Universidade de Trás-os-Montes e Alto Douro

Evaluation of Survival of Pathogenic Microorganisms on Animal Manure Under Ambient Conditions. A One Health Approach

PhD Thesis in Veterinary Sciences

Ana Sofia Pereira Carvalho Soares

Supervisors: Profª. Doutora Ana Cláudia Correia Coelho
Prof. Doutor Henrique Manuel da Fonseca Trindade

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Declaration

I, Ana Sofia Pereira Carvalho Soares, declare that the work present in the thesis is my own. Where the information has been derived from other sources, there is an indication on the thesis.

“If I cannot do great things, I can do small things in a great way”

Napoleon Hill

To my Family and Friends

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Resumo

Os estrumes e chorumes originários na produção bovina são reservatório de elevadas concentrações de microrganismos alguns dos quais com capacidade patogénica, o que constitui, atualmente, uma séria preocupação em termos de saúde pública, medicina veterinária e proteção ambiental. A emissão de gases de efeito estufa e a contaminação ambiental são um dos principais tópicos da ordem do dia encontram-se indiretamente associados à produção animal. Recentemente, assistiu-se ao desenvolvimento de técnicas para criar novas estratégias de maneio de efluentes animais, contudo, nem todas estas metodologias se apresentam promissoras. A quantidade de microrganismos presentes nos efluentes animais é variável, dependendo do tipo de efluente, das condições de armazenamento e de fatores ambientais. Alguns destes microrganismos tem capacidade patogénica para o homem e animais, o que requer atenção aos possíveis efeitos nocivos na área da saúde numa abordagem do tipo "Uma Só Saúde" ou "One Health". Nos últimos anos, com as alterações climáticas em curso, novos tipos de doenças emergem e outras reemergem. Por esse motivo, as abordagens do tipo One Health aos problemas ambientais tiveram um crescimento sem precedentes. Atualmente, existem métodos para reduzir microrganismos no chorume e estrumes, mas existe falta de conhecimento sobre o seu real efeito sobre a sobrevivência destes agentes. O principal objetivo deste trabalho consistiu na contribuição para a compreensão da sobrevivência de microrganismos patogénicos nos efluentes animais em condições ambientais, tendo em consideração a perspetiva One Health. O presente trabalho está estruturado em sete capítulos:

O capítulo I apresenta uma revisão geral do controle de microrganismos em efluentes animais e sua importância em uma abordagem de uma só saúde.

O capítulo II identifica os principais objetivos desta tese.

O capítulo III apresenta um estudo para avaliar o impacto de diferentes tratamentos na sobrevivência de *Escherichia coli* durante o armazenamento de estrume de bovinos leiteiros. Os resultados obtidos mostraram que os diferentes tratamentos aplicados que incluíam acidificação do chorume, apresentaram as médias mais elevadas de UFC/mL. Estes resultados podem contribuir para a melhoria da qualidade do chorume aplicado nos solos, beneficiando a agricultura, mas também a saúde pública

No capítulo IV é apresentado um estudo que consistiu na avaliação da sobrevivência de *Enterococcus* spp. em chorume vacas leiteiras durante o

armazenamento. Os diferentes tratamentos aplicados ao chorume de acordo com as diferentes datas de amostragem (30, 60 e 90 dias) apresentaram diferenças estatisticamente significativas para a UFC/mL (P <0,001). Segundo a literatura, o tratamento do chorume com ácido tem o objetivo de manter baixas as emissões de gases com efeito de estufa, no entanto, neste estudo, os tratamentos contendo adição de ácido apresentaram o maior crescimento bacteriano durante os três meses de armazenamento.

No capítulo V é abordada a sobrevivência bacteriana, em termos de presença ou ausência de *Salmonella enterica* no chorume de vaca tratado, durante o seu armazenamento a 4 ° C e 20 ° C. Os resultados sugerem que a temperatura mais elevada (20 ° C) foi mais eficaz na eliminação de *Salmonella enterica*. O tratamento contendo ácido sulfúrico foi o menos eficiente para eliminar o microrganismo patogénico quando armazenado a 20 ° C.

O capítulo VI descreve um estudo epidemiológico da ocorrência de má qualidade da água em explorações leiteiras em Portugal. A ocorrência de má qualidade da água foi registrada em 19 (59,4%) explorações. As bactérias isoladas foram *Escherichia coli* (20,0%), *Enterococcus faecalis* (25,0%), coliformes totais (65,6%) e coliformes fecais (43,8%). As explorações que não realizavam rotineiramente desinfeção apresentaram pior qualidade da água (70,8%) quando comparadas a explorações que realizavam desinfeção (25,0%) (P = 0,022). Esta pesquisa revelou a importância da analise da qualidade da água de abeberamento de vacas, o que poderia contribuir para melhorar a saúde animal, humana e ambiental segundo uma abordagem One Heath.

O capítulo final (capítulo VII - Considerações finais) é uma conclusão geral da nossa investigação, resumindo os dados mais relevantes e significativos. Os estudos apresentados neste capítulo fornecem novas perspetivas sobre a importância da gestão de resíduos de animais em termos do seu impacto em saúde pública e sanidade animal, originando conhecimento sobre a capacidade de sobrevivência de microrganismos patogénicos, contribuindo para o controle desses agentes e melhorando as condições de saúde animal, humana e ambiental, o que na prática é uma abordagem One Health.

Abstract

Manure and slurry originated in cattle production are a reservoir of high concentrations of microorganisms, some of which are pathogenic, which is currently a serious concern in terms of public health, veterinary medicine and environmental protection. Greenhouse gas emissions and environmental contamination are one of the main topics on the agenda and are indirectly associated with animal production. In recent years, there has been the development of techniques to create new strategies for the management of animal effluents, however, not all of these methodologies are promising. The amount of microorganisms present in animal effluents varies, depending on the type of effluent, storage conditions and environmental factors. Some of these microorganisms are pathogenic to humans and animals, which requires attention to the possible adverse health effects in "One Health" approach. In recent years, with climate change underway, new types of diseases are emerging and others re-emerging. For this reason, One Health approaches to environmental problems have seen unprecedented growth. There are currently methods for reducing microorganisms in manure and slurry, but there is a lack of knowledge about their real effect on the survival of these agents. The main objective of this work is to contribute to understanding the survival of pathogenic microorganisms in animal effluents under environmental conditions, taking into account the One Health approach. This thesis is structured in seven chapters:

Chapter I presents a general review of microorganisms control in animal effluents and its importance in a One Health approach.

Chapter II presents the major objectives of this thesis.

Chapter III is a study to test the impact of different treatments on the survival of *Escherichia coli* during the storage of manure from dairy cattle. Its results showed that the treatments that include the acidification of slurry have the highest averages of CFU/ml. These results can contribute to the improvement of the quality of slurry applied on soils, beneficiating agriculture but also public health and animal health.

Chapter IV is a study to assess the survival of *Enterococcus* spp. in slurry of dairy cows during storage. The different treatments applied to slurry according to the different sampling dates (30, 60 and 90 days) showed statistically significant differences for the CFU/ml ($P < 0.001$). In the literature, the treatment of slurry with acid has the propose to maintain low the GHG emissions, however, in this study the treatments

containing added acid have the highest bacterial growth during the three months of storage.

Chapter V also focuses on bacterial survival, in this case the presence or absence of *Salmonella* in previously treated dairy manure. The aim of this study was to evaluate the presence/absence of *Salmonella enterica* in treated cow slurry during storage at 4 °C and 20 °C. Its results suggest that the highest temperature (20 °C) was more effective on the elimination of *Salmonella enterica*. The treatment containing Sulphuric acid was the less efficient to eliminate the pathogen when stored at 20 °C.

Chapter VI describes an epidemiological study of the occurrence of poor water quality in dairy farms in Portugal. The occurrence of bad water quality was recorded at 19 (59.4%) cattle farms. Bacteria isolated included *Escherichia coli* (20.0%), *Enterococcus faecalis* (25.0%), total coliforms (65.6%) and faecal coliforms (43.8%). In farms that did not routinely performed disinfection had worst water quality (70.8%) when compared with herds that performed disinfection (25.0%) ($P = 0.022$). This research revealed the importance of screening the quality of drinking water in cattle, which could contribute to improve animal, human and environmental health in a One Heath approach.

The final chapter (chapter VII - Concluding remarks) is a general conclusion of our research, summarizing the most relevant and significant data. The studies presented in this chapter provide more information on the importance of animal waste management in terms of its ability to influence public health and animal health. Providing new insights into the survivability of pathogens, contributing to the control of these agents and improving animal, human and environmental health conditions, which is practice in a One Health approach.

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List of Abbreviations

BIO - Biochar

$Cacl_2$ - Calcium chloride

CFU - Colony forming unit

DM- dry matter

EC- electrical conductivity

GHG - Greenhouse gases emissions

H_2SO_4 - Sulphuric acid

$HClO_4$ - Perchloric Acid

LF - liquid fraction

NH4+- Ammonium

NO_3^- - Nitrate

SF - Solid fraction

UTAD - Universidade de Trás os Montes e Alto Douro

VTECs - Verocytotoxic *Escherichia coli*

WS - Whole slurry

Chapter I

Microorganisms Control in Animal Effluents is This Important? A One Health Approach

I. Microorganisms control in animal effluents is this important? A One Health approach

Soares A.S., Miranda C., Trindade H., Coelho A.C. (2019). Microorganisms Control in Animal Effluents is This Important? A One Health Approach.

