BRUCELLOSIS EPIDEMIOLOGY, VIRULENCE FACTORS, CONTROL AND MOLECULAR TARGETS TO PREVENT BACTERIAL INFECTIOUS DISEASES

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Brucellosis Epidemiology, virulence factors, control and molecular targets to prevent bacterial infectious diseases

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MASTER OF SCIENCE

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ABSTRACT

Brucellosis is a bacterial zoonosis that infects both professional phagocytic and non-phagocytic cells in the hosts. *Brucella* intracellular survival is important for its virulence. In a study to establish the seroprevalence and risk factors of brucellosis in livestock in Kazo and Buremba sub-counties of Kiruhura district, Uganda, fifty goat and 112 bovine serum samples were tested for *Brucella* antibodies. The prevalence of *Brucella* antibodies in goats and cattle was 26.0% and 38.4% respectively, while individual seroprevalence rates by livestock breeds were 10.7% (cross-breed goats), 45.5% (local goat breeds), 49.1% (cross-breed cattle), 31.0% (local cattle breeds), and 17.4% (exotic cattle breeds) ($p = 0.001$). Sharing of watering points, using surface water for livestock, presence of wildlife on pasture, lack of vaccination was significantly correlated with *Brucella* seropositivity in livestock.

The molecular study on biofilm in *Escherichia coli* included in this paper revealed that *pflA* knock out mutations had a significant effect on biofilm amounts when biofilms formed on D-serine and acetate. The *ldhA* formed generally high bacterial biofilm amounts on all carbon sources as compared to the wild type.

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DEDICATION

I dedicate this work first and foremost to God, my parents Mr. Patrick Kiwoomya and Merab Nalweyiso, my relatives and siblings.

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1. INTRODUCTION

Brucellosis is one of the most common bacterial zoonoses worldwide and it poses a major threat to human health, animal health, and animal production (48). Brucellosis is caused by *Brucella* species, which is a facultative intracellular gram-negative bacterial pathogen of many vertebrate species including man.

Brucella are small, aerobic, cocobacilli, non-motile, and do not produce spores. The different *Brucella* spp. includes *Brucella melitensis*, *Brucella abortus*, *Brucella canis* and *Brucella suis* and their preferential hosts are sheep/goats, cattle/bison, dogs, and pigs, respectively. However, cross-species infections can occur; for example, cattle can be affected by both *B*. *arbotus* and *B*. *melitensis* at the same time (1). *Brucella neotomae* which affects desert rat is not associated with human infections, *Brucella pinnipedialis* and *Brucella ceti* that were recently isolated from marine animals can also cause disease in humans (54). *Brucella melitensis* is the most virulent and most widely encountered of all the species (7). The *Brucella* bacteria are capable of invading and surviving in phagocytic and none phagocytic cells (14).

In humans, Brucellosis is life threatening and presents with nonspecific symptoms, including intermittent fever, weight loss, depression, hepatomegaly, and splenomegaly. Arthritis, spondylitis, osteomyelitis, epididymitis, and orchitis, as well as other more severe complications such as neurobrucellosis, liver abscesses, and endocarditis, are common in some patients (7). Neurobrucellosis in humans can occur in the form of meningitis and meningoencephalitis. In domestic animals, *Brucella* infects the reticuloendothelial system and genital organs causing chronic infection and abortion (especially in the last trimester), stillbirth, and infertility, which significantly affect farmers economically due to loss of production. Epididymo-ochitis is

common in males, and the females that have aborted show necropulurent placentitis and endometrititis. Lesions in the placenta cause edema of the chorionic stroma and multifocal necrosis of allantochorion. These lesions are cardinal in the induction of abortion and eventual infertility. This is accompanied by large accumulation of neutrophils and degenerate leukocytes. In addition, large number of tiny gram-positive cocobacilli *Brucella* can be seen in trophoblasts (7).

The consumption of contaminated dairy products has been widely documented as an important route of *Brucella* transmission. In particular, unpasteurized dairy products from infected animals have been considered a source of infection for the general population, especially in developing countries where disease control infrastructure is limited (20). Contaminated carcasses have been cited as a major source of infection for workers in the meat packing industry (20). Veterinarians have been reported to acquire brucellosis from assisting births in infected livestock, as well as accidental exposure to live vaccines (19). Contact with contaminated products of aborted animals has been shown to significantly influence the transmission of brucellosis to humans (19) while airborne transmission of bacteria to humans has also been documented in clinical laboratories and abattoirs (6). Brucellosis has been eradicated in most developed countries that have implemented a tight control program like test and slaughter (36). The increase in business and leisure travel to brucellosis endemic countries has led to importation of the disease into non-endemic areas.

The epidemiology of the Brucellosis in livestock and humans is poorly understood (33). Although the disease has a worldwide geographical distribution, it remains a major public health problem in Mediterranean region, western Asia, Africa and Latin America. Brucellosis remains

widespread in the livestock populations and presents a great economic and public health problem in African countries (18). In the sub-Saharan Africa for example, the average seroprevalence of brucellosis in cattle populations varies from 10% to 16%. Previously, a seroprevalence of 15.8% and 10.3% were reported in the southwestern and western Uganda respectively (18). The high seroprevalence of brucellosis in some parts of the country (Uganda) in cattle and goats might be attributed to limited and poorly funded animal disease surveillance systems in the country including but not limited to lack of diagnostic laboratories. A bovine brucellosis seroprevalence of 41.0% has been reported in Togo (16) another Sub-Saharan tropical country with rudimentary animal disease control systems not dissimilar to Uganda. Lack of a robust surveillance system makes it hard to recognize presence of the disease in animals further augmenting the threat brucellosis poses to other animals, farmers, veterinarians and humans (22).

2. OBJECTIVES OF THE MS PAPER

I. To summarize epidemiological data on Brucellosis in Uganda

II. To review the major virulence factors of *Brucella*

III. To review current control and prevention strategies of Brucellosis

IV. To present experimental data on *E.coli* as a model that has the ultimate goal of developing novel prevention techniques of bacterial pathogens

2.1. Epidemiologic research work on brucellosis in Uganda

2.1.1. Study area and design

This was a creative project (MICR 794) to shed further light into the poorly understood epidemiology of brucellosis and identify novel risk factors that may be of help to the Uganda farmers. As one specific example, we determined the seroprevalence of brucellosis, risk factors in cattle and goats in Kiruhura District, which is one of the districts in the *"cattle corridor"* in the western region of Uganda (Fig. 1). The study based on screening 10 herds of cattle and goats in Kazo and Buremba sub-counties. Herds which had 10-30, 30-100 and >100 animals were categorized as "small", "medium" and "large" respectively. Five herds were selected from each sub-county; two large, two medium, and one small herd were studied. Five, 10 and 15 milking cows from small, medium and large herds, respectively, were selected and a uniform number of five goats selected from each herd. A total of 50 goats and 112 cattle were sampled from the ten herds. A structured questionnaire was administered to the owner of each herd for assessing the risk factors that influence the occurrence of the disease in each herd. A total of 10 questionnaires were administered; that is one questionnaire per selected herd

Fig.1. Map of Uganda showing the study area (Arrow) **(**writersagency.blogspot.com**)**

2.1.2. Sample collection, handling, and testing

Approximately 8ml of blood was collected from the jugular vein of each animal in the selected herds using plain vacutainer tubes without anticoagulant (Becton Dickson, UK). Each sample was labeled using codes describing each animal and herd. Each tube was then tilted on a table at room temperature (rT) to allow clotting, and then centrifuged to obtain clear serum. The serum was tested immediately with Rose Bengal Plate Test (RBPT) in a laboratory in Kazo Sub County in Kiruhura district and the remainder stored at -20°C. This was done according to the procedure recommended by *Organization Mondial de la SanteAnimale* (OIE). Briefly, 30µl of RBPT antigen and 30µl of serum were placed on a plate and thoroughly mixed. The plate was rocked for 4 min. The degree of agglutination for each test sample was compared to the positive and negative controls that were part of the RBPT test kit. The statistical analysis of the data was done with SPSS (statistical package for social scientists) Version 17.A chi square test was done to compare the prevalence of brucellosis (in percent) between different counties (analysis 1) and different breeds (analysis 2). The difference was considered statistically significant if the *p*-value was < 0.05.

