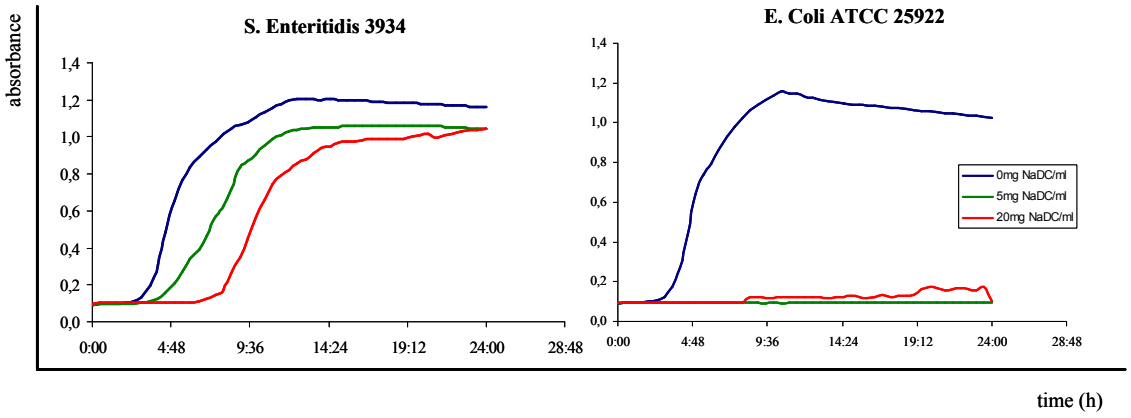


Figure 33: Growth curve of S. Enteritidis and *E. coli* ATCC 25922 in presence of various concentrations of NaDC.

S. Enteritidis 3934 growth is inhibited by presence of NaDC. With increasing concentration of NaDC, the growth possibilities are decreasing. *E. coli* 25922 is not growing in any concentration of NaDC (Fig. 33).

- **Growth curves of mutant strains of Salmonella Typhi 567 in presence of NaDC**

Special honeycomb microplate with cover for Bioscreen C were prepared with mutant strains of *S.* Typhi 567 (Tab. 5) and the media according to the protocol. Concentration of NaDC: 5 mg/ml and 0 mg/ml.