I. Abstract

Utilization of organic manures such as cow slurry is the most economic, practical, environmentally beneficial and useful option for improving soil quality and fertility. In the specific case of land-spreading of bovine manure, that is a common fertilization practice, this effluent is recognised to contain human pathogenic bacteria, that may persist for several weeks in soil, so this practise needs to be done correctly. Spreading fresh manures directly to land presents complex risks in many levels, including a higher risk of pathogen transfer to the food chain, for example the liquid fraction of bovine slurry contains an important part of the total bacteria of slurry (>80%). This information was being used during the years to improve guidelines on the management of manures to minimise the risks of pathogen transfer from animal effluents to the human food chain or water systems. The effectiveness of manure management systems needs to concern environmental transmission pathways by which zoonotic pathogens presents in animal manures may be transported to the environment. So, these management systems are planned to reduce the concentrations of microbes found in manure (by 90 to 99% or more). Antibiotics used in farms are excreted by the animals and end up in their effluents, being animal effluents (manure or slurry) used as fertilizers, the antibiotics residues will be transferred to the soil and in some cases can contaminate ground water. With the amount of slurry applied on the soils as fertiliser every year, there is a need of studies to measure the leaching of pathogenic agents, antibiotics residues normally present in slurry, and their fate in the environment using for that a One Health approach. This paper is a review of the scientific literature on the viability of some microorganisms in animal effluents and the effects of different manure treatments on the microbial concentrations.

I.1 Introduction

One of the main problems of intensive animal production is the quantity of animal effluents created in small areas. Every year, one billion tons of animal manure and slurry are produced in the Europe, therefore, the management of slurry was been converted in one of the central dairy activities in farms (Fangueiro et al., 2014). Manure represents one of the most valuable organic fertilizers, but it could contain a large number of microorganisms that can have pathogenic potential as *Escherichia coli*, *Enterococcus* spp. or *Salmonella* sp. (Ferens and Hovde, 2011; Nolan et al., 2018). Livestock effluents have long been used as organic fertilizers. However, in the last years, the growth of application of animal production in certain areas has been responsible for the production of large volumes of livestock effluents that if where not managed properly can have significant risks that can have consequences to humans, animals and the environment (Collins et al., 2005; Cardoso et al., 2012). Introduction of new intensive animal housing practices results in the increased amount of manure produced with a higher share of slurry (Uvarov et al., 2019). Utilization of organic manures such as cow slurry is the most economic, practical, environmentally beneficial and useful option for improving soil quality and fertility, and at the same time providing an extra source of nutrients for growing plants (Nicholson et al., 2005; Semenov et al., 2009). Effluents from farming processes include raw manure, untreated slurry (a mixture of manure, urine, split feed, and water that is held without aeration), and treated slurry (that is filtered to separate the solid fraction from the liquid fraction). Livestock manure is constituted by animal excreta (faeces and urine), water, bedding, secretions, blood and skin, nutrients and organic matter used to improve soil properties, but may also contain a variety of pathogens with potential negative impact on human health including zoonosis. Animal effluents can be a reservoir of a huge number of microorganisms as faecal streptococci, *E. coli*, particularly *E. coli* O157:H7 and *Salmonella* sp. but it is also possible the isolation or detection of other pathogenic microorganisms such as multiple virus like rotavirus group A (RV-A), hepatitis E virus (HEV), *Brucella*, *Mycobacterium*, *Leptospira*, *Chlamydia*, *Campylobacter* as well parasites (Guan and Holley, 2003; Bornay-Llinares et al., 2006; Ziemer et al., 2010; Olszewska and Skowron, 2013a; Krog et al., 2017; Nolan et al., 2018). The concerns about its management are in the most of the cases only fixated in the environmental effects of is nutrients (Nitrogen, Phosphate, Carbon) and the presence of microorganisms is the most of the cases not must studied (Pell,1997; Gerba and Smith,

2005). Areas with a high density of animal production are documented as having a higher risk for contaminating fresh produce, however, the specific pathway of contamination remains unproven. Suspected sources include inappropriately composted manure/wastewater and wild animals (Jacobsen and Bech, 2012). It is possible that surface application of manure may decline the risk of contamination of groundwater and plant roots compared to injection of slurry (Semenov et al., 2009). Furthermore, it has been shown that *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may be detected in the surface of plants growing in soil corrected with contaminated manure (Natvig et al., 2002; Islam et al., 2004) and could even be internalized by the plants (Solomon et al., 2002; Semenov et al., 2009). Animal wastes like manure are largely recycled to agricultural land as the most economical and environmentally sustainable means of treatment and reuse. These materials have a beneficial fertilizer value (nitrogen-phosphate-potassium) and can help maintain soil quality and fertility. However, animal manures frequently contain enteric pathogenic microorganisms and land spreading can lead to pathogen entry into the food chain (Islam et al., 2004). In consequence of this, with the amount of slurry applied on the soils as fertiliser every year, there is a need of studies to measure the leaching of microbial agents normally presents in slurry (Hodgson et al., 2016; Krog et al., 2017).

I.2 Microorganisms in manure, slurry and effluents

The danger of pathogen transmission by land application of untreated animal manures is documented for more than 100 years. Around 150 pathogens are identified with capacity to be transmitted from animals do humans by different ways. Once manure is correctly treated it is a safe and effective fertilizer, but when it is untreated or incorrectly treated, it may contain pathogens like *Bacillus anthracis*, *Brucell*a spp., *Campylobacter* spp., *Chlamydia* spp., *Escherichia coli*, *Leptospira* spp., *Listeria monocytogenes*, *Mycobacterium* spp., *Salmonella* sp., or *Yersinia* spp. that are capable of contaminate the field or even water supplies (Gerba and Smith, 2005). In the specific case of land-spreading of bovine manure, that is a common fertilization practice. This effluent is recognised to occasionally contain human pathogenic bacteria, such as *Escherichia coli* O157:H7 and *Salmonella* sp. that may persist for several weeks in soil, so the practise of spread manure in the land needs to be done correctly (Lau and Ingham, 2001). In the past, *E. coli* O157:H7 and *Campylobacter jejuni* liberated to the environment, originating from contaminated manure, resulted in a large waterborne

disease outbreak in Walkerton, Canada, related to the consume of contaminated drinking water (Hrudey et al., 2003; Krog et al., 2017). Determining the environmental fate of pathogens from manure is very difficult. Biological variables include pathogen liberation by individual animals; microbial interactions with the stored manure; inoculation of stored manure each time an animal liberates pathogens; interactions with water, organic matter, exchanges with plants, nematodes, and soil microorganisms after land application. Physical variables that can influence include type of manure storage, temperature and humidity during storage, soil type, temperature, moisture, water, pH, salinity, and rainfall events. There is a need for research to determine the factors that affect the environmental survival and persistence of microbial pathogens in manures (Unc and Goss, 2004; Ziemer et al., 2010). Other studies demonstrate that pathogens prevalence and their levels in manures are affected by animals age (with higher levels in the faeces of young animals), dietary variations (for example when cattle are moved between housing and grazing), and with fasting or other forms of stress (Nicholson et al., 2005). Other studies have revealed that inoculum cell conditions, exposure of inoculated soil to sunlight, protozoan predation, frost and temperature may also be important to pathogen survival (Lau and Ingham, 2001). Studies show that *E. coli* O157:H7, *Salmonella*, *Campylobacter* and *Listeria* are capable of survive in stored slurries and dirty water for up to three months. However, all these pathogens survived for less than one month in solid manure heaps where temperatures are superior to 55 ºC (Jacobsen and Bech, 2012; Nicholson et al., 2005). Survival of pathogens may range from several days (turned composted manure) to more than a year (nonrated manure) (Semenov et al., 2009). Food-borne illness affects lots of people around the world including in developed countries, between the main causative agents are bacteria, mainly Verocytotoxic *Escherichia coli* (VTECs), *Salmonella*, *Campylobacter* and *Listeria*. One-way explanation for this is the application of contaminated animal effluents to agricultural soil (Nicholson et al., 2005). The anaerobic conditions in the slurry storage have revealed ability to increase survival for *E. coli* O157:H7. Though, for *S.* Typhimurium identical survival in aerobic and anaerobic slurry was similarly appreciated (Semenov et al., 2011). Also, *S.* Typhimurium has been shown to be more resistant to environmental stresses and survive for longer periods of time in natural substrates compared to *E. coli* O157:H7 (Franz et al., 2005; Sinton et al., 2007; Jacobsen and Bech, 2012). Survival and transport of pathogens from manure to the environment depends on a number of multifaceted phenomena, like seasonal effects.