2.1.3. Analysis 1:Seroprevalence of brucellosis in goats and cattle

The overall prevalence of *Brucella* antibodies among the 50 goats was 26.0% (13/50) with Kazo showing a slightly higher seroprevalence [28.0% (7/25)] than Buremba sub-county [24.0% (6/25)]; the difference in the seroprevalence of brucellosis between the two sub counties was not statistically significant (p=0.41). Of the 112 bovine serum samples, 38.4% (43/112) tested positive for *Brucella* antibodies. There was no statistically significant difference in seroprevalance between the two counties (p=0.34).

2.1.4. Analysis 2:Seroprevalence of brucellosis among the different goat and cattle breeds

The cross-bred goats and local goat breeds showed a statistically significant (p=0.009) difference in brucellosis seroprevalence of 10.7% and 45.5%, respectively. Among the different cattle breeds, the mixed breeds or "crosses" had a seroprevalence of 49.1%, *Bos indicus* or local cattle breeds 31.0%, and *Bos taurus* (exotic) breeds 17.4%. These differences were statistically significant with a p-value of 0.001.

2.1.5. Questionnaire results

Factor	Seroprevalence (%)	p-value
Raise their own stock	39.3	0.387
Do not raise their stock	20.0	
Vaccinate	27.3	0.004*
Do not vaccinate	54.3	
Had abortion on farm	33.0	0.314
No abortion	42.6	

Table 1. The role of different risk factors in the seroprevalence of bovine brucellosis in Kiruhura district, western Uganda. A variable highlighted in asterisk (vaccination) was statistically significant.

Factor	Brucellosis prevalence	*p*-value
Abortion on farm	14.6	0.186
No abortion on farm	50.0	
Graze with wildlife	34.3	0.041*
Don't graze	6.0	
Use open surface water for goats	36	0.009*
Do not use it	0.0	
Share water with others	30.0	0.000*
Do not share	13.3	
Raise own stock	84.0	0.452
Get elsewhere sometimes	91.8	
Vaccinate	7.1	0.049*
Do not vaccinate	33.3	
Seek vet services	26.6	0.747
Do not seek	20.0	

Table 2. The role of different risk factors in the seroprevalence of caprine brucellosis in Kiruhura district, western Uganda. Grazing with wildlife, use of surface water and sharing of water were statistically significant (In asterisk).

The answers to the questions could help to direct us towards the factors that influence the presence of brucellosis in the herds. For cattle, vaccination was the only factor that yielded a statistically different seroprevalence between vaccinated and non-vaccinated animals. For goats,

the statistically significant risk factors were grazing with wild life, use of open surface water, sharing of water with other herds and vaccination.

The overall seroprevalence rates of *Brucella* antibodies in cattle (38.4%) and goats (26.0%) in Kiruhura district further corroborate previous reports on brucellosis in Ugandan livestock (23,27,37). A 38.4% seroprevalence rate suggests that cattle may be playing an important role in the epidemiology of brucellosis in the district. The seroprevalence of bovine brucellosis was higher among the cross-breeds (49.1%) than the local (31. %) and the exotic (17.4%) breeds (Figure 1) ($p<0.05$). The lower seroprevalence among the "exotics" could be attributed to better disease management. However, this needs to be elucidated with a bigger sample size. In goats, the seroprevalence was significantly higher ($p<0.05$) among local breeds (45.5%) versus cross-breeds (10.7%).

Seventy five per cent of the respondents did not vaccinate their animals against brucellosis (Table 1 and 2). This result is not surprising since a poor vaccination practice is an important brucellosis risk factor (31). Sharing of surface water, mixed farming, and presence of wild animals on the pastures were also significantly associated with seropositivity. Mixed farming and wild life have been associated with increases in risk to brucellosis (23, 39). Future studies based on high sample size and more sensitive tests may corroborate these findings.

In conclusion, Brucellosis is still a big challenge in the two sub counties of Kazo and Buremba with an overall seroprevalence of 38.4% and 26% in cows and goats respectively using RBPT. Lack of vaccination, presence of wild animals on the grazing land, mixed farming and use of surface water for cattle and goats have been noted as some factors that influence the presence of the disease in the area. Therefore, reducing the burden of brucellosis in the area

requires combined effort between the government, area veterinarians, wildlife conservationists and intensive sensitization of the farmers. Seroprevalence of the disease in the wild animals needs to be evaluated.

2.2. Major virulence factors that contribute to brucellosis pathogenicity

The high occurrence of brucellosis in Kiruhura in Uganda indicates a need to develop novel prevention and treatment techniques for the disease. In order to successfully control Brucellosis, an understanding of the virulence factors that contribute to disease is needed. *Brucella* have a predilection for macrophages, dendritic cells (DCs) and trophoblasts (6). The bacteria can enter, survive, and replicate within these cells and cause disease (15). *Brucella* gain access to the host through inhalation, conjunctiva, skin abrasions and ingestion (52). In the gastrointestinal tract, the organisms are engulfed by lympho-epithelial cells of gut associated lymphoid tissue and gain access to the submucosa. The pathogens are later ingested by polymorphonuclear leukocytes and macrophages (2). The ingested bacteria are transported to lymphoid tissue draining the infection site, and may finally localize in lymph nodes, liver, spleen, mammary gland, bone marrow and reproductive tract (54). According to Seleem et al, 2008, *Brucella*, as compared to other bacteria lack the classical virulence factors like exotoxins, fimbria, capsules, plasmids, lysogenic phages, drug resistant forms, antigenic variation and endotoxic lipopolysacharide. Brucella pathogenesis is governed by their ability to invade the host cell, intracellular survival and evasion of the immune system. Some of the virulence factors (lipopolysaccharide, two-component system, type 4 secretion system and cyclic β 1, 2 glucans (CβP) responsible for these mechanisms are reviewed in this paper.

In macrophages, *Brucella* may inhibit fusion of phagosomes and lysosomes and replicate in the phagosomes (54). In a few cases some bacteria are destroyed by the host cell bactericidal action of free radicals of oxygen, and lysosomal enzymes (54). However, a certain number of bacteria resist these bactericidal effects and can still replicate within the cells. This results into destruction of the host cells and ultimate infection of other cells and dissemination to reproductive tract and other organs (2). On the other hand, *Brucella* can also replicate in host tissues leading to granuloma formation and caseous necrosis. Trophoblasts are non-phagocytic cells that are key targets for *Brucella* infection during the late phase of gestation in ruminants (53). Growth of the bacteria inside trophoblasts is enhanced due the presence of high concentration of steroid hormones and erythritol during the final third of gestation. This compromises the integrity of the placenta and finally infection of the fetus ensues resulting in abortion and stillbirth. These intracellular pathogens use different mechanisms to survive and replicate in the intracellular environment and evasion of the host immune system. Intracellular survival and immune evasion underlie the pathogenesis of the disease.

2.2.1. Two-component systems

For successful establishment of infection, *Brucella* must gain entry into the host cell (30). *Brucella* has a two-component BvrR/BvrS gene regulation system that acts through a cascade of phosphorylation to modulate bacterial gene expression (20). This system is believed to be involved in modulation of binding to and penetration of the host cell. BvrR is a response regulator protein whereas BvrS is a sensor protein with histidine- kinase activity. This regulator system is required for recruitment of GTPase and actin filaments and for maintaining the integrity of the bacterial outer membrane (30, 54). It is postulated that this system modulates the

outer membrane, which is necessary for binding, cell invasion and resistance to lethal cationic peptides (52). This system has a significant effect on the expression of the surface proteins Omp25 and Omp22. It is believed that expression of such surface proteins allows *Brucella* to bind to and penetrate host cells, while escaping from the lysosomal pathway. Mutants that are defective in this system have impaired cellular penetration and increased destruction by phagolysosomes.