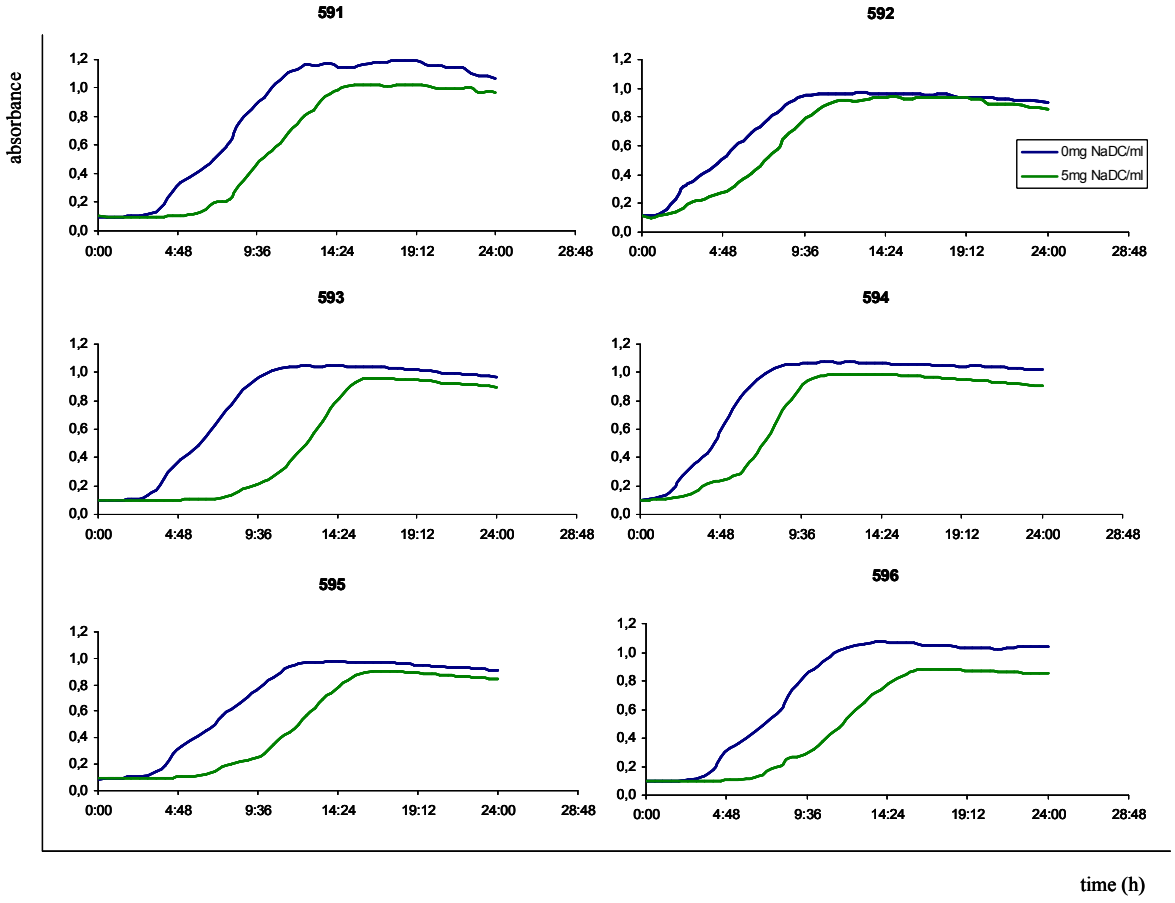


Figure 34: Graphs of mutant strains of *S.* Typhi 567, blue curves (control – no NaDC, green curves - 5 mg/ml of NaDC)

The mutants of *S.* Typhi 567 more or less respond to behaviour of the wild strain in presence of NaDC. Even if they do not over grow the control sample, they have a longer lag-phase and lately grow almost as much as the control (except of 591 and 596, which growth is inhibited in presence of NaDC (Fig. 34).

- **Growth curves of Salmonella Typhi 567 in presence of NaDC and 1% glucose.**

Special honeycomb microplate with cover for Bioscreen C was prepared with *S.* Typhi 567 and the media according to the protocol.

Concentration of NaDC: 20 mg/ml, 5 mg/ml and 0 mg/ml. Prepare the same concentration line also with 1% of glucose in each concentration.

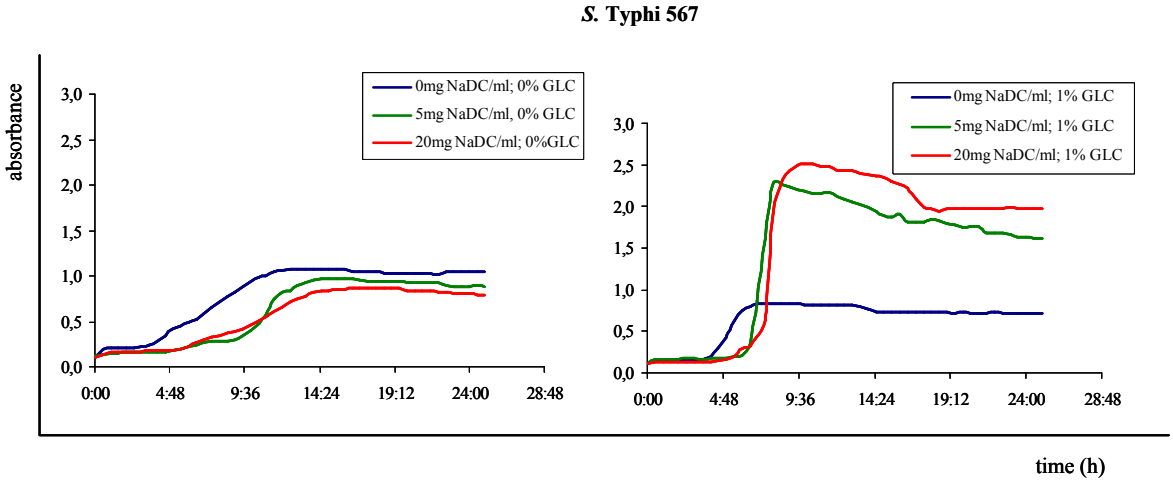


Figure 35: Growth curves of *S.* Typhi 567 in presence of NaDC (left) and in presence of NaDC and glucose (right).

S. Typhi 567 in presence of NaDC without glucose grows as was described before (in this case it does not overgrow the control, but in the lag phase it is still possible to see, that it needs time to adapt and then grows to the same level as the control). In presence of 1% glucose without NaDC also grows standardly. But in presence of both the glucose and the NaDC grows extremely rapidly, the lag phase is prolonged (Fig. 35).

- **Growth curves of various bacteria strains in presence of NaDC and 1% glucose.**

Special honeycomb microplate with cover for Bioscreen C were prepared with various strains of bacteria (*S.* Enteritidis, *E. coli*, *Shigella sonnei*, *Yersinia enterocolitica*) and media according to the protocol.

Concentration of NaDC: 20 mg/ml, 5 mg/ml and 0 mg/ml. Prepare the same concentration line also with 1% of glucose in each concentration.