After infiltrating the soil, the retaining of bacteria depends on the physical structure of soil, the soil chemistry, and the properties of the bacterial cells (Lau and Ingham, 2001; Unc and Goss, 2004;Natvig et al., 2002). Between soil types, the sandy soil showed to be the best for *E. coli* survival and the nastiest for *Enterococcus* spp. survival. However, *Enterococcus* spp. survived best in the loamy soil. For both bacterial species, an inferior incubation temperature (5 ºC) and a higher soil moisture content (100% Field Capacity) improved survival (Cools et al., 2001a). While the threat of contamination by nutrients leaching from manure-treated fields is well recognized, the threat by leaching of zoonotic pathogens from the manure has received much less attention (Krog et al., 2017). This information was being used during the years to improve guidelines on the management of manures to minimise the risks of pathogen transfer from animal effluents to the human food chain or water systems, to reduce the potential costs and environmental concerns (e.g. on ammonia and nitrous oxide emissions to air, nitrate leaching losses to water) or to implement practical measures to control the spread of manure-borne pathogens (Nicholson et al., 2005). Studies refer also that to lower the risk of manure-borne pathogens, such as *Salmonella* serovar Typhimurium and *E. coli* O157:H7 contaminate vegetables grown in manure-fertilized soil, it is necessary to establish appropriate time limits between the application of the manure (if non treated) and the vegetable harvest (Natvig et al., 2002). To avoid all of these problems, farm effluents should be stored in holding tanks with proper aeration for suitable lengths of time (1 to 3 months or as required) before being used as fertilizers. Improperly incubation and/or storage of slurry can serve as a way for environmental spread and dissemination of pathogens (Stocker et al., 2018).

I.2.1 *Escherichia coli*

The liquid fraction of slurry contains an important part of the total bacteria of slurry (>80%), including the pathogen indicator organisms such as *Escherichia coli (E. coli).* This bacteria is predominant in animal wastes and consequently serves as an indicator of faecal contamination (Stocker et al., 2018), with epidemiological data indicating that the serotype *E. coli* O157:H7 may be existing in up to 8.3% of dairy and beef cattle and that being asymptomatically in the faeces (Faith et al., 1996; Solomon et al., 2002). Since it was first recognised as a human pathogen in 1982, Vero cytotoxin-producing *Escherichia coli* (VTEC) serogroup O157 has been gradually recognized as a cause of haemorrhagic colitis, including severe abdominal pain, diarrhoea and blood in

the stools. Transmission of VTEC O157 is often foodborne mainly from undercooked contaminated beef or raw milk, and contact with farmed or companion animals it has also caused human infections. The source of VTEC O157 is the gastrointestinal tract of animals, including humans, cattle have been recognised as a main reservoir (Chalmers et al., 2000). Bovine faeces are a potential way for transmitting *E. coli* O157:H7 to cattle, food and the environment, with the pathogen holding its ability to produce verotoxins. *E. coli* is capable to survive much longer in soil than in the manure representing a major risk to contaminate water or vegetables (Islam et al., 2004; Hruby et al., 2018). Studies refereed also that *E. coli* survives longer than other slurry pathogens on slurry storage processes (Biswas et al., 2016). According to Hodgson et al. (2016), a significant increase of *E. coli* was observed when slurry was applied to grassland via shallow injection compared to surface application and the decay rates were significantly higher for slurry applied to grassland in spring relative to summer and autumn (Hodgson et al., 2016). Some experiments showed, that the survival rate of *E. coli* in the soil was related to the local pH. Moreover, *E. coli* O157:H7 owns systems for survival at low pH (Sang et al., 2000; Van Elsas et al., 2011). The animal manures including poultry, cattle, and swine are the primary source of water resources contamination by water-borne pathogens, as well as *E. coli* or *Salmonella* sp. (Collins et al., 2005; Cardoso et al., 2012). Research has established the long-term survival of *E. coli* O157:H7 in manure. It may survive at least 19 weeks at 9±21 °C in bovine manure/soil. This survival held under a variety of conditions and may result from the application of manure containing viable *E. coli* O157:H7 to production fields (Wang et al., 1996; Lau and Ingham, 2001). Another way by which *E. coli* O157:H7 may be introduced in the environment is flood irrigation with water contaminated with cattle faeces or contact with contaminated surface runoff, studies have also demonstrated the capacity for this pathogen to survive for extended periods in water (Wang and Doyle, 1998; Chalmers et al., 2000). Some *E. coli* O157:H7 outbreaks have been linked to contaminated water by the application of contaminated manure to the production field or irrigation with contaminated water, because this bacteria can enter the lettuce plant over the root system and migrate to the edible part of the plant (Solomon et al., 2002). An outbreak of *E. coli* O157:H7 with 23 confirmed cases was related with the drinking of freshly pressed apple cider that was made from fallen apples at a farm. It was suspected that the apples had fallen in to soil that had been contaminated by cow manure (Besser et al., 1993).

I.2.2 *Salmonella* sp.

Salmonellosis, caused by *Salmonella enterica* with several serovars is considered one of the most widespread zoonosis in the world. Such as the transmission cycle of *Salmonella* involves practically all vertebrates, their control poses a test to public health in both developed and developing countries. Most *Salmonella* serovars associated with pathologies in animals are the strains: *S.* Dublin (bovines), *S.* Choleraesuis and *S.* Typhisuis (swine), *S.* Abortusequi (equines), *S.* Pullorum and *S.* Gallinarum (birds). However, since *Salmonella* is a zoonotic agent, some of its strains cause undifferentiated infection between humans and other animal species (Maurer, 2007). Being *Salmonella* sp. the agent of an infection associated with production animals, it have a negative economic impact on the commercialization of these animals and their products, because when an animal present in a herd is considered positive, normally, the entire herd is treated as positive (Warnick et al., 2003; Magwedere et al., 2015). As this infection have long periods of latency, it is possible that occurs release of bacterial cells from faeces of the infected animals, contaminating environment, food and water (Wakchaure and Ganguly, 2015; Tohidi et al., 2017). Cattle is considered one of the main disseminator of this pathogen, because of that, among the years some studies have been done to better understand the survival of this pathogen on cattle manures (Shinohara et al., 2008). In the study of Diao et al. (2015), *S.* Typhimurium was survived in dairy efluents with various particle sizes after a 30 days of storage under greenhouse condition (Diao et al., 2015). Chen et al. (2018) have observed that dairy compost can provide long-term survival conditions for *Salmonella enterica*, including survival for more than 168 days, at 5 ºC with high inoculum levels (Chen et al., 2018). In the study of Biswas et al. (2016) it was showed a clear relation among the temperatures of storage and the *Salmonella* survival. In last decades, numerous studies have been done and showed that *Salmonella* sp. survival is influenced by storage temperature, suspended solids content and pH value of the slurry (Guan and Holley, 2003; Arrus et al., 2006; Côté et al., 2006; Olszewska and Skowron, 2013; Biswas et al., 2016). Being, high temperature of storage the factor that presents better results in the elimination of *Salmonella* sp. (Plachá et al., 2001; Côté et al., 2006; Olszewska and Skowron, 2013b; Soares et al., 2019). Outbreaks of this pathogen are progressively related to consumption of fresh produce. An understanding of soil survival of *Salmonella* and transfer to water and to fresh produce is needed to control salmonellosis

(Jacobsen and Bech, 2012). Numerous studies have isolated *Salmonella* sp. together in farm animals and environmental samples (Sandvang et al., 2000; Baloda et al., 2001; Dorr et al., 2009; Weigel et al., 2007). What proves that this pathogen can persist in the farm environment for prolonged periods of time, circulating inside the farm between different reservoirs such as animals, stools, soil and plants (Kupriianov et al., 2010; Jacobsen and Bech, 2012;). The content of this pathogen in farm environment is dependent of several factors including: herd health, herd size, age and housing environment (Ojha and Kostrzynska, 2007; Payne et al., 2007; Rajić et al., 2007). *Salmonella* sp. may contaminate the soil from various ways, however application of contaminated manure remains the most common source (Jacobsen and Bech, 2012). After being introduced to the soil, the pathogen survival has been revealed to be manly influenced by method of introduction, temperature, predation by soil protozoa, temperature, moisture, soil type, presence of plants, exposure to sun (UV) light, and the initial number of microorganisms present. *Salmonella* sp. has been described to survive from a few days up to 332 days in manure-amended soils (Islam et al., 2004; You et al., 2006). Surface spreading of manure is leading to air pollution problems and therefore injection of manure into the soil has been in the last years studied. This application method might increase the survival of *Salmonella* sp. (Jacobsen and Bech, 2012).

I.2.3 *Enterococcus* spp.