2.2.2. Lipopolysaccharide (LPS)

Brucella bacteria like other gram-negative bacteria have lipopolysaccharide (LPS) (Fig. 2), which is a virulence factor essential for the functional and structural integrity of the outer membrane (12). The LPS in *Brucella* is unique relative to that present in Enterobacteriaceae such as *Escherichia coli* and it has been identified as a major virulence determinant. The LPS phenotype of Brucella species is either smooth or rough if they posses or lack the surface exposed O-polysaccharides (O-PS) chain respectively. The O-PS plays a major role in virulence associated with smooth LPS (S-LPS) in that mutant smooth strains fail to survive in macrophages (20,54). The LPS is smooth in *B.melitensis, B.abortus and B.suis* and rough in *B. canis*. *Brucella* have a unique ability to inhibit phagosome maturation through engagement of S-LPS which inhibits the phagosome–lysosome fusion although the exact mechanism how the inhibition is achieved is not yet elucidated (13,41). Formation of the phagolysosme is paramount in the killing of engulfed bacteria. In addition, S-LPS confers resistance to nitric oxide, free radicals and lysozyme, which are important antimicrobial mechanisms of macrophages and neutrophils (19). Further still, smooth LPS prevent the synthesis of immune mediators and have less potential to induce host release of inflammatory cytokines. This is due to its failure to be

detected by Toll Like Receptors (pathogen recognition receptors) of the innate immune system because of its low endotoxic properties (28). Through this mechanism, it prevents stimulation of the innate immune system that would otherwise facilitate the killing of the pathogens. The LPS is postulated to alter the capacity of infected cell to present foreign antigens to CD4+ T cells, hence preventing attack and killing of infected cell by the immune system (21,29). As mentioned, macrophages and dendritic cells are some of the target cells, which are well known

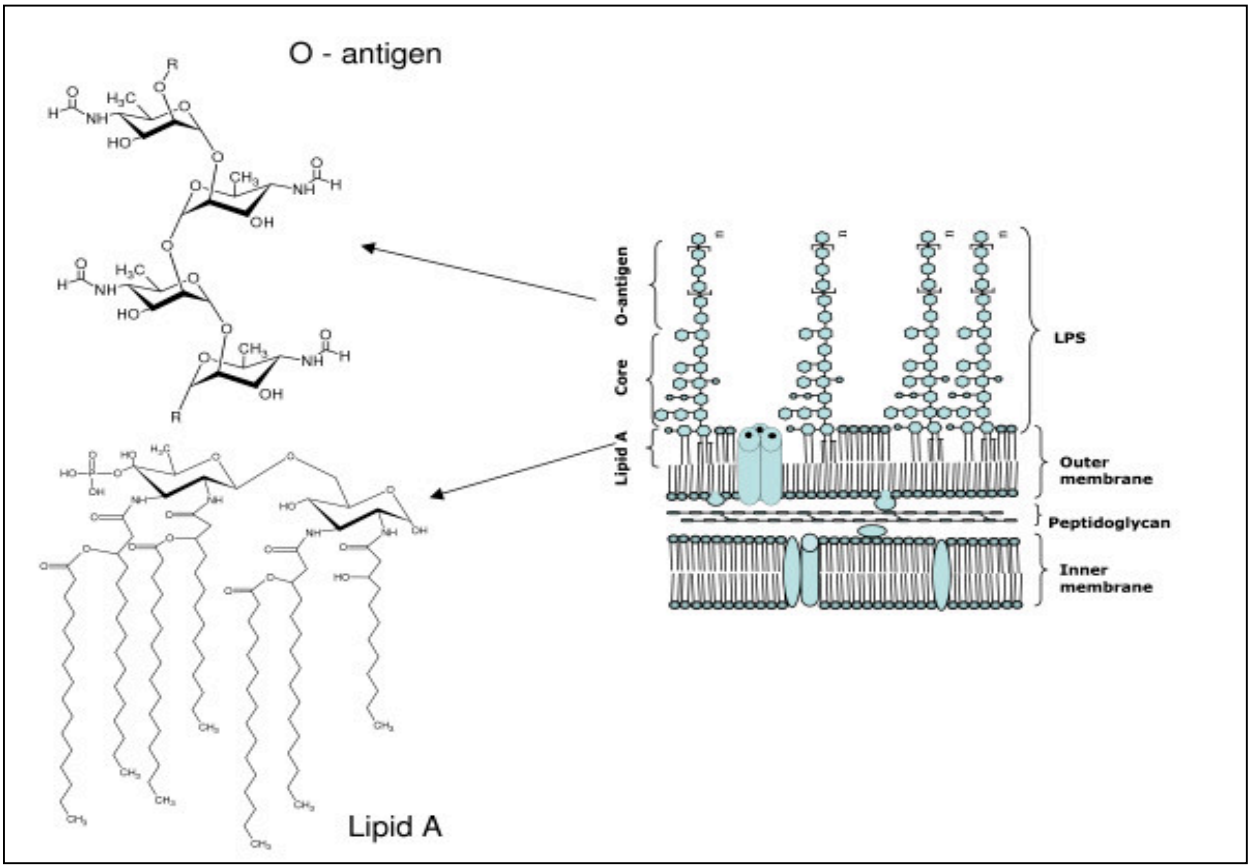

Fig. 2. Schematic drawing of the *Brucella* lipopolysaccharide (LPS)

for antigen presentation. In the same manner, smooth LPS is involved in the inhibition of apoptosis. Resistance to apoptosis of infected cells has been seen in patients with acute and chronic disease. Further still, *Brucellae* do not activate the alternative complement system and

have relatively low endotoxicity. This makes them further poor inducers of some inflammatory cytokines such as tumor necrosis factor (TNF) and interferons (54). Interferon gamma activates macrophages to enhance killing of internalized bacteria (41). The intracellular lifestyle makes the bacteria evade the immune system (antibodies) in the extracellular milieu.

2.2.3. Cyclic β 1, 2 glucans (CβP)

In *Brucella*, the cyclic β 1, 2 glucans (CβP) is produced by cyclic β 1-2 glucan synthase encoded by *cgs* gene (25). Glucans are constituents of the bacterial periplasm with osmoregulatory and cholesterol sequestering activity and are required for survival of the bacteria in phagocytic and non-phagocytic cells. Glucans of *Brucella* prevent phagosome maturation by interfering with lipid rafts and eventually altering protein expression in vacuolar membrane and excluding lysosomal proteins from *Brucella* containing vacuole (4). *Brucella* pathogens avoid fusion of the *Brucella* containing vacuole with lysozomes in the macrophages by use of cyclic β 1, 2 glucans (4). Studies by Arellano- Retnoso et al, 2005 indicated inability to prevent phagosome-lysosome fusion among the CβP deficient mutants and replication was abrogated. This suggests that CβP is one of the *Brucella* virulence factors, which interferes with lipid rafts on host cells and contributes to pathogen survival.

2.2.4. Type 4-secretion system

According to Franco et al, 2007, a type IV secretory system is defined as system that is responsible for the selective transport of proteins and other macromolecules across membranes in some pathogens (*Helicobacter pylori*, *Bordetella pertussis*, *Legionella pneumophilia*). The molecules transported could be classical virulence factors such as toxins that are inoculated into the blood stream or into the host cells (15). The *Brucella* type 4 secretion system (T4SS)

encoded by the VirB1-VirB12 genes is responsible partly for the bacterial intracellular growth in phagocytic and non-phagocytic cells (15). It transports bacterial effector proteins into the host cells; the effector molecules play a role in trafficking of the *Brucella* containing vacuoles towards the replication site (20). A VirB mutant strain of *B. abortus* is not capable of invading trophoblast *in vivo* (11).

In conclusion, various virulence factors play a significant role for the intracellular survival of *Brucella*, this underlies the pathogenesis of the disease. Research is needed to design control strategies targeting these virulence factors especially the smooth LPS.