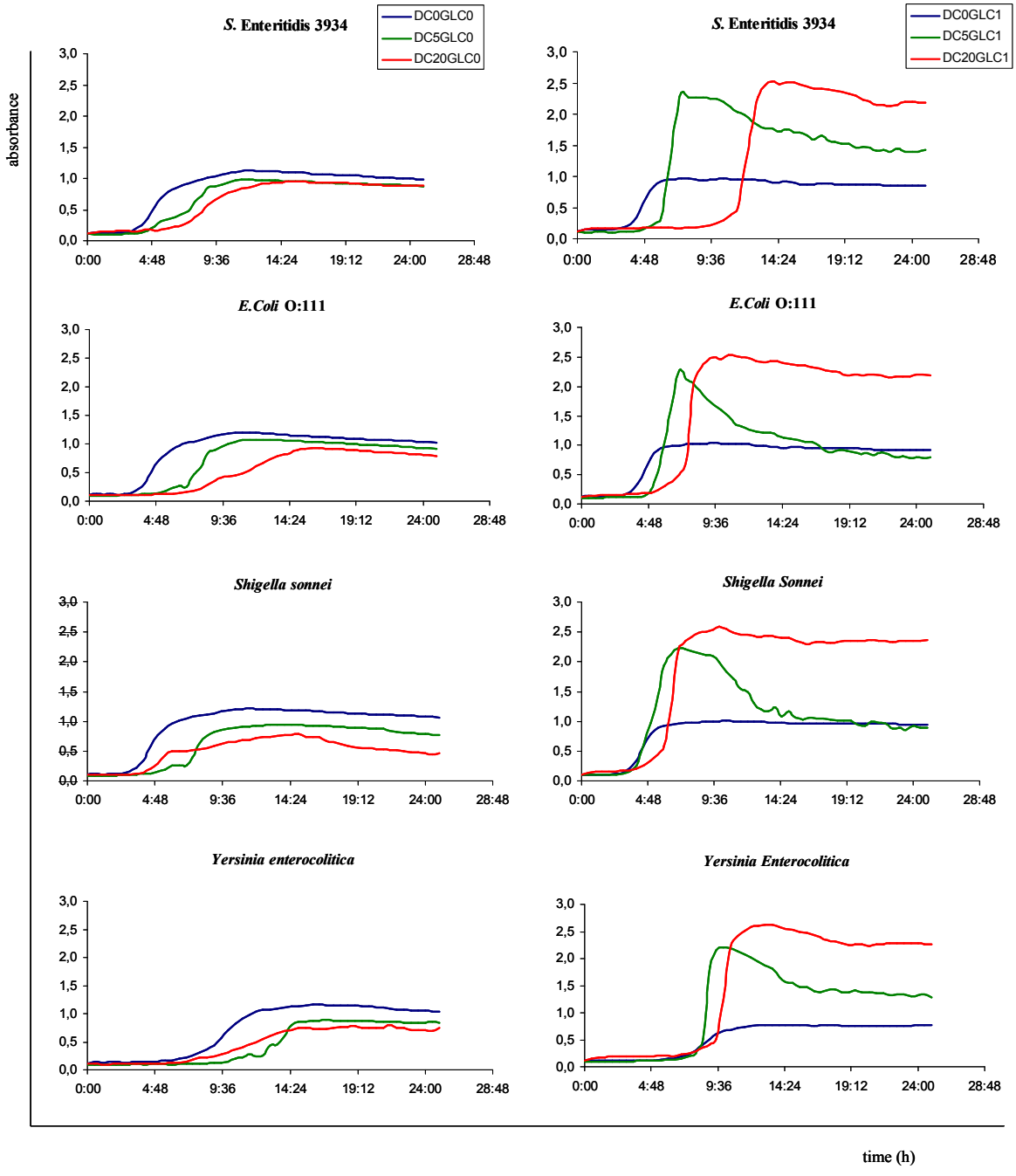


Figure 36: Growth curves of various strains of bacteria influenced by presence of NaDC.

The phenomenon of rapid bacterial growth in the presence of both NaDC and glucose is observed in all bacteria strains used in the experiment. Though this phenomenon is interesting, it is not specific for S. Typhi as was supposed.

4.7 No significant expression differences between single samples by SDS-PAGE and following Silver staining are observable.

Samples were prepared according to the protocol. Bacteria mass for samples was taken from Bioscreen C plate, exact after experiment was stopped.

M. Rainbow marker

C. Extract of LPS (SAO Rv6, Universidad de Navarra)

1. *S.* Typhi 567 in LB
2. *S.* Typhi 567 in LB, 5 mg/ml NaDC
3. *S.* Typhi 567 in LB, 20 mg/ml NaDC
4. *S.* Typhi 567 in LB, 1% glucose
5. *S.* Typhi 567 in LB, 5 mg/ml NaDC, 1% glucose
6. *S.* Typhi 567 in LB, 20 mg/ml NaDC, 1% glucose

SDS-PAGE and Silver staining were done according to the protocol (Fig. 37, 38)