Enterococcus spp. have long been known as commensals of the gastrointestinal tract and as one of the present bacteria in the environment. Between the multiple set of bacteria that constitutes the intestinal microbiota of animals and humans the most of these microorganisms are beneficial to intestinal motility (Fisher and Phillips, 2009; van Schaik and Willems, 2010; John and Carvalho, 2011). *Enterococcus* spp. are facultative anaerobic organisms that can survive in high salt concentrations and survive in pH extremes (as low as 4.8 and as high as 9.6) (Huycke et al., 1998; Fisher and Phillips, 2009; van Schaik and Willems, 2010). Although these bacterias do not produce toxins, have virulence factors in the form of aggregation substances, so they can cause disease based in the way they adhere to the host tissues. Based on this virulence factors production and there high antibiotic resistance *Enterococcus* are now considered emerging pathogens (Vu and Carvalho, 2011; Aslam et al., 2012; Guerrero-Ramos et al., 2016; Ahmed and Baptiste, 2017). Based on their abundance in faeces of mammals and their long survival in the environment *Enterococci* are commonly used as faecal

indicators in water management studies and in studies focused in the source of faecal pollution. The presence and abundance of *Enterococcus* in slurry´s is a known fact (Manero et al., 2002), because of that, when slurry, resultant from animal production is used to fertilize fields, this bacteria and its antibiotic resistance genes presents in the slurry can be transmitted to the environment (Sengeløv et al., 2001; Wallace et al., 2018).

I.3 Manure treatments

Soil adjustment with animal slurry (a mixture of urine, feces and water), is a cost-effective and viable option for reloading nutrients and organic matter in cropped soils, constituting a sustaining option for recycling nutrients at the farm level (Hjorth et al., 2010). With the crescent demands from de worldwide population, the selection of proper treatment and manure disposal methods constitutes a challenge. In order to properly utilise cattle manure, this waste needs to be characterised to evaluate its value as feedstock, elemental composition, heavy metal content, and GHG emissions (Font-Palma., 2019).

Spreading fresh manures directly to land presents complex risks in many levels, including a higher risk of pathogen transfer to the food chain (Nicholson et al., 2005). The contaminants of slurry may take different pathways to groundwater and/or surface water bodies via direct runoff, subsurface flow, leaching and percolation depending on the local hydrology (Kjaer et al.,2007; Bech et al., 2014). The effectiveness of manure management systems need to concern not only GHG emissions but also environmental transmission pathways by which zoonotic pathogens presents in animal manures may be transported in environment including to water resources (Figure I.1).

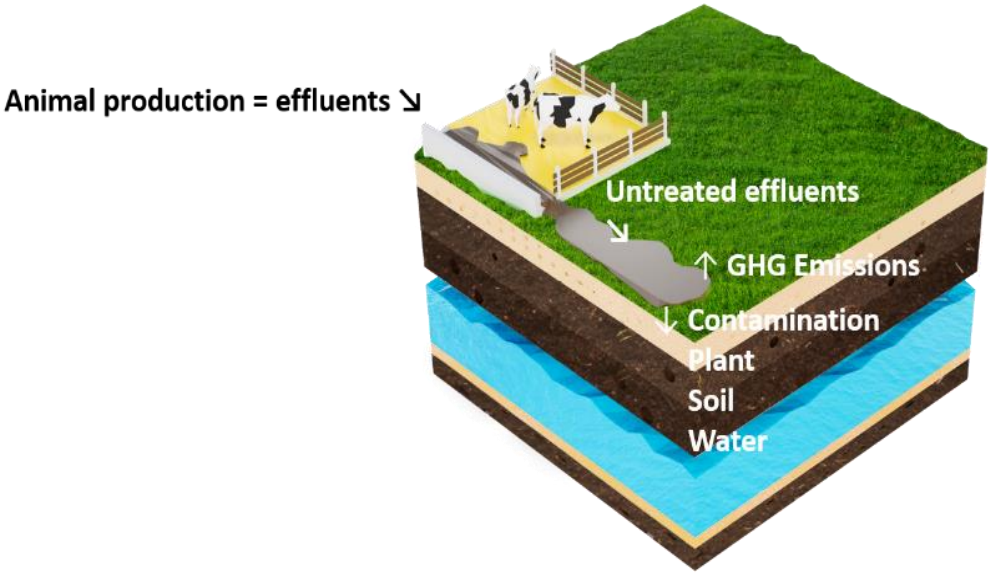

Figure I. 1 - Diagram summarizing animal effluents disposal and consequences without treatment application (Soares JA®).

This management systems are planned to reduce the concentrations of microbes found in manure (by 90 to 99% or more) and to prevent off-farm transport of manure materials (e.g., no discharge systems) (Ziemer et al., 2010). In the past, animal wastes and residues from bedding were composted for several days, with the compost reaching temperatures of 70 °C or more before being used as fertilizer.

Composting and drying of manure is recognised to be effective in reducing the number of viable pathogens that could be present in the effluent, there continued a small risk of pathogen survival in the cooler exterior or drier parts of manure heaps (Nicholson et al., 2005). However, composting is not an effective approach for processing cattle manure. Progressions in mechanized farming have led to large numbers of animals per farm and most of those large farms wash animal faeces, urine, and spilt feed into a slurry mixture. The most common practise for storing slurry is storage in tanks, away from the animal housing and under anaerobic degradation processes (untreated slurry) for more than one month before disposal (Figure I.2).

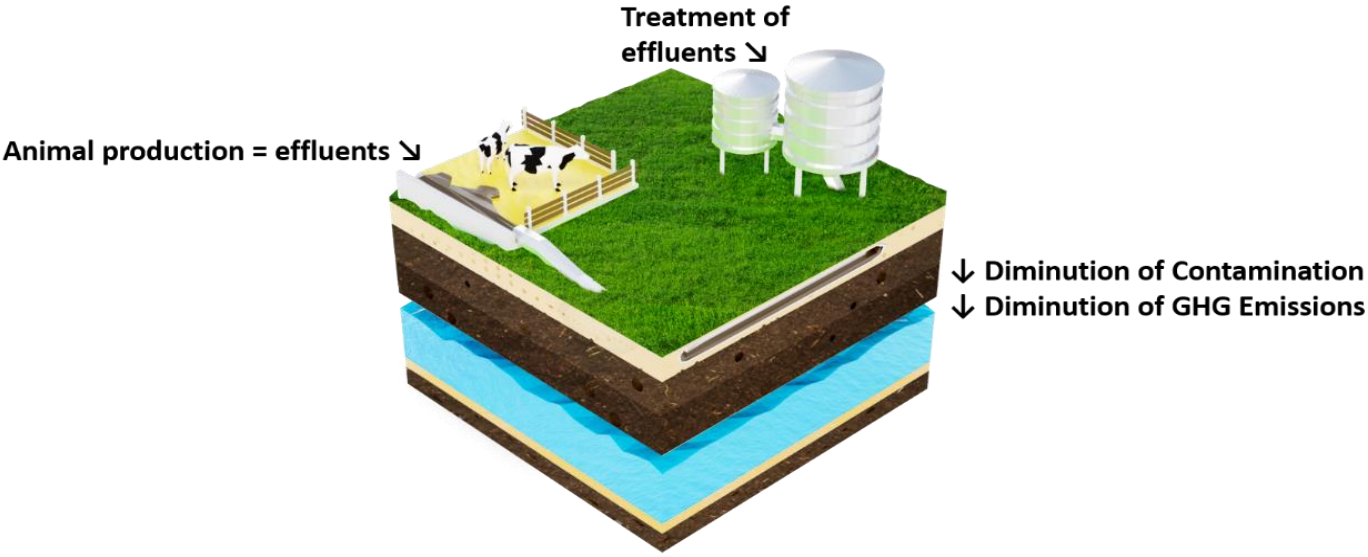

Figure I.2 - Diagram summarizing animal effluents disposal and consequences when there is a previous treatment application (Soares JA®).

In addition, some farmers reduce the amount of untreated slurry by using a mechanical/aeration technique that separates the solid and liquid portions of the slurry. With the main propose of recycling some of the solids to made beds and to composting (Font-Palma, 2019). Slurry differs from manure by having less dry matter, lower pH, less nitrogen, more dissolved organic matter and less diversity in microbial community. Previous studies showed that pathogens have been shown to survive longer in the slurry than in farm yard manure (Nicholson et al., 2005; Semenov et al., 2009; Jacobsen and Bech, 2012). The pathogen declined for example of *Salmonela*, *Mycobacterium paratuberculosis*, *E. coli* O157:H7 and some viruses (rotavirus, herpesvirus) in stored effluents depends on management and storage conditions, temperature, aeration, pH, dry matter content and the length of time that manure or slurry is stored before it is applied to soil (Kudva et al., 1997; Himathongkham and Riemann, 1999; Nicholson et al., 2005). Studies show that even at low to moderate temperatures pathogen numbers can decline over time if there are presence of dry conditions and UV light radiation