2.3. Prevention and treatment techniques for Brucellosis

Brucellosis is the most common zoonosis worldwide with over 500,000 cases every year (50). In general, prevention of brucellosis like any other zoonoses begins by elimination of the pathogens in animals. The following methods are crucial in the control of zoonoses (i) maintenance of occupational hygiene among the veterinarians and herdsmen and health education (ii) test and slaughter though this might not be applicable in developing countries (iii) vaccination of animals (38). Limited vaccination in particular was determined a major risk factor for brucellosis in cattle by our own study. In addition, vaccination is also considered the most economical. In shoats (sheep and goats) and cattle, the *B. melitensis* strain Rev1 vaccine and *B. arbotus* strain 19 are used, respectively. Vaccination increases immunity to infection, thus minimizing the risk of abortion and spread of the infection. Another important control strategy is improving the quality of veterinary services and implementing appropriate diagnostic services. This includes standardization of quality control of diagnostic kits/ reagents and vaccines (40). Further more, sero-surveillance of infection in animals is important. Identification and reporting

of sick animals is necessary for risk analysis and monitoring of control programs. The surveillance and reporting system should include both domestic and wild animals (45). Human brucellosis is a severely debilitating disease that requires prolonged treatment with a combination of antimicrobial (Doxycycline and rivampicin) agents and often leaves permanent and disabling sequel, considerable medical expenses in addition to diminished income due to loss of working hours.

2.4. Biofilms as a drug target mechanism to combat bacterial infectious disease

Current treatments of bacterial infectious diseases, such as most antibiotics, aim at reducing the growth rate of the bacteria, which puts them under selective pressure and induce resistance. A new generation of treatment techniques is currently being researched which aims at interfering with the signal transduction pathways in the bacteria to impact certain cellular processes. These treatments will decrease the selective pressure on the bacteria and reduce the occurrence of resistance. One such mechanism is quorum sensing, a mechanism of cell-to-cell communication between bacteria that form a biofilm. A second mechanism, by means of two-component signaling systems, which receive their environmental signals from acetate metabolism, is being investigated in the laboratory of Dr. Pruess in the model organism *Escherichia coli* K-12.

Biofilms complicate the disease progression and contributes to bacterial resistance towards antibiotics and disinfection. Biofilms are bacterial communities that form a slime layer on liquid/solid and air/liquid interfaces and are enclosed in a polymeric matrix. Biofilm formation is of public health importance in that they can form on teeth (56), medical implants (32), gastrointestinal tract, (24) and catheters that act as source of infection. Progress of many

bacterial infections from acute to chronic is often associated with biofilms (3). It is estimated that 99% of all bacteria can form biofilms and over 65% of human microbial infections involve biofilms (42). In *Brucella*, biofilm formation has been suspected to occur and contribute to the disease progression (50).

2.5. *Escherichia coli* as a model organism to understand bacterial biofilms

The formation of biofilms was first studied in *Pseudomonas aeruginosa* and later in *E. coli*. In both bacteria, the transition from the planktonic form of life into a community is a multistep process mediated by bacterial surface appendages (46). In *E. coli,* the first step in biofilm formation, reversible attachment is mediated by flagella. The first step is followed by irreversible attachment, in which the bacteria firmly adhere to surfaces. This is the function of the type 1 fimbriae and curli and is also characterized by the loss of flagella. The third step (maturation phase) involves production of an extracellular polymeric matrix, made up of a complexity of exopolysaccharides, including colanic acid. The matrix allows the biofilm to form a three dimensional structure. The last step in biofilm formation is the dispersion of the flagellated bacteria, which will then colonize other niches (51). At a molecular level, quorum sensing (cell to cell communication) has been studied as a drug target mechanism, aiming at the prevention of biofilm formation in *E. coli* (44).

Modern approaches to develop novel prevention techniques of biofilm associated infectious disease are based on a thorough understanding of the environmental conditions and genetic control that are responsible for biofilm formation. Early biofilm research implicated nutrient changes, PH, oxygen concentration, and osmolarity to trigger biofilm formation (34). In a recent study (43), temperature and nutrients were ranked high to favor biofilm formation. The

same study revealed that carbon sources metabolized to acetyl-coA and acetyl phosphate supported high biofilm biomass. This suggests that acetate metabolism could contribute to biofilm formation. The hypothesis of the second study was that enzymes involved in acetate metabolism might have an effect on biofilm formation on certain carbon sources. The ultimate goal of this research is to identify small molecules that inhibit biofilm formation through acetate metabolism.

As the next step towards this goal, the second MICR794 creative project compared biofilm amounts between an *E. coli* K-12 strain and several mutants in acetate metabolism. This was done with bacteria that were grown on different carbon sources. The *ldhA* gene encodes a fermentative enzyme, lactate dehydrogenase that is responsible for the conversion of pyruvate to lactate. *pflA* and *pflB* encode pyruvate formate lyase, the enzyme that converts pyruvate to formic acid and acetyl Co-A (35,55). The objective of this creative project was to determine the effect of these enzymes on biofilm amounts that formed in the presence of different carbon sources.

2.5.1. Acetic acid and D-serine permitted the formation of reduced biofilm amounts in the *pflA* mutants while, *ldhA* mutants formed significantly higher biofilm amounts on all carbon sources**.**

For this creative project, we tested the ability to form biofilm by the *E. coli*K-12 strain AJW678 and several mutants in acetate metabolism on numerous carbon sources. AJW678 is characterized by its high ability to form biofilm (26). Mutants in *ldhA, pflA,* and *pflB* were isogenic to their AJW678 parent. BP1299 (AJW678 *ldhA*) was cultured on Phenotype MicroArray^{TM}(PM)plates in order to determine biofilm amounts formed by this strain in the presence of different carbon sources. The PM technology from Biolog (Hayward, CA) consists

of 96 well plates in which a single nutrient is dried onto the bottom of each well. When used in combination with a tetrazolium dye, respiration can be monitored which is indicative of growth (8,9,20). For this particular study, PM1 plates were used which consist of 95 different carbon sources and a negative control.

The *E. coli* were first grown on R2A agar plates in order to deplete nutrient sources, collected with a nylon flocked swab, and then diluted in IFO-a GN/GP base inoculating fluid to a final concentration of 0.6 at an OD_{600}. Threonine, methionine, leucine and thiamine were used as supplements each at a concentration of 20 µl/mg to allow growth of the auxotrophic mutant strains. Two ml of the diluted bacteria were added to 10 ml of solution containing supplements and tetrazolium dye in IF-Oa. The rationale was to dilute the *E. coli* to a final concentration of 0.1 for inoculation of PM1 plates. To each well, 100µl of the inoculum solution was added. The plates were wrapped in parafilm and incubated at 37°C for 48 hours. Four independent experiments were done per each strain. *E.coli* growth on different carbon sources was determined as an optical density (OD_{600}) from the plate that was inoculated with tetrazolium dye using a micro plate reader

To quantify the biofilm amounts formed by the *ldhA* mutant on different carbon sources, ATP assay was used as previously explained (49). In this assay, ATP is converted by luciferase into a bioluminescence signal. The bioluminescence is assumed to be directly proportional to biofilm biomass. The experimental set up is similar to the one for growth evaluation, except the tetrazolium dye is omitted. Briefly, the growth medium from the previously incubated plate was carefully removed using a multichannel pipette in order to maintain the biofilms that had formed at the bottom of wells. The plates were washed twice with 100 µl phosphate buffered saline

(PBS). The plates were allowed to air dry for 15 minutes. 100 µl of BacTiterGlo™ (Promega, Madison, WI) were added to each well. Bioluminescence was measured after 10 min incubation at room temperature with a TD 20-20 bioluminometer from Turner Design (Sunnyvale, CA). Biofilm amounts formed on each well were measured as relative bioluminescence units (RLU) (Fig 3).