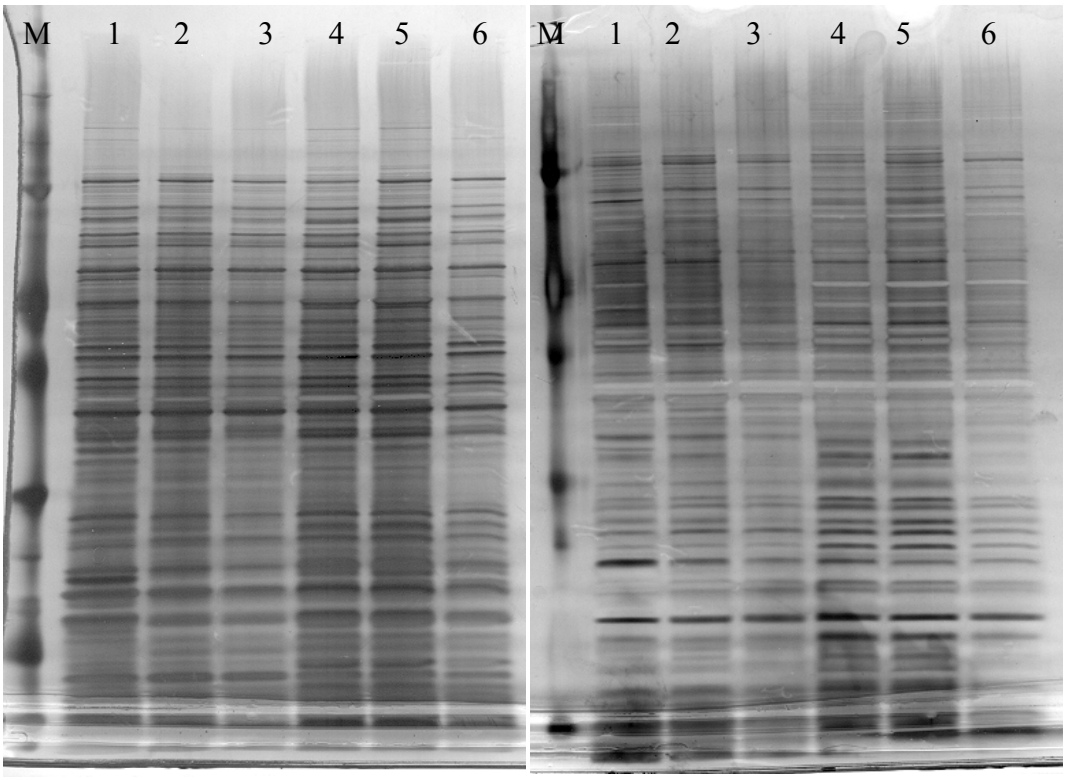


Figure 37: Silver staining, (a) – proteins, (b) – lipopolysaccharides (LPS)

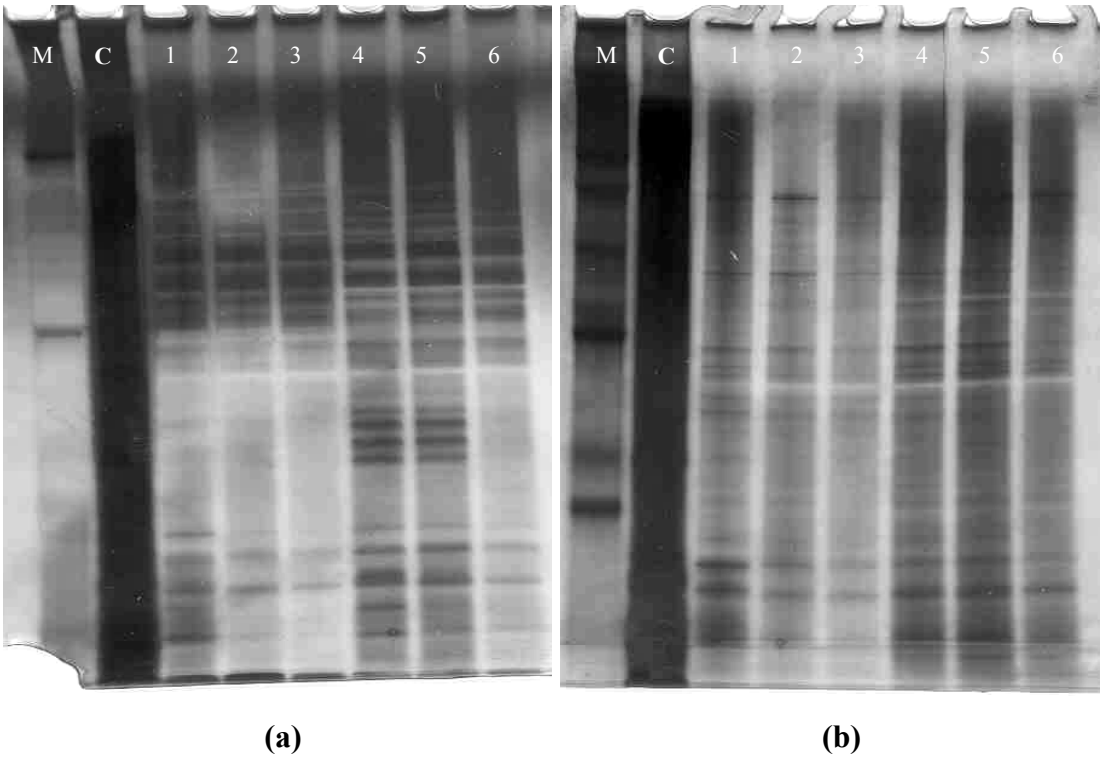


Figure 38: Silver staining, (a) – proteins, (b) – lipopolysaccharides (LPS)

There were observed some variations in proteins and LPS expression between single samples, but there must be other studies done to find out if they are significant or not.