exposure. The bacteria can survive up to three months in slurry stored in laboratory conditions that have been reported (Nicholson et al., 2005; Soares et al., 2019). Pathogen survival in slurry stored outdoors is longer during winter than in summer, because of inferior ambient temperature (Plachá et al., 2001). In recent years, slurry has achieved a greatest importance between the natural fertilizers. Yet, incorrect practices of management makes it a potential microbiological risk for environmental and public health (Owusu-Twum et al., 2017a; Nolan et al., 2018). The long-term capacity of survival of microorganisms in animal effluents highlights the need for appropriate farm waste management to limit environmental spread of this microorganism. Because of that, since a few years manure-handling guidelines stated that a composting/storage period before application of the effluent to a field as fertilizer needs to be done (Solomon et al., 2002). Slurry treatment has been used as a solution to minimize its environmental impact, greater convenience because of farms storage capacity and to increase its agronomic value for crop utilization (Owusu-Twum et al., 2017a). Environmental problems may rise on livestock farms, which produce an excess of slurry in relation to field requirements. Separation of this slurry into a liquid fraction and a solid fraction, followed by transferring the one of the fractions to farms with less animals, may lighten the problem (Møller et al., 2000). To guarantee that the slurry applied to fields matches the nutrient necessities of the crops, some treatment techniques have been established. Slurry separation is now used in some dairy farms to improve slurry management, specifically the recycling of slurry nutrients on farms. After the use of slurry separation systems, the high dry-matter solid fraction (SF) obtained may be transferred out of the farm and may be composted while the low dry-matter liquid fraction (LF) may be applied on farm for example may be used for fertigation instead of the raw slurry (Hjorth et al., 2010; Owusu-Twum et al., 2017a). Mechanical slurry separation has been extensively used in Asia (Hjorth et al., 2010). On the other hand, such practise was less established in Europe and North America where slurry treatment has been considered as cumulative in production costs (Petersen et al., 2007). Studies refer that treatment costs can be reduced if the amount of manure that treated is augmented, both by having a larger livestock production on farms or by processing manure from several farms at a centralized separation system (Møller et al., 2000). The particle size of the slurry fractions obtained depends on the type of the mechanical separation technique (centrifugation, screening, or screw-press), the filter size and the slurry type (Fangueiro et al., 2010).

Biochar, is a carbonaceous solid resultant from pyrogenic material that have a substantial scientific attention in line for to its potential in climate change mitigation. But also for waste management, soil fertility development and crop growth promotion (Chan et al., 2008; Hossain et al., 2011; Jeffery et al., 2015). When biochar is applied to soil its major benefits are associated with increased carbon (C) sequestration and suppression of greenhouse gas (GHG) emissions (Lin et al., 2015; Subedi et al., 2016). Studies show that 90% of the biochar used in Europe goes into animal farming. Wherever less studied, when it is used in feeding, litter or for slurry treatment, the farmers rapidly notice less smell (Schmidt and Wilson, 2014) making it a new technique to be applicate directly in to slurry. Application of concentrated acid to slurry has been a common practice in some countries since 2010 based on its efficiency to the minimization of NH_3 emissions (Fangueiro et al., 2015). This minimization is based on the decrease of pH of the effluent, and has the propose to reduce the greenhouse gases emissions, and improve the nutrient value of the slurry (Fangueiro et al., 2015; Owusu-Twum et al., 2017b). Lowering pH may affect chemical and biological routes in slurry, as well as slurry composition. In result, the fertilizer value of slurry as well as the N dynamics from acidified slurry may differ for non-acidified slurry after soil application (Ottosen et al., 2009; Hjorth et al., 2010; Owusu-Twum et al., 2017a). A study reported also a postponement in nitrification of ammonium (NH_{4+}) and subsequent reduction in nitrous oxide (N_2O) emissions from soils amended with acidified slurries relative to non-acidified ones (Fangueiro et al., 2010). In the study of Ottosen et al. (2009) is was described that slurry acidification is capable to reduce the microbial activity, however, in the study of Zhang et al. (2011) was mentioned that in some conditions, acidification can have a weaker reducing effect on pathogenic bacteria (Ottosen et al., 2009; Zhang et al., 2011).

I.4 Cattle manure and slurry as an emergent concern in the One Health approach

The “One Health” approach is based on the premise that man shares habitat with animals resulting in a measure between human medicine and veterinary medicine to promote interdisciplinary collaboration to achieve knowledge about common diseases. But also promoting interventions about health as something more than the absence of disease. The American Veterinary Medical Association (2008) has defined “One Health” as an effort to collaborate across disciplines - working locally, nationally and globally - to achieve health for humans, animals and the environment (Figure I.3).

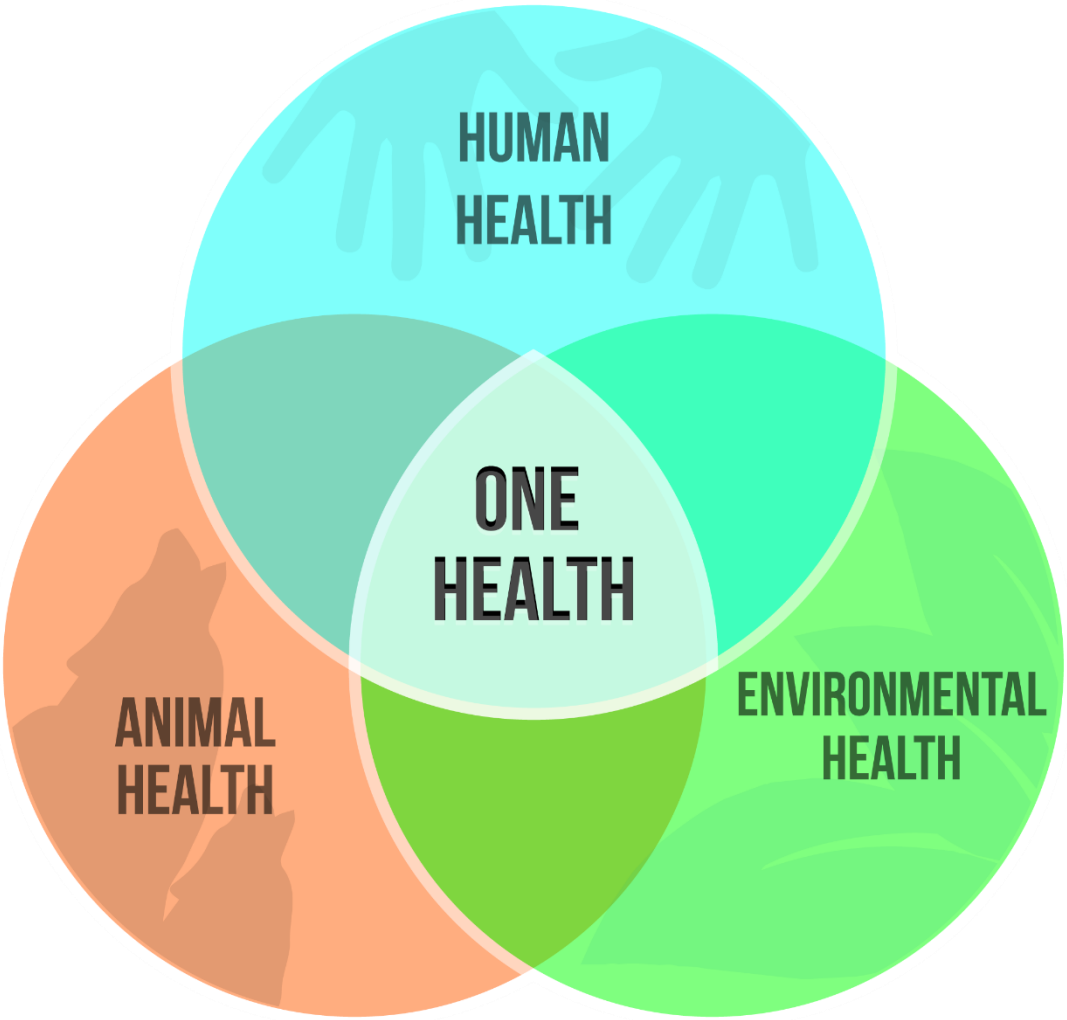

Figure I.3 - Diagram of the One Health concept (Soares JA®).

Understanding and addressing the health problems that rise in the middle of these links is the basis of this concept. The extensive use of antibiotics in animal production has started the concern of antibiotic resistance spread from animals to humans, and studies estimate that the most common route of bacterial transmission is through the food chain, characteristically due to an absence of hygiene while handling animals or animal products. The use of high doses of antibiotics in animal feed increases the number of antibiotic-resistant bacteria that may spread their genes into the environment after slurry disposal on fields (Cools et al., 2001a). Because of that, animal manure is considered a major source of antimicrobial resistant bacteria entering the environment (Aarestrup et al., 1996; Binh et al,. 2007; Ghosh and LaPara 2007). Related do that, studying resistance to different groups of antibiotics in a sentinel species like *E. coli* can give an

indication of the reservoir of resistances that is present in the farm bacterial population (Ibrahim et al., 2016). Also, Enterobacteriaceae owning genes for producing extended-spectrum β-lactamases (ESBL) are an emerging problem, and with great importance for public health. Because these bacteria show resistance to β-lactam antimicrobials, including third- and fourth-generation cephalosporins, which its categorized by the World Health Organization as critically significant for human medicine (WHO, 2012). Antibiotics used in farms for veterinary purposes or as growth promoters are excreted by the animals and end up in their effluents. Being animal effluents manure or slurry used as fertilizers, the antibiotics residues that can be presents will be transferred to the soil and in some cases can contaminate ground water (Xie et al., 2018). Soil antibiotic resistances could be related with clinical incidences of resistant infections (Graham et al., 2016). It´s not clear what is the fate of antimicrobials released in the environment and the mechanisms to contain or detoxify the environment from antimicrobials. However, it´s clear that farmers have an important paper, being their education on the subject essential (Bicudo and Goyal, 2003) The One Health approach may play a catalytic role for new research discoveries, the achievement of effective prevention responses, the development of health care education and better preparedness for zoonotic disease surveillance (Osburn et al., 2009; Rabozzi et al., 2012).