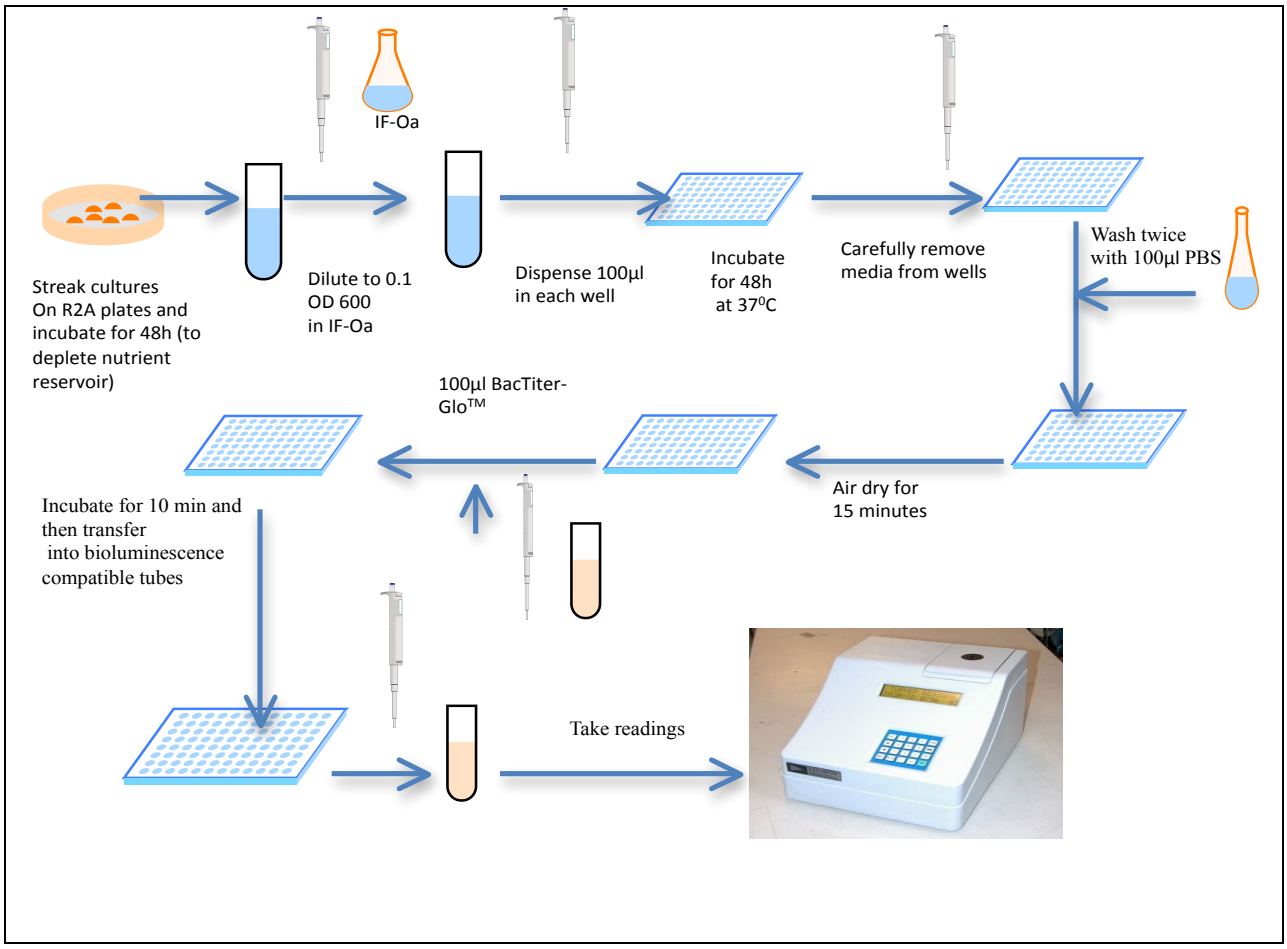

Fig.3. Workflow for the determination of biofilm amounts

Bioluminescence values were added for all the carbon sources from one experiment (one plate). A fold variation was calculated using the smallest experiment as the norm (1 fold). The

remaining experiments were normalized to the smallest one. Biofilm amounts formed on the different carbon sources by the *ldhA* mutant were compared to those previously determined for the wild-type AJW678 strain (43). The same comparison was performed between the *pflA* mutant (Leith, Horne, and Pruess, unpublished) and AJW678. This was done as previously described (43), carbon sources that permitted biofilm formation equivalent to more than 1,300 RLU were considered good promoters of biofilm. Carbon sources were considered moderate supporters of biofilm if they permitted biofilm amounts equivalent of 800 to 1,300 RLU. For this analysis, we used those carbon sources that followed the above criteria for the wild-type (43). Biofilm amounts from both mutants were compared to those of the wild-type.

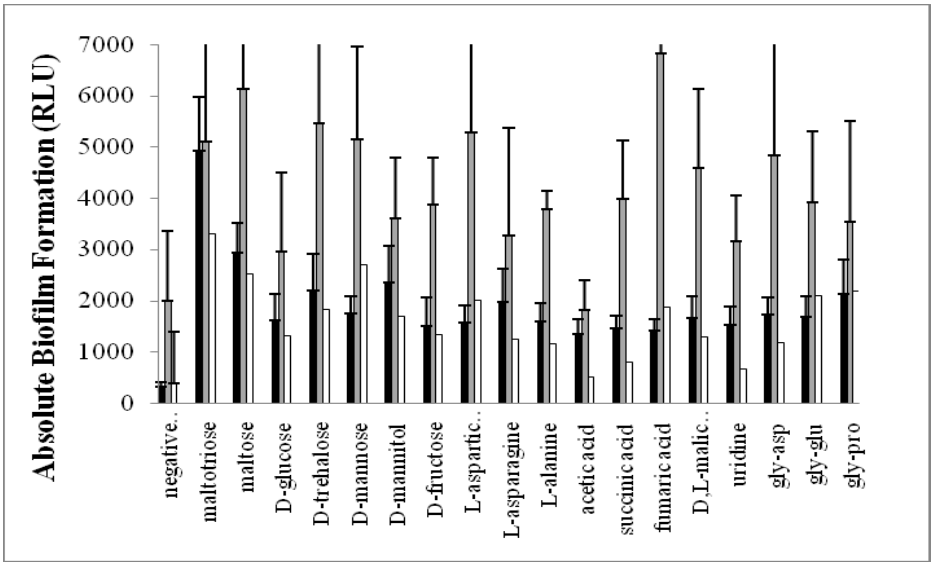

Fig. 4. Comparison of biofilms amounts on carbon sources that formed at least 1,300 RLU biomass in the wild-type to those of the mutants. The black bars represent the wild-type strain, the gray bars the *ldhA* mutant and the white bars the *pflA* mutant. Data are presented as average over 4 replicate experiments. The error bars indicate the standard deviation. All the carbon sources permitted growth to at least 0.5 OD_{600} in all strains.

The first graph (Fig.4) compared biofilm amounts formed on carbon sources that permitted a biofilm amount equivalent to at least 1,300RLU in the wild-type strain to those of the *ldhA* and *pflA* mutants. All the carbon sources permitted higher amounts of biofilm in the *ldhA* mutant (grey bars) as compared to the wild type (black bars). Biofilm amounts formed by the wild-type on maltotriose was slightly lower (4,935 RLU) than biofilm formed by the *ldhA* mutant (5,095 RLU) on this carbon source. Acetic acid permitted 1,349 RLU and 517 RLU biofilm biomass in the wild-type and *pflA* mutant (white bars), respectively. This is a 2.4 fold reduction in biofilm amounts by the *pflA* mutation.

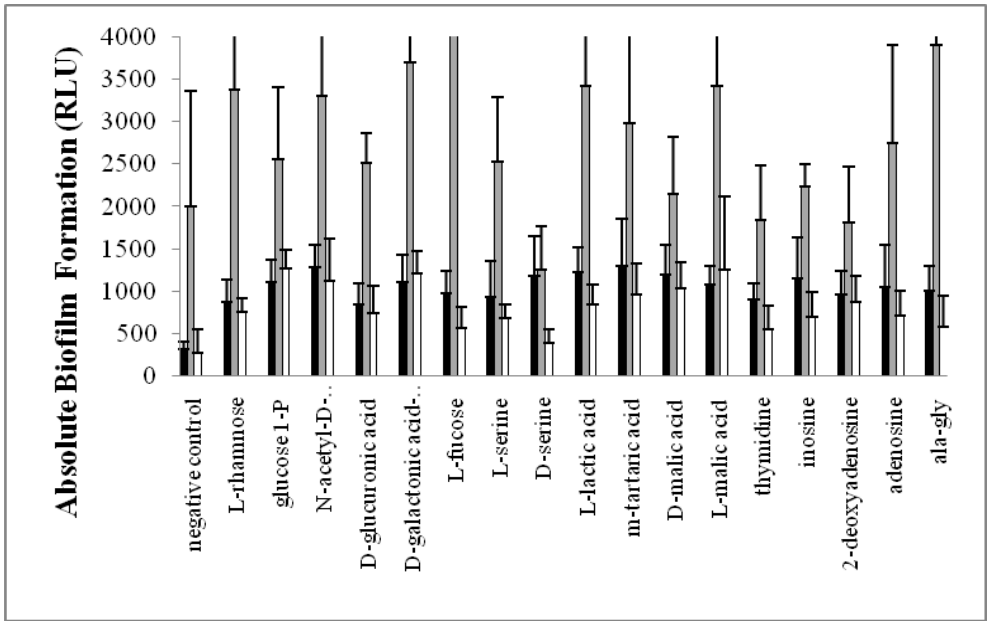

Fig 5. Comparison of biofilms amounts on carbon sources that formed 800 to 1,300 RLU biofilm biomass in the wild-type to those of the mutants. The black bars represent the wild type strain, the gray bars the *ldhA* mutant and the white bars the *pflA* mutants. The error bars indicate the standard deviations. All the carbon sources permitted growth to at least 0.5 O_{600} in the strains.