4.8 Comparisons of proteins expression by Immunoblotting do not prove any variations.

Immuno-blotting was running according to the protocol with the same samples and marker, as the Silver staining experiment. The primary antibodies used were:

1. anti-bapA (rabbit, 1:1000, Universidad Publica de Navarra)
2. anti-groel (rabbit, 1:100, Universidad de Navarra)
3. anti-humano (human, 1:50, Universidad de Navarra)

Secondary antibodies used were:

1. anti-conejo-PO (rabbit, 1:1000, GantiIgG(Fc)/PO, Nordic)
2. anti-humano-PO (human, 1:500, GAHu/IgG(H+L)/PO, Nordic)

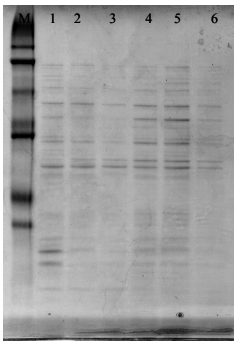


Figure 39: SDS-PAGE gel stained with Coomassie blue, after transblotting.

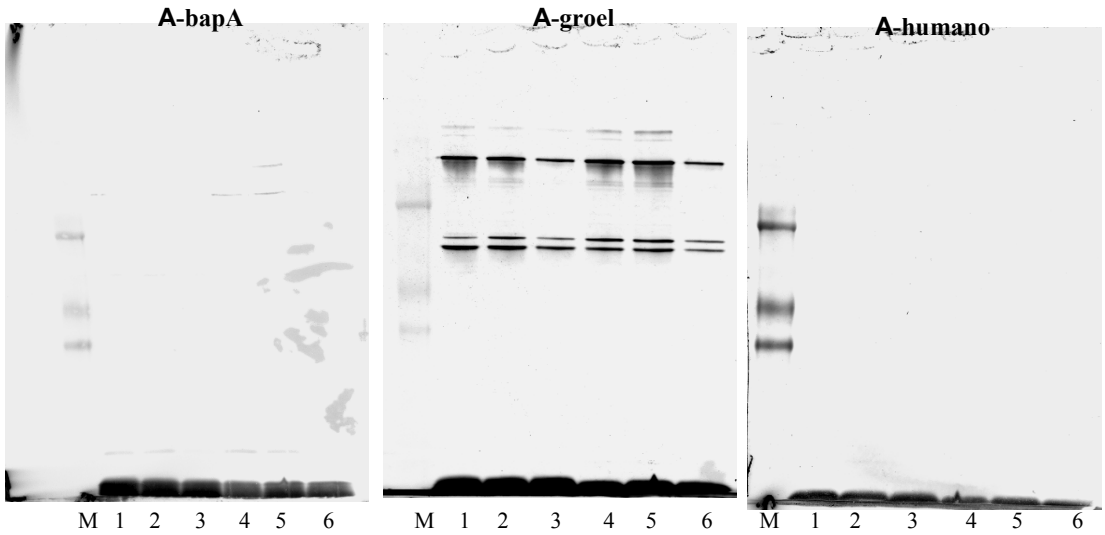


Figure 40: Immuno blotting membranes.

Results were not conclusive, since we were not able to detect significant variations among the different samples (Fig 39, 40).

4.9 Another interesting phenomenon occurs in incubation of S. Typhi, *in vitro*.

During incubation of *S.* Typhi in tubes containing LB and NaDC, another phenomenon was observed. Occasionally, under the unspecified conditions, small aggregations of bacteria were observed in the gel part of medium. These aggregations resembled to small dots or snowflakes (Fig. 41).

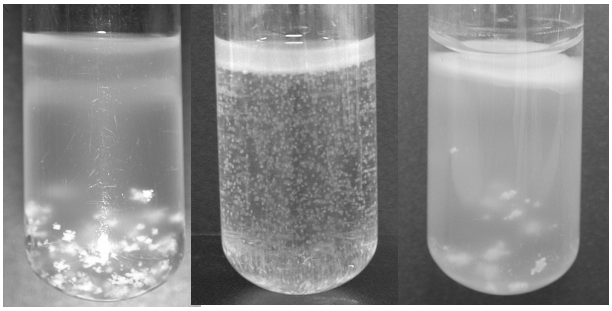


Figure 41: *Salmonella* Typhi in various conditions and eldery of samples, occurrence of aggregation. LB medium, 0.5% NaDC, 37°C, no agitation.