I.5 Conclusions

Adult milking cows can produce 7-8% of their body weight as manure per day (Font-Palma, 2019). Cattle manure ends mostly in the form of slurry. The best way to recycle this effluent is its application into the soil. This process has many advantages, but also can be a source of contaminations, the effectiveness of management systems needs to concern not only the reduction of GHG emissions but also environmental transmission of pathogens that can be present in animal effluents and transmitted to the environment. Because of that, proper manure management systems must be used to minimize environmental impacts. The best way to fix and to do research on this field is to consider the interdisciplinary collaboration to achieve health for humans, animals and the environment.

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Chapter II

Objectives

Objectives

The main objective of this research is to contribute to the understanding of survival of the pathogenic microorganisms on animal manure under ambient conditions. To accomplish this general objective, several specific goals were achieved, particularly:

1. Evaluate the impact on *Escherichia coli* capacity of survival in treated cow slurry during storage. This goal was accomplished by performing one laboratory essay during three months.

2. Evaluate *Enterococcus* survival in treated cow slurry during storage. This aim was accomplished by performing one laboratory essay during three months.

3. Evaluate *Salmonella enterica* presence or absence in treated cow slurry during storage process. This specific aim was achieved by performing one laboratory essay during three months simulating slurry storage at two different temperatures.

4. Perform one screening of water quality in dairy farms. This objective was accomplished by performing assessing water quality in thirty-two different dairy farms in four regions of Portugal.

Chapter III

Impact of Different Treatments on *Escherichia Coli* During Storage of Cattle Slurry

III. Impact of different treatments on *Escherichia coli* during storage of cattle slurry

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III. Abstract

There are different types of effluents from farming operations including untreated slurry (a mixture of manure, urine, split feed, and water), and treated slurry that normally is filtered to separate the solid fraction from the liquid fraction. With the amount of slurry applied on the soils as fertiliser every year, there are necessary to measure the leaching of microbial capable of transmitting infective agents that can be normally on slurry, because slurry can be a potential biohazard capable of transmitting infective agents. The aim of this study was to investigate the presence and survival of *Escherichia coli* (*E. coli*) on liquid fraction of dairy slurry, with the addition of different treatments during storage, such as addition of Biochar, beneficial microorganisms, sulphuric acid and the combinations of them. All the applied treatments to slurry show statistically significant differences ($P < 0.001$), according to the different sampling dates. Results showed that there are conditions and treatments that benefit the survival probability of *E. coli*, the treatments that include the acidification of slurry have the highest averages of CFU/ml (243288.3 at 37 °C; 136584 at 44 °C). These results can contribute to the improvement of the quality of slurry applied on soils, beneficiating agriculture but also public health.

Keywords: *Escherichia coli*, survival analysis, cattle, slurry

III.1 Introduction

Livestock effluents have long been used as organic fertilizers. However, in recent years, the growth of concentration of animal production in certain areas has been responsible for the production of large volumes of livestock effluents that if where not managed correctly there can be significant risks that can effect humans, animals and the environment. The animal manures including poultry, cattle, and swine are the primary source of water resources contamination by water-borne pathogens, as well as *Escherichia coli* (*E. coli*) or *Salmonella* sp. (Collins et al., 2005; Cardoso et al., 2012). The concentration of fecal indicator microorganisms contained in animal manures are variable depending on the manure type, the storage conditions and environmental factors (Hodgson et al., 2016). Every year, one billion tones of animal manure and slurry are produced in the Europe, consequently, in the last years the management of slurry was become one of the central dairy activities in farms (Fangueiro et al., 2014). With the amount of slurry applied on the soils as fertiliser every year, there is a need of studies to measure the leaching of microbial agents normally presents in slurry (Hodgson et al., 2016; Krog et al., 2017). The contamination with gastrointestinal pathogenic bacteria is a serious public health concern, because exposure to these microorganisms can result in severe animal and human diseases (Kudva et al., 1998; Mead et al., 1999; Cardoso et al., 2012).

The aim of this study is to investigate the presence and pathogen survival of *E. coli* on liquid fraction of dairy slurry during the storage process for which they were applied different treatments such as addition of Biochar, beneficial microorganisms and acidification.

III.2 Material and Methods

Slurry

The cattle slurry was collected from a dairy farm in Vila Real (Portugal). A hundred Kg of whole slurry (WS) was subjected to solid-liquid separation by screeners to obtain two fractions, a liquid (LF) and a solid fraction (SF). The LF was preserved at 4 °C in plastic barrels until required. This fraction was analysed for the presence of *E. coli*, total coliforms and *Salmonella* sp., prior to inoculation to ensure that they were *Salmonella* free.

Slurry Treatments

The liquid fraction obtained was placed in 2 L Kilner jars. Each jar was filled with 1 L of LF and a total of seven treatments were considered in this study:

1. LF + Biochar + *E. coli*
2. LF + Sulphuric acid + *E. coli*
3. LF + Beneficial microorganisms + *E. coli*
4. LF + Biochar + Beneficial microorganisms + *E. coli*
5. LF + Biochar + Sulphuric acid + *E. coli*
6. LF + Sulphuric acid + Beneficial microorganisms + *E. coli*
7. LF + Biochar + Sulphuric acid + Beneficial microorganisms + *E. coli*

Two controls were used one control was constituted by untreated liquid slurry and the other containing *E. coli*. All controls were maintained in the same conditions as the jars with the treated slurry. Three replicates of each treatment and controls were considered for microbiological analysis. In the treated slurries with Biochar (Piroeco Bioenergy, S.L®), this was added 4.5% in slurries and it was derived from wood shavings (2 mm diameter) pyrolised in a muffle furnace at 900 °C. The acidification of the liquid fractions to pH 5.5, were performed by addition of concentrated sulphuric acid (H_2SO_4, 96%) at a rate of 4.5 ml kg-1 slurry. Continuous stirring and pH measurement were performed during the acidification process. The addition of beneficial microorganisms (Bio buster, Bio-Systems Europe, England) was performed following the manufacturer's instructions. In the combined treatments Biochar, beneficial microorganisms and concentrated sulphuric acid added as previously described, being acidification made after the addition of the other treatments.

Slurry Inoculation with E. coli

To inoculate liquid slurry with *E. coli*, an isolated colony was used. Which it was previously characterized as *E. coli* with hemolysis by microbiological methods, obtained from the microbial analysis applied to slurry before the treatments. For the determination of the presence *E. coli* in slurry, 1 ml of the sample were diluted in 9 ml of sterile saline solution (1:10, w/v). Each diluted sample was plated in Brilliance™ *E. coli*/coliform Selective Agar (OXOID®) by incorporation and incubated at 37 °C for

24h and confirmed by API 20E (bioMérieux®, Marcy l'Etoile, France). *Escherichia coli* concentration in agar plates was calculated to create suspensions in sterile saline solution (1:10, w/v) with concentration 0.5 in McFarland standard scale (109 CFU/ml) using DensiCHEK® Plus 21255-P1ML1 instrument and then 1 ml of the culture was inoculated in each treatment. Using suspensions of such high concentrations allowed observation of the sanitization effectiveness of the selected methods under conditions of the extreme level of slurry contamination (Bauza-Kaszewska et al., 2015). *Escherichia. coli* was inoculated in liquid fraction of dairy slurry after the addition of Biochar, sulphuric acid and beneficial microorganisms. *E. coli* suspension was introduced directly into the jars, except in control jar only with LF. The jars were kept at 20 °C during the storage.

Microbiological Analysis

Samples for the microbiological analysis were collected before the storage and in different time intervals for the period of 90 days (0, 3, 30, 60 and 90 days). From the obtained initial liquid fraction and for each treatment, 2 g were placed in a sterile flask and immediately transported in a cooled (4 °C) transport box to the microbiology laboratory.

The screening for the presence/absence of *Salmonella* sp. was determined only from the liquid fraction of the cattle slurry before the addition of treatments, according to standard procedures (ISO 6579:2002). First, the sample was inoculated in Buffered peptone water (BPW - pre-enrichment in non-selective liquid medium) at room temperature, following the incubation in Modified semi-solid Rappaport- Vassiliadis (MSRV medium, Scharlau® - Enrichment on selective semisolid medium) contained 20 mg/l of novobiocin (OXOID®) at 42 °C (Olszewska and Skowron., 2013). From the culture obtained, selective solid media are inoculated: Xylose Lysine Desoxycholate Modified agar (VWR®), Hektoen Enteric agar (SIGMA-ALDRICH®) to verify the growth of *Salmonella* sp. In addition, the samples were incubated in MacConkey agar (VWR®), for total coliforms, which the pure culture of *E. coli* produced lactose fermenting colonies and pure culture of *Salmonella* sp. produced colourless colonies.