We further compared biofilm amounts on carbon sources that permitted biofilm amounts from 800 to 1,300RLU (Fig.5). This second set of carbon sources also consistently permitted high biofilm amounts in the *ldhA* mutant as compared to the wild type. The only carbon source that

permitted a relatively reduced biofilm amounts in the *pflA* mutants was D-serine. In the wild-type strain (black bars), D-serine permitted 1,187 RLU and 390 RLU in the *pflA* mutant strain (white bars). This was over a four-fold decrease in biofilm amounts.

2.5.2. Metabolic modeling

Metabolic pathways were formulated for carbon sources that were poor biofilm performers in the mutants, but permitted ample amounts of biofilm in the wild-type. Maltotriose feeds directly into the glycolytic pathway. D-serine feeds into the glycolytic pathway at the level of pyruvic acid. These carbon sources are metabolized to acetyl-CoA, acetyl phosphate and acetate. Acetate is one of the final products of the glycolytic pathway (Fig. 6).

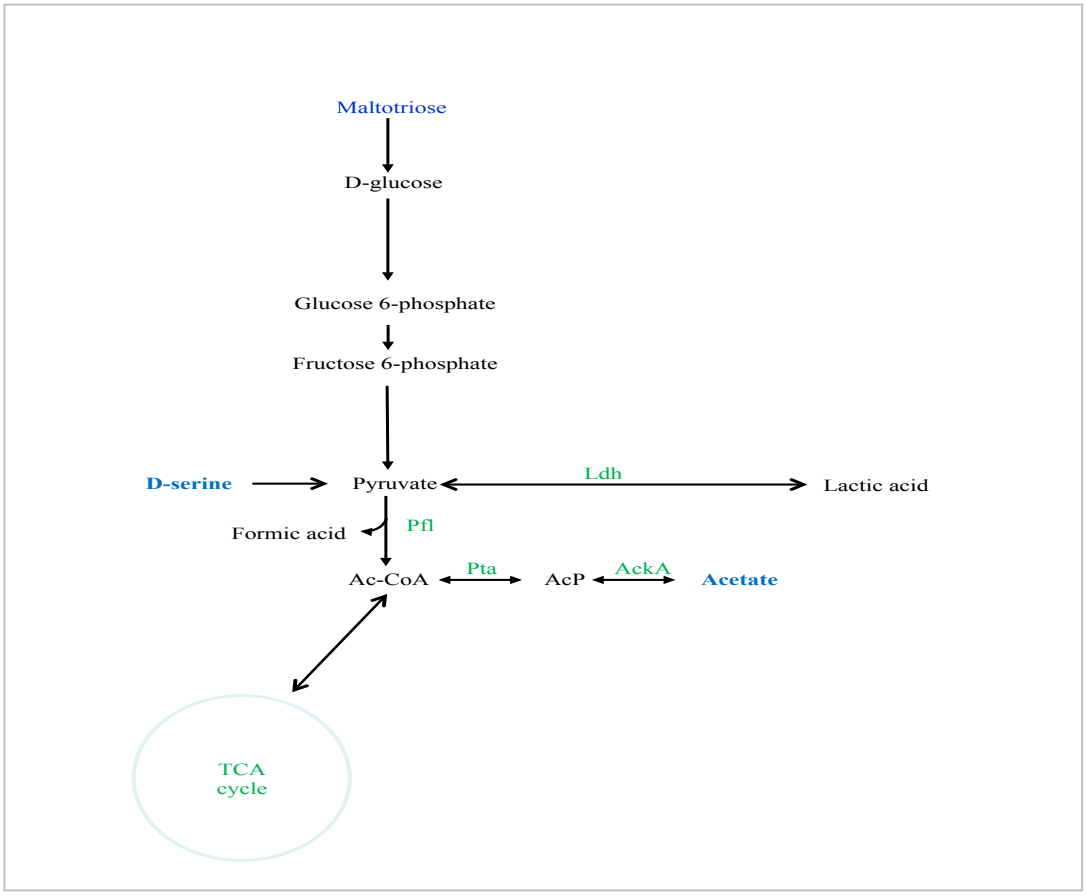

Fig. 6. Metabolic pathway to acetate.

Carbon sources in blue reduced biofilm amounts in the *pflA* mutant. Pta and AckA represent phosphotransacetylase and acetate kinase respectively. LdhA and PflA stand for lactate dehydrogenase and pyruvate formatelyase.

2.5.3. Scanning Electron Microscopy

Biofilms were prepared for scanning electron microscopy (SEM) according to Sule et al 2009. Biofilms were produced from the parental strain AJW678 and the *ldhA*, *pflA*, and *pflB* mutants on 12 mm cover slips (Assistant Germany) in 6 well plates. Briefly, 40 µl of bacteria from an overnight culture were added to each well containing the cover slips and 4 ml of Luria-Bertani broth (LB). The plate was incubated at 32°C for 48 h in a non-shaking incubator. The medium was removed carefully and the biofilms were washed twice with PBS. After the washing, the biofilms on the cover slips were allowed to air dry and fixed with 2 ml of 2.5% glutaralydehyde (Tousimis, Research cooperation Rockville MD) in 0.1 mol 1-1 phosphate buffer. Biofilms were rinsed in the same buffer and deionized water, and dehydrated using a graded alcohol series (15 minutes each in 30%, 50%, 70%, 90% and two changes of 100% ethanol). Samples were critically point dried in an Autosamdri-810 critical point drier (Tousimis, Rockville MD) with liquid carbon dioxide as transitional fluid. The cover slips were then attached to aluminum mounts with adhesive carbon tabs or silver paint and coated with gold/palladium using a Balzers SCD 030 sputter coater (Balzers Union Ltd., Liechtenstein). Images were obtained with a JEOLJSM-6490LV scanning electron microscope (JOEL Ltd Japan) at 1,000X, 3,000X, and 6,500X magnification. The experiments were repeated at least three times per strain. Between 24 and 27 images were obtained per strain. Representative images were selected (Fig 7). Generally, all the strains formed significant biofilm amounts

suggesting that the mutations could be having minimal effect on the ability of *E.coli* to form biofilms on a mixed amino acid medium. There may be a difference in the three dimensional structure of the biofilms, when comparing the *ldhA* mutant strain with the *pfl* mutants.