Microscope studies confirmed that they are formed from huge amount of bacteria that are close to each other and have special general conformation (Fig. 42).

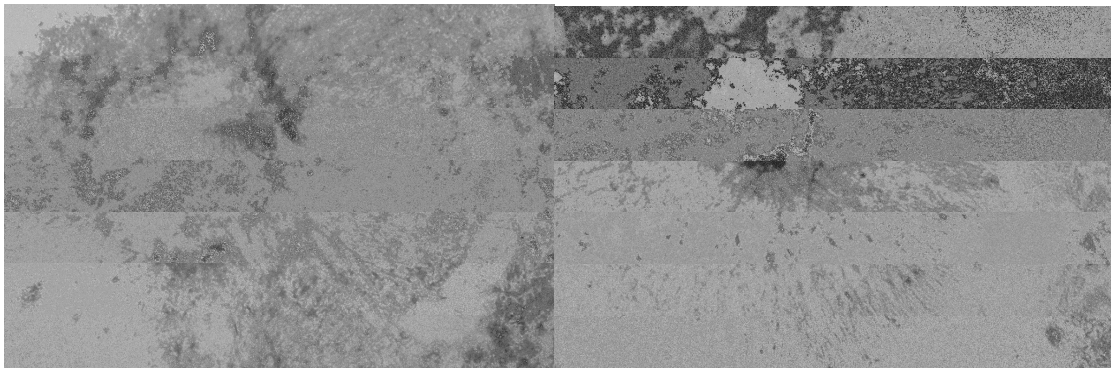


Figure 42: *Salmonella* Typhi, Gram staining of a specific aggregation.

5. DISCUSSION

Microorganisms can exist both as a planctonic independent cells or operate as a part of a complex system with higher level of organisation known as a biofilm. Many important bacterial pathogens produce biofilms during their infective cycle.

Results of this thesis indicate that *S.* Typhi is not able to form biofilm in the classical *in vitro* conditions described for other bacteria (*S.* Enteritidis, LB broth medium). However, we found that *S.* Typhi produced a biofilm in LB broth medium in the presence of NaDC, one of main compound of human bile. This result may be related to the fact that *S.* Typhi is able to produce biofilm on gallstones and only in the presence of bile (Prouty A. M., 2002).

Another interesting result was that apart of forming a biofilm, gel was also observed. It is well known that deoxycholic acid may form a gel in the presence of chemical factors (Mukhopadhyay, *S.* et al., 2004; Subuddhi, U. et al., 2007; Peng Q. et al., 1995). However, it was not described that bacteria may also be able to induce such phenomenon.

NaDC mixed with LB medium turn to gel, with thixotropic character (Mukhopadhyay, S. et al., 2004), stable at room temperature. Rigidity of this gel depends on concentrations of NaDC, generally 0.12% of NaDC forms very soft gel, 0.5% concentration forms rigid gel, it is possible to turn the tube with this gel up-side down and the gel remains on its place. Thixotropic means that it is possible to liquidize this gel by increasing temperature, strong agitation and other ways of supplying energy. At 37°C this system is liquid. After inoculation with *S.* Typhi, in a few hours (about 4, depends on the amount of initial inoculum), gelification appears at 37°C. Medium becomes turbid, because of multiplying bacteria and on the liquid: gas interface, a thin layer of biofilm begins to generate. Gram staining and Alcian blue staining was done to confirm this. In the biofilm area (upper part), a bluish matrix surrounding the bacteria was observed, in the rest of medium (gel), only aggregated cells of *S.* Typhi were observed. Gel formed by *S.* Typhi is resistant to treatment of boiling temperature and proteinase K, and disappears after 2 - 4 days if air circulation is available. Gel passes through microbial filters for DNA isolation (remain gel-like), but does not pass trough 300.000 MWCO filters (changes to liquid).

Biofilm of *S.* Enteritidis is mostly composed of cellulose (Alcian blue positive) (Solano C., 2002). It has not been demonstrated yet that the matrix of *S.* Typhi also contains cellulose. In fact, our experiments with mutants in the biosynthesis of cellulose indicated that these mutants were also able to gelificate the deoxycholic medium.

It was necessary for bacteria to be alive to render this effect. After inoculating samples with dead bacteria, the gel was not formed at 37°C. This leaded to the hypothesis that the gelator factors are compounds produced actively by the bacteria. In order to confirm it, filtered bacterial cultures were used as gel-inducers and positive results were obtained.

After that, those supernatants were treated with heat (100ºC), proteinase K or filtered through special hydrophobic membranes. The results indicated that the gel-inducing factor (or factors) were not proteins, were resistant to boiling and were retained on hydrophobic membranes. Currently, new experiments are being conducted in order to know the nature of the gelating factors.

Finally, although dead bacteria did not induce the gel, the presence of some structures of the bacteria may be acting as stabilizers, since a mutant defective in a large surface protein (BapA) was gel-negative after 3 days of incubation.