As previously described, the determination of the presence *E. coli* in slurry was performed using the Brilliance™ *E. coli*/coliform Selective Agar (OXOID®). The plates were incubated under aerobic conditions at 37 °C (total coliforms/*E. coli*) and 44 °C (*E. coli*), for 48h. The number of microorganisms was expressed as colony forming units

(CFU) per millilitre of slurry. Isolates were characterized phenotypically by Gram staining and biochemical characterization and species identification were performed using IMViC (Indole, Methyl red, Voges-Proskauer and Citrate), triple sugar iron agar, nitrate reduction, urease, oxidase and catalase tests. In addition, API 20E identification system (bioMérieux®, Marcy l'Etoile, France) was also performed, according to the manufacturer's instructions.

Slurry Analysis

Whole slurry and fractions obtained were analysed in terms of dry mater DM content, pH value, electrical conductivity (EC), nitrogen (N), NH_4^+ content, NO_3^- content, carbon (C), sulphur (S), potassium (K) and phosphorus (P). The DM content was set by drying samples in an oven at 105 °C for 48 h. The pH value was analysed with a pH meter using fresh samples extracted with distilled water (1:5 w/v) and EC was measured in the same extract (filtered through Whatman No. 2 filter paper). The mineral N content was extracted with 0.01M $CaCl_2$ (1:10 w/v). The NH_4+ and NO_3^- contents in the extracts were measured by segmented flow analysis (San Plus, Skalar, Breda, NL), based on the Barthelot reaction for NH_4+ and on the Griess-Ilosvay's reagent after the reduction to NO_2 in a cadmium column for the NO_3^- (Houba et al., 1995). Total carbon was determined by dry combustion; there after near infrared detection was used with a Carbon-Nitrogen/Protein analyser (Primacs SN 22, Slakar, Breda; NL). Sulphur was determined after digestion with concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) followed by turbi-dimetric segmented flow analysis. Potassium content was quantified after hydrochloric acid (HCl) treatment of the ash through graphite furnace atomic absorption spectrophotometry (Unicam M Series™). Phosphorous was determined using the ammonium vanadomolybdate method by molecular absorption spectrophotometry (Hitachi High-Tech Science Corporation, Japan 2000™). These screenings were also performed in three replicates of each treatment and controls after 90 days of slurry storage.

Statistical Analysis

Data from *E. coli* plate count after log10 transformed was subjected to ANOVA complete randomized design and using a split plot layout, with treatment type as main plot factor and dates as the subplot factor. Slurry data was also subjected to ANOVA considering a complete randomized design with a single factor (treatment types). When

factors and their interaction were statistically significant ($P < 0.05$) means were separated by the post hoc comparisons Tukey test ($P < 0.05$). All these analysis were performed using Statistix 10 (Analitycal Software version™ 10.0 (2005), Tallahassee, FL).

III.3 Results and Discussion

Before the beginning of the experiment, the main physic-chemical characteristics of whole slurry, liquid fraction and solid fraction, were respectively: 8.48, 4.55 and 13.69% of DM; a pH value similar in the three materials ≈8; an electrical conductivity of 4.73, 3.46 and 2.12 mS/cm; a total N of 2.70, 1.79 and 2.79 g N L^{-1}; 213.1, 159.0 and 77.7 mg NH_4^+ L^{-1}; an NO_3^-content below the detectable level (< 0.01); a carbon content of 397.5, 351.2 and 392.2 g kg^{-1}; 5.33, 8.44 and 3.73 g P kg^{-1}; 34.2, 59.1 and 18.5 g K kg^{-1}; 3.7, 5.7 and 3.6 g $kg^{-1}$of (S). At the end of the storage period, the same parameters have been evaluated for each treatment and controls. The different treatments applied to slurry showed statistically significant differences for the parameters analysed (DM; pH; EC; N; NH_4^+; C; P; K and S) ($P < 0.005$) (Table III.1). As expected the pH value was lower and S content was higher for the treatments where sulphuric acid was applied.

Those treatments showed the highest means for NH_4^+ and total N, data that corroborate other studies saying that slurry acidification can increase its fertiliser value (Fangueiro et al., 2014; Owusu-Twum et al., 2017; Kai et al., 2008). However, the highest P values were detected in the two controls of the experiment. In addition, the most conservative treatment for K was the treatment with beneficial microorganisms (65.5 g kg^{-1}), which showed to be significantly different from treatments combining Biochar + Acid (with or without Beneficial microorganisms).

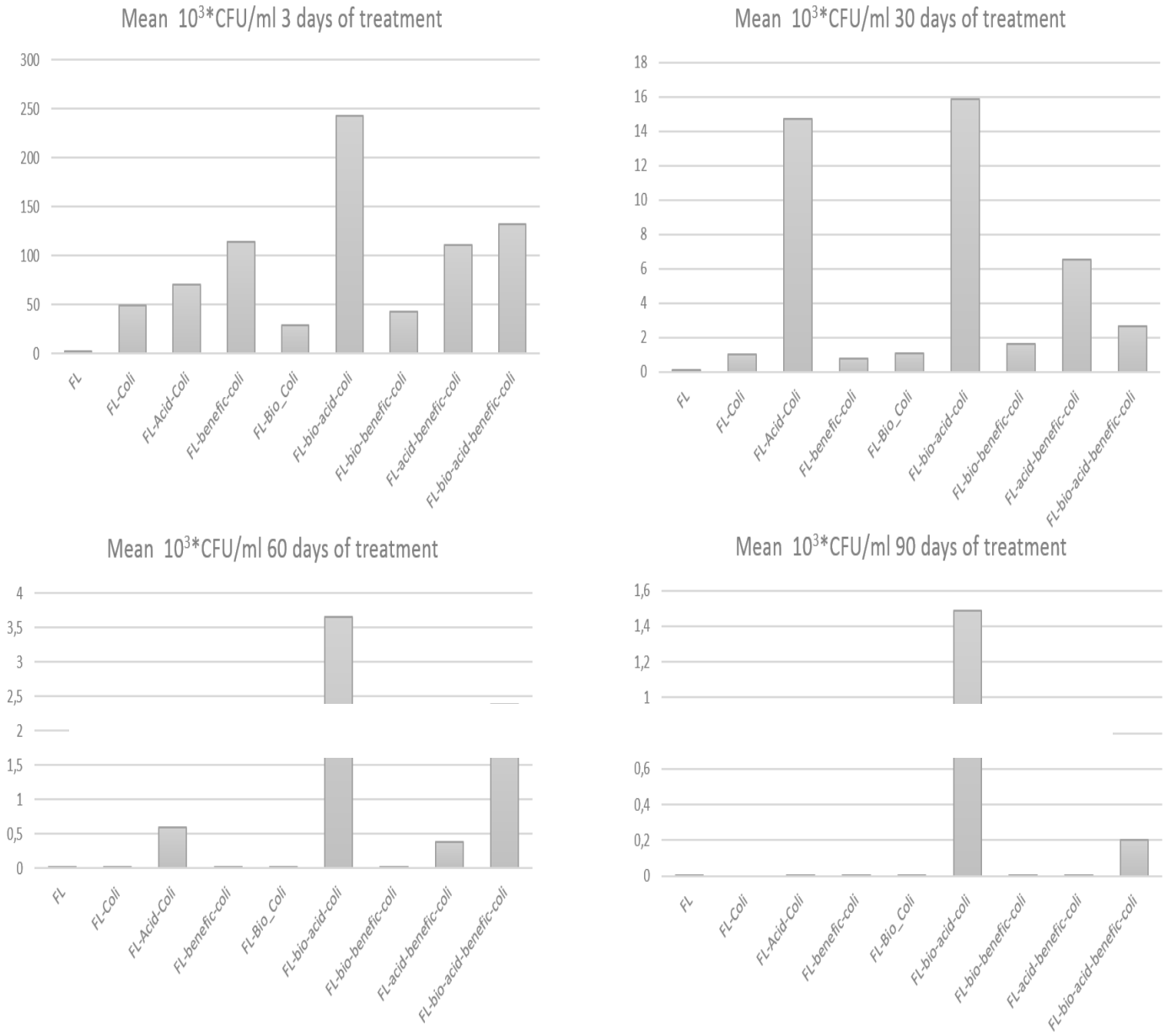
In the analysis made to LF of slurry before treatments and storage, there was no evidence of the presence of *Salmonella* sp.; however, for *E. coli*, a count of 60 000 CFU/ml was obtained. During the three months of cattle-storage, there was a marked decrease in CFU/ml for survival of *E. coli*, for all treatments (Figures. II.1 and II.2). In the different sampling dates, the different treatments applied to slurry revealed statistically significant differences for the CFU/ml ($P < 0.001$) (Tables III.2 and III.3).

Table III.1- Main physical-chemical characteristics of the effluents at the end of the experiment (90 days of storage).