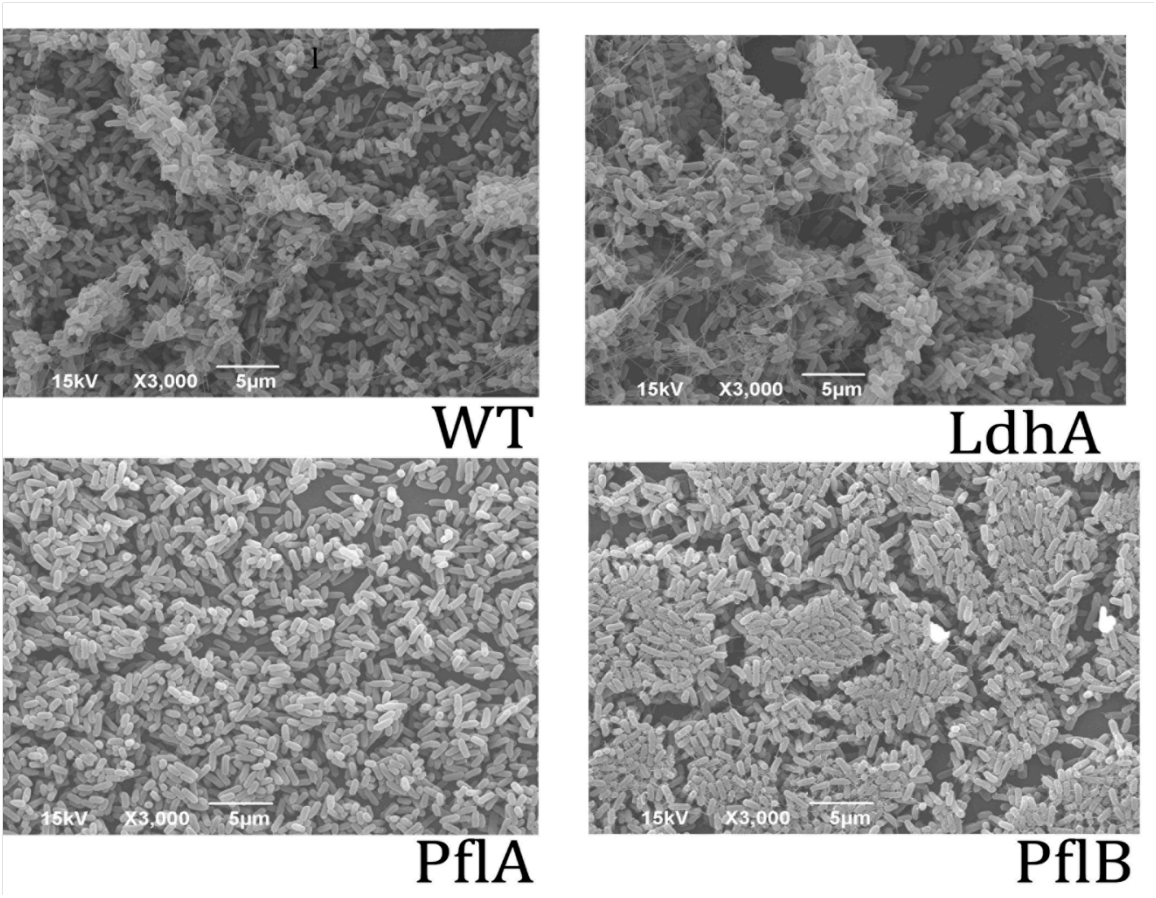

Fig. 7. Electron Microscopy at 3000-fold magnification of the three strains

Findings from this biofilm study indicate that mutations in *ldhA* had a significant effect on biofilm amounts when *E.coli* bacteria were grown on all carbon sources, whereas *pflA* significantly affect biofilm amounts on acetic acid and D-serine. A previous study had postulated that acetate metabolism was a metabolic sensor that related information about *E. coli*'s environment to the formation of biofilms (43). In the current study, the effect of acetate metabolism on biofilm amounts was detailed by using single carbon sources rather than the

previously used mixed amino acid medium, as well as mutations in additional genes that contribute to acetate metabolism (*ldhA*, *pflA*). Mutation in *ldhA* would lead to an accumulation of acetyl-CoA. In contrast, knocking out *PflA* would lead to a decrease in intracellular acetyl-CoA levels. *Escherichia coli* deficient in the fermentative lactate dehydrogenase have been studied previously (17). The *ldhA* mutants have been observed to grow with mild effect under anaerobic conditions as compared to the parental strain. Bacterial strain with a double deletion of lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pfl*) however could not grow on glucose and other sugars (fructose, rhamnose, gluconate, xylose and sorbitol) (17), even when acetate was supplemented. In summary, *ldhA* and *pflA* knock out has significant effect on biofilm amounts formed on all carbon sources generally and on D-serine and acetate respectively. We did not see this on a mixture of amino acids (EM), when compared with the wild type. Accumulation of acetyl Co-A may lead to increased biofilm amounts.

3. GENERAL CONCLUSION

Zoonotic pathogens are still a big burden in the world. Control of zoonoses begins with elimination of the diseases in animals and maintenance of biosecurity. According to my first MICR794 project, vaccination, separation of domestic animals from wildlife, and maintenance of occupational hygiene rank among the important factors in preventing brucellosis. Pathogenesis of any infectious disease is governed by a variety of virulence factors, some of which could be used as drug targets. More specifically, virulence factors, which enable Brucella to survive in the intracellular milieu, are very important and further research is needed in this area. Among many virulence factors, biofilm formation especially complicates treatment of animals and humans infected by some of the zoonotic pathogens. Bacterial biofilms are resistant to antibiotics. Molecular studies are crucial in designing molecular targets to control pathogens, including the ones studied in this paper (*E.coli* and Brucella). From our studies, acetate metabolism may one day be seen as one of the drug target mechanisms for controlling biofilm formation in *E.coli.* Findings from the second MICR794 project study give an insight on how some gene mutations could impact biofilm amounts by modulating the levels of certain metabolic intermediates, such as acetyl-CoA. Instead of mutating genes, the addition of nutrients or chemicals that lead to depletion of acetyl-CoA would decrease biofilm formation. The extent to which this knowledge can be applied to other biofilm forming pathogens remains to be determined.

4. REFERENCES

1. Abdussalam M, Fein DA (1976). Brucellosis as a world problem. Developments in Biological Standardization. 31:9-23
2. Ackermann MR, Cheville NF, Deyoe BL (1988). Bovine ileal dome lymphoepithelial cells: Endocytosis and transport of *Brucella abortus* strain 19. Veterinary Pathology. 25:28–35
3. Aparna, Madhu S, Sarita Y (2008). Biofilms: Microbes and Disease. The Brazilian Journal of Infectious Diseases 12: 526-530
4. Arellano-Reynoso B, Lapaque N, Salcedo S, *et al* (2005). Cyclic β-1,2 –glucan is a Brucella virulence factor required for intracellular survival. Nature. Immunology.6:618-625
5. Bayram Y, Korkoca H, Aypak C, Parlak M, Cikman A, Kilik S, Berkas M (2011). Antibiotic susceptibility of Brucella isolates from various clinical specimens. International Journal of Medical Science 8:198-202
6. Billard E, Cazevieille C, Dornand J, *et al* (2005). High susceptibility of human dendritic cells to invasion by the intracellular pathogens *Brucellasuis, B. abortus,* and *B. melitensis*. Infection and Immunity 73: 8418-24.
7. Bingöl A, Yücemen N, Meço O (1999). Medically treated intraspinal "*Brucella*" granuloma. Surgical Neurology. 52:570–576. doi: 10.1016/S0090-3019(99)00110-X
8. Bochner BR (2008). Global phenotype characterization of bacteria. FEMS Microbiological Reviews 33:191-205
9. Bochner BR, Gadzinski P, Panomitros E (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Research 11:1246-1255