Bioscreen method proved that *S.* Typhi is particularly resistant to NaDC. Interestingly, at low concentrations (0.5%) although the lag phase of growth was longer, after the period of adaptation they reached the same level of growth as the untreated cells, or even higher. Higher concentrations (above 10%) were inhibiting bacterial growth. In any case, these bacteria were more resistant than other species (*S.* Enteritidis, *E. coli*, *Yersenia enterocolitica*, *Shigella sonnei*). This result also confirms the fact of particular resistance of *S.* Typhi to bile and supports the hypothesis of the role of NaDC as a „helper“in *S.* Typhi's life cycle in gallbladder. Currently, other experiments are being conducted to investigate this increasing bacterial resistance.

An interesting point to see is the bacterial behaviour in presence of both bile salt and glucose. Bacteria in presence of glucose were growing as expected, but in the presence of both glucose and NaDC, the lag phase is extremely longer and the exponential phase extremely steep, rendering higher growth rates than the no-deoxycholate treated cells. Our hypothesis is that the bacteria sense the stress (conceive suspect) in the presence of NaDC, and rather than decrease growth and multiplying, it starts to use glucose as another source of nourishment which is during normal

conditions idle. Because of the fact, that this phenomenon was not specific for *S.* Typhi and was observed in other bacterial strains too, and also due to time limitation of work period, this phenomenon was not studied in more details.

The studies of gene expression in this thesis were done as a qualitative analysis to compare possible differences between gene expressions of variously treated bacteria. Our results were not significant as we expected, although it was possible to see there some changes, results were not clear enough and thus not quantified. The possible influence of BapA protein in gelification was not confirmed.

Last observation was focused on interesting snowflake-like aggregations, which appear in standardly performed incubation of S. Typhi 567 under the unspecified conditions. These shapes are noticeably similar to formations described in the manuscript of Peng Q. et al., 1995 (Periodic and chaotic precipitation phenomena in bile salt system related to gallstone formation, Fig.8 in Introduction part). Authors claimed the possible relation of these forms due to the precipitation of NaDC with metal ions and the gallstone formation.

6. CONCLUSION

- *S.* Enteritidis 3934 is able to produce a biofilm in LB broth on interface liquid: air after 2 - 3 days at room temperature, which *S.* Typhi is not. *S.* Typhi is able to produce a biofilm in LB broth and in presence of NaDC in 37 °C.

- NaDC gelificates LB broth medium and creates a thixotropic gel, which is stable at room temperature. Living bacteria of *S.* Typhi support this gelification and stabilize gel at 37°C. According to the unspecified obtained results, it is suspected that stabilization of gel is induced by complex activity of S. Typhi.

- Mutant strains of *S.* Typhi 567 also provide efficient conditions to biofilm production and gelification of NaDC in LB broth. Only the mutant ΔbapA :: Km of *Salmonella* Typhi 567 strain appeared to be defective in the ability to form gel, but following experiments did not confirm it.

- Experiments made for gelificate medium at 37°C by physico-chemical factors did not have positive results. It was proved that gelification of NaDC in LB broth is pH-dependent although there was not found any relevance between pH-dependence and the gel stabilization by S. Typhi.

- The resistance of *S.* Typhi in presence of low concentration of NaDC was approved. The influence of NaDC on other tested bacterial strains was inhibiting. Presence of both NaDC and glucose in medium provides interesting conversion in growth curves of bacteria, multiplying is run extremely fast. This phenomenon is not specific for *S.* Typhi.

- Differences in gene expression of samples of bacteria growing under various conditions were not so huge, as we expected. Their significance was not investigated. Mainly, due to the time limitation.

- The results obtained in this study support the hypothesis that *S.* Typhi could be related to the gallstone generation in the gall-bladder environment.

7. SUMMARY IN CZECH

Salmonella Enterica serovar. Typhi je G- tyčka s bičíky ze třídy *Enterobacteriaceae.* Je intracelulárním parazitem a napadá výhradně člověka. Přenos infekce probíhá orofekální cestou a způsobuje tyfovou horečku, která byla jednou z hlavních příčin úmrtí v 19. století. Ačkoli se zlepšením hygienických podmínek počet nemocných rapidně klesl, v rozvojových zemích je dodnes častá a běžná. Choroba se projevuje vysokou horečkou a střevními potížemi. Zácpa a průjmy jsou zastoupeny ve stejné míře. Charakteristické jsou také rudé skvrny na kůži, ze kterých je možné bakterii kultivovat. Jako prevenční opatření se doporučuje vyvarovat se nedokonale připraveným pokrmům v zemích s vysokým výskytem nemoci. Nejlepším prevenčním prostředkem je však vakcinace, a to nejméně jeden týden před předpokládaným pobytem v rizikovém přostředí. 3-5% nakažených zůstane přenašeči bakterie.