Treatments	**DM %**	**PH**	**EC**	**NH_4^+ (mg L^{-1})**	**NO_3^- (mg L^{-1})**	**N total (g L^{-1})**	**C org (g kg^{-1})**	**S (g kg^{-1})**	**P (g kg^{-1})**	**K (g kg^{-1})**
LF	5.14^{B}	8.82^{A}	3.10^{B}	45.19^{BC}	< dl	0.65^{B}	300.30^{B}	6.07^{C}	9.04^{A}	60.41^{AB}
LF+ *E.coli*	5.76^{AB}	8.86^{A}	2.44^{B}	9.88^{C}	< dl	0.67^{B}	307.91^{B}	6.30^{C}	9.18^{A}	56.72^{ABC}
LF+Acid+Benefic + *E.coli*	6.00^{AB}	6.47^{B}	5.49^{A}	131.11^{AB}	< dl	1.90^{A}	263.82^{B}	52.88^{A}	5.83^{BC}	52.50^{ABC}
LF+Acid+ *E.coli*	6.81^{AB}	6.50^{B}	4.12^{AB}	154.02^{A}	< dl	2.08^{A}	246.56^{B}	56.34^{A}	6.54^{B}	52.64^{ABC}
LF+Bio+Acid+Benefic+ *E.coli*	10.65^{A}	6.71^{B}	5.65^{A}	131.59^{AB}	< dl	1.58^{A}	475.38^{A}	35.20^{B}	3.64^{D}	38.13^{BC}
LF+Bio+Acid+ *E.coli*	9.33^{AB}	6.87^{B}	4.90^{A}	78.12^{ABC}	< dl	1.58^{A}	493.90^{A}	30.55^{B}	3.64^{D}	33.23^{C}
LF+Bio+Benefic+ *E.coli*	7.32^{AB}	8.98^{A}	3.13^{B}	10.23^{C}	< dl	0.93^{B}	507.96^{A}	5.19^{C}	5.29^{BC}	51.97^{ABC}
LF+Bio+ *E.coli*	8.72^{AB}	8.99^{A}	2.74^{B}	11.62^{C}	< dl	0.97^{B}	529.83^{A}	4.82^{C}	4.83^{CD}	45.54^{ABC}
LF+Benefic+ *E.coli*	5.01^{B}	8.91^{A}	2.75^{B}	12.76^{C}	< dl	0.60^{B}	305.94^{B}	6.53^{C}	8.47^{A}	65.49^{A}

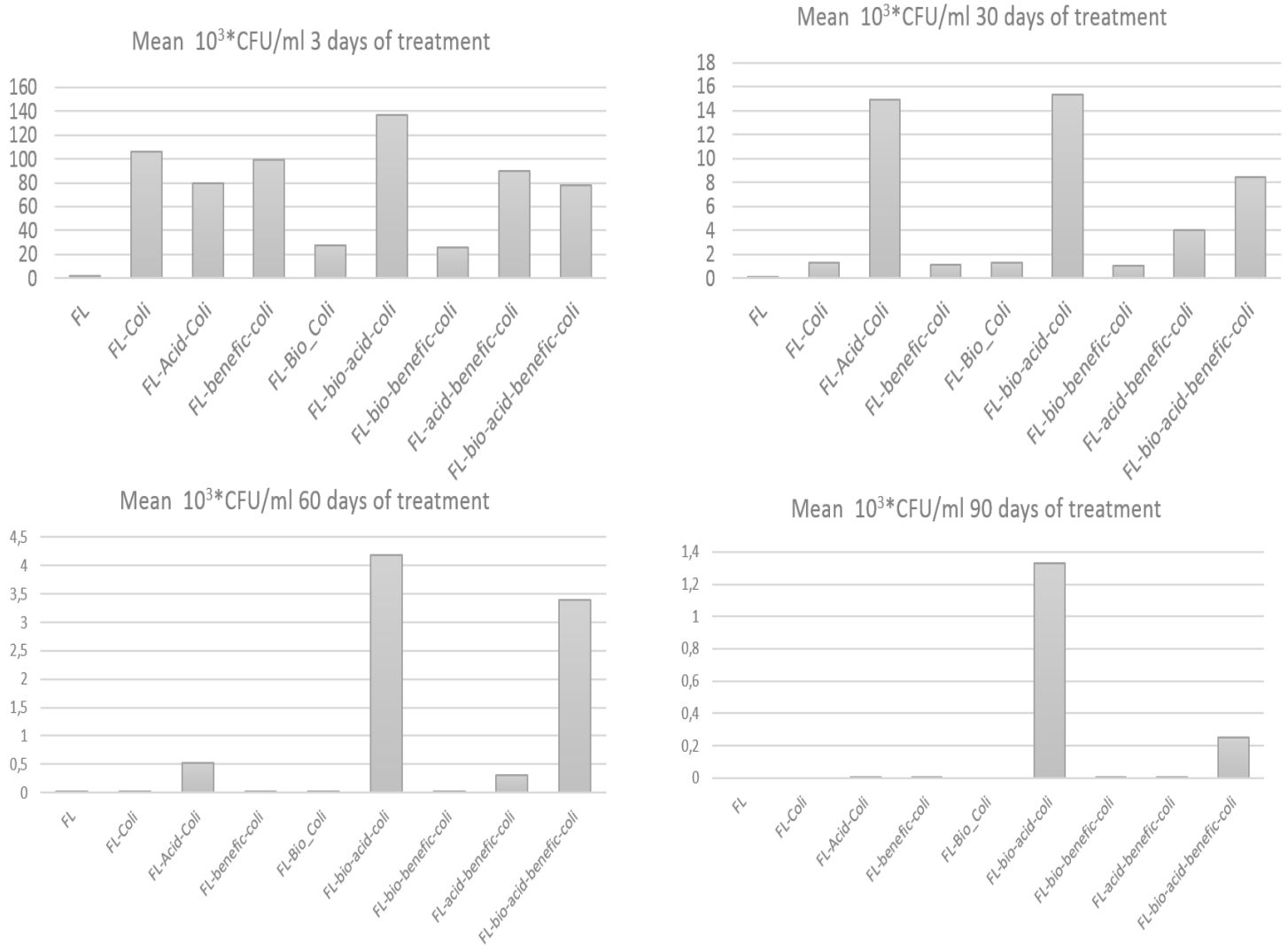
LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; *E. coli*: *Escherichia coli*; DM: Dry Matter Content; <dl: bellow detectable levels Values are the mean of three replicates; Means followed by different letters are significantly different (P<0.05) using HSD Tuckey mean separation test.

However, these results showed that there are conditions and treatments that benefit the survival probability of *E. coli*. The samples incubated at 37 ºC like LF treated with Biochar + Sulfuric acid demonstrated the highest means, 243 288 CFU/ml, 15 874 CFU/ml, 3 654 CFU/ml and 1 486 CFU/ml, at 3, 30, 60, 90 days of storage, respectively (Figure III.1). Similar results were obtained in the treatment with LF + Biochar + Sulfuric acid for the *E. coli* survival at 44º C – 136 584 CFU/ml, 15 326 CFU/ml, 4 175 CFU/ml and 1 328 CFU/ml at 3, 30, 60, 90 days after the storage, respectively (Figure III.2). In this study, the treatments that include the acidification of slurry have the highest averages of CFU/ml, corroborating with studies that tested the survival and resistance of *E. coli* on acid mediums and in processes based on pH reduction (Conner and Kotrola, 1995; Lin et al., 1996; Presser et al., 1997 Nyambe et al., 2017).



FL: Liquid fraction Acid: acidification; benefic: beneficial microorganisms; bio: Biochar.

Figure II. 1 - Graphic representation of the means of 10^3* CUF/ml for *E. coli* at 3, 30, 60, 90 days of slurry storage, incubated at 37 °C.



FL: Liquid fraction Acid: acidification; benefic: beneficial microorganisms; bio: Biochar

Figure II.2 - Graphic representation of the means of 10^3* CUF/ml for *E. coli* at 3, 30, 60, 90 days of slurry storage, incubated at 44 °C.

Table III.2 - Results of log means CFU of *E. coli*/ml of slurry (37 °C) in the different sampling dates.

Treatment	**3 days**	**30 days**	**60 days**	**90 days**
LF+Bio+Acid+*E.coli*	5.39^{A}	4.20^{A}	3.56^{A}	3.17^{A}
LF+Bio+Acid+Benefic+ *E.coli*	5.12^{A}	3.43^{AB}	3.38^{A}	2.31^{A}
LF+Benefic+ *E.coli*	5.06^{A}	2.87^{BC}	0.64^{BC}	0.14^{B}
LF+Acid+Benefic+ *E.coli*	5.04^{A}	3.82^{AB}	2.58^{A}	0.48^{B}
LF+Acid+ *E.coli*	4.85^{A}	4.17^{A}	2.77^{A}	0.26^{B}
LF+ *E.coli*	4.69^{A}	3.00^{BC}	0.04^{C}	0.00^{B}
LF+Bio+Benefic+ *E.coli*	4.63^{A}	3.20^{AB}	0.70^{BC}	0.10^{B}
LF+Bio+ *E.coli*	4.46^{A}	3.03^{BC}