10. Bochner BR, Giovannetti L, Viti C (2008). Important discoveries from analyzing bacterial phenotypes. Molecular Microbiology 70:274-280
11. Boschiroli ML, Ouahrani-Bettache S, Foulongne V *et al* (2002). Type IV secretion and *Brucella* virulence. Veterinary Microbiology. 90: 341-8
12. Cardoso PG, Macedo GC, Azevedo V, Oliveira SC (2006). Brucella spp non canonical LPS: structure, biogenesis, and interaction with host immune system. Microbial Cell Factory 5:13
13. CarvalhoNeta AV, Mol JPS, Xavier MN, Paixao TA, Lage AP, Santos RL. 2010. Pathogenesis of bovine brucellosis. The Veterinary Journal 184:146-155
14. Celli J (2006). Surviving inside a macrophage: the many ways of *Brucella*. Research in Microbiology 157: 93-8
15. Delrue RM, Lestrate P, Tibor A, Letesson JJ, Bolle XD (2004). Brucella Pathogenesis, genes identified from random scale screens. FEMS Microbiology Letters. 231:1-12
16. Domingo, A.M (2000). Current status of some zoonoses in Togo. ActaTropica 76:65-69
17. Fairoz Mat-Jan, Kiswar Y Alam, and David P Clark (1989). Mutants of *Escherichia coli* deficient in the Fermentative Lactate Dehydrogenase. Journal of Bacteriology 171:342-348
18. Faye B, Castel V, Lesnoff M, Rutabinda D, Dhalwa J (2005). Tubercullosis and Brucellosis prevalence on dairy cattle in Mbarara milk basin (Uganda). Preventive Veterinary Medicine 67: 267-281
19. Fernandez-Prada CM, Zelazowska EB, Nikolich M, et al (2003). Interactions between *Brucella melitensis* and human phagocytes:bacterial surface O-Polysaccharide inhibits

phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infection and Immunology 71:2110-9

20. Franco MP, Mulder M, Gilman RH, Smits HL (2007). Review: Human Brucellosis. Lancet of Infectous Diseases 7:775-86

21. Harmon BG, Adams LG, Frey M (1988). Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. American Journal of Veterinary Research 49:1092–1097

22. Henk LS, Sally JC (2004). Contributions of biotechnology to control and prevention of brucellosis in Africa. African Journal of Biotechnology 3:631-636.

23. Kabagambe EK, Elzer PH, Geaghan JP, Opuda-Asibo J, Scholl DT, and Miller JE (2001). Risk factors for Brucella seropositivity in goat herds in eastern and western Uganda. Preventive Veterinary Medicine 52: 267-281. Elsevier science

24. Kleessen B, Blaut M (2005). Modulation of mucosal biofilms. British Journal of nutrition 93, suppl.1, S35-S40

25. Ko J, Splitter GA. 2003. Molecular Host-pathogen Interaction in Brucellosis: Current Understanding and Future approaches to Vaccine Development for Mice and Humans. Journal of Clinical Microbiology16:65-78

26. Kumari S, Beatty CM, Browning DF, Busby SJ, Simel EJ, Hovel minter G, Wolfe AJ (2000). Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. Journal of Bacteriology 182:4173-179.

27. Kungu JM, Okwee-Acai J, Ayebazibwe C, Okech SG, Erume J (2010). Seroprevalence and risk factors for brucellosis in cattle in Gulu and Amuru districts, Northern Uganda. African Journal of Animal and Biomedical Sciences *5*:36-42

28. Lapaque N, Forquet F, de Chastellier C, *et al* (2006). Characterization of *Brucella abortus* lipopolysaccharide macrodomains as mega rafts. Cell Microbiology 8: 197-206.

29. Lecaroz C, Blanco-Prieto MJ, Burrell MA, *et al* (2006). Intracellular killing of Brucella melitensis in human macrophages with microsphere-encapsulated gentamicin. Journal of Antimicrobial Chemotherapy. 58:549-56

30. Lopez-Goni I, Guzman-Verri C, Manterola L, *et al* (2002). Regulation of brucella virulence by the two-component system BvrR/BvrS. Veterinary Microbiology90: 329–39

31. Luna-Martínez JE, Mejía-Terán C (2002). Brucellosis in Mexico: current status and trends. Veterinary Microbiology 90:19–30

32. Mack D, Ronde H, Harris LG, Davies AP, Horstkotte MA, Knobloch JKM (2006). Biofilm formation in medical device related infection. International Journal of Artificial Organs 29: 343-59

33. McDermont JJ, Arimi SM (2002). Brucellosis in sub-Saharan Africa: epidemiology, control and impact. Veterinary Microbiology 20:111-134, doi 10:1016/S0378 (02) 00249-3

34. Mohamed JA and Huang DB (2007) Biofilm formation by enterococci. Journal of Medical Microbiology 12:1581-1588

35. Mohiuddin K, Ho PY, Shimizu K (2005). Effect of *ldhA* gene deletion on the metabolism of *Escherichia coli* based on gene expression, enzyme activity, intracellular metabolite concentrations, and metabolic flux distribution. Biochemical Engineering Journal 26:1-11

36. Mwebe M, Nakavuma J, Moriyon I (2011). Brucellosis seroprevalence in livestock in Uganda from 1999 to 2008: a retrospective study. Tropical Animal Health and Production 43:603-608

37. Nakavuma, J, Kibirige-Sebunya I, and Opuda-Asibo J (1999). Serosurvey of *Brucellaarbotus* in cattle and goats in central and southern Uganda. Uganda Journal of Agricultural Sciences, 4:13-18

38. Nicolleti P (2010). Brucellosis Past, Present and future. Prilozi 31: 21-32

39. Omer MK, Skjerve E, Holstad G, Woldehiwet Z, Macmillan AP (2000). Prevalence of antibodies to *Brucella* spp. in cattle, sheep, goats, horses and camels in the State of Eritrea; influence of husbandry systems. Epidemiology and Infection 125:447–453

40. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV (2006). The new global map of human brucellosis. Lancet of Infectious Diseases 6:91-9

41. Porte F, Naroeni A, Ouahrani-Bettache S, *et al* (2003). Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome–lysosome fusion in murine macrophages. Infection and Immunology 71: 1481-90.

42. Prakash B, Veeregowda BM, Krisnappa G (2003). Biofilms: A survival strategy of bacteria. Current science Vol. 85, No. 9

43. Pruess BM, Verma K, Samanta P, Sule P et al (2010). Environmental and genetic factors that contribute to *Escherichia coli* K-12 biofilm formation. Archives of Microbiology 192:715-72

44. Raffa RB, Iannuzo JR, Levine DR et al (2005). Bacterial communication (Quorum sensing) via ligands and receptors. A novel pharmacological target for the design of antibiotic drugs. Journal of Pharmacology and Experimental Therapeutics 312:417-423

45. Rao V (2010). Integrated regional bioengagement framework to combat brucellosis. Prilozi. 31:191-207

46. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002). Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. Journal of Bacteriology 184:1140-1154.

47. Seleem MN, Boyle SM, Sriranganathan N (2008). Brucella: A pathogen without classical Virulence factors. Veterinary Microbiology129: 1-14

48. Seleem MN, Boyle SM, Sriranganathan N (2010). Brucellosis: A re-emerging zoonosis. Veterinary Microbiology. 140:392-398

49. Sule P, Wadhawan T, Carr NJ, Horne SM, Wolf AJ, Pruess BM (2009). A combination of assays reveals biomass differences in biofilms formed by *Escherichia coli* mutants. Letters in Applied Microbiology 49:299-304

50. Uzureau S, Godefroid M, Deschamps C, Lemaire J, De Bolle X, Letesson JJ (2007). Mutations of the quorum sensing-dependent regulator VjbR lead to drastic surface modifications in *Brucella Melitensis.* Journal of Bacteriology 189:6035-47

51. Van Houdt R and Michiels CW (2005). Role of bacterial surface structures in *Escherichia coli* biofilm formation. Research in Microbiology 156:626-633

52. Vassalos CM, Economou V, Vassalou E and Papadopoulou C (2009). Brucellosis in Humans: Why is it elusive? Reviews in Medical Microbiology 20:63-73

53. Xavier MN, Paixão TA, Poester FP, *et al*. (2009). Pathology,immunohistochemistry, and bacteriology of tissues and milk of cows and fetuses experimentally infected with *Brucella abortus*. Journal of Comparative Pathology 140: 149-57

54. Xavier MN, PaixaoTA, Hartigh AB D, Tsolis RM, Santos RL. 2010. Pathogenesis of Brucellosis. The open Veterinary Science Journal. 4:109-118

55. Zhu J and Shimizu K (2004). The effect of pfl gene knockout on the metabolism for optically pure D-lactate production by *Escherichia coli*. Applied Microbiology and Biotechnology 64:367-375

56. Zijnge V, Van Leeuwen MBM, Degener JE, Abbas F, Thurnheer T *et al*, (2010). Oral biofilms Achitecture on natural teeth. Plos One 5(2): e9321.do1.1371/journal.pone.0009321