Deoxycholát sodný je jednou z hlavních žlučových solí a významně se podílí na hlavní funkci žluči, což je trávení tuků. Žluč má také micelizační schopnost a nespecifický antibakteriální účinek. Je skladována ve žlučníku. Při nerovnováze obsažených látek mohou ve žlučníku vzniknout pevné abnormality - žlučníkové kameny (cholelythiasis). V dnešní době je velmi diskutovaná také možnost vzniku kamenů v důsledku přítomností bakterií. Biofilm je hmota vytvářená bakterií na mezifázi dvou skupenství, je strukturně velmi různorodý a poskytuje bakterii ochranu před vnějšími vlivy. Může způsobit zvýšení rezistence vůči léčbě, např. antibiotiky.

Výchozím podkladem pro tuto práci byl fakt, že *Salmonella* Typhi vytváří za přítomnosti žluči biofilm na žlučníkových kamenech, tím se stává rezistentní k léčbě a onemocnění a přechází do chronicity (přenašečství). Dosud jediným řešení tohoto jevu je chirurgické vynětí žlučníkových kamenů. Hlavním cílem práce je posoudit úlohu deoxycholátu sodného (NaDC) na tomto jevu a studium ostatních jevů inkubace *S.* Typhi v přítomnosti této soli, *in vitro*.

S. Typhi byla inkubována ve skleněných zkumavkách v kapalném médiu Luria-Bretani a vystavena různým inkubačním podmínkám, zejména za přítomnosti NaDC

v médiu. Dalšími metodami využívanými při práci byla metoda Bioscreen C, která spolehlivě stanovuje růstové křivky bakterie měřením měnící se absorbance média v čase. Metody SDS-PAGE a Immunoblotting byly užity pro stanovení změn v expresi proteinů a lipopolysacharidů u bakterií vystavených různým inkubačním podmínkám.

Podařilo se potvrdit, že NaDC je nezbytně nutný pro tvorbu biofilmu *S.* Typhi, *in vitro*. Pokud NaDC není obsažen, bakterie se množí (zvyšuje se zakalení ve zkumavce), ale biofilm se nevytváří.

Během pokusů byl zaznamenán zajímavý jev, NaDC je schopný gelifikovat kapalné živné médium. Tento gel je tixotropního charakteru, je stabilní v pokojové teplotě, ale v teplotě 37°C (teplotě lidského těla) se mění opět v kapalinu. Ovšem po naočkování vzorku *S.* Typhi nejenže dochází ke tvorbě biofilmu, ale také ke vzniku gelu stabilního ve 37°C. Ústředním tématem práce se tedy stal tento jev, neboť podporuje domněnku o vzniku abnormalit ve žlučníku vlivem přítomnosti bakterie.

Gel byl podroben mnoha zkouškám, prokázalo se například, že samotná přítomnost bakterie není nutná, neboť pevný gel se vytvoří i po přidání soli do filtrátu média, ve kterém byla bakterie inkubována. Bohužel se nepodařilo odhalit mechanismus stabilizace gelu vlivem metabolitů bakterie, proto se domníváme, že tento jev je způsoben komplexním působením metabolitů.

Zajímavým jevem je též vznik drobných vločkovitých sraženin ve zgelovatělém médiu při inkubaci *S.* Typhi. Morfologické zkoumání prokázalo, že tyto sraženiny jsou vytvořené obrovským množstvím bakteriálních buněk, které jsou navzájem v těsné blízkosti. Tyto sraženiny mají dokonce vyšší paprsčitou konformaci (viz obr.42). Po prostudování vědeckých článků byl tento jev shledán velice podobný s jevem popisujícím vznik sraženin NaDC s ionty některých kovů, vědci zde potvrzují možnost vzniku žlučníkových kamenů z tohoto komplexu. Tento poznatek podporuje hypotézu o *S.* Typhi jako potencionální příčině vzniku žlučníkových kamenů.

8. ABBREVIATIONS

AIDS – acquired immune deficiency

BapA (biofilm associated protein A) – a protein required to biofilm formation

cfu – colony forming unit

CMC - critical micelle concentration

$CoCl_2$ - cobalt dichloride

cyclic AMP – cyclic aminophosphate

E. coli – Escherichia coli

EDTA – sodium ethylendiamine tetraacetate

EPS - extracellular polymeric substance

G- - gram negative

ID_{50} - the minimum infectious dose, which affect 50 respondents

LB – Luria Bretani broth

LPS – lipopolysaccharides

M cells - microfold enterocytes

NaDC - sodium deoxycholate

OD – optical density

RT – room temperature

S. Enteritidis – *Salmonella enterica* serovar. Enteritidis

S. Paratyphi - *Salmonella enterica* serovar. Paratyphi

S. Typhi – *Salmonella enterica* serovar. Typhi

S. Typhimirium - *Salmonella enterica* serovar. Typhimirium

SDS-PAGE - sodium dodecylsulphate-polyacrylamide gel electrophoresis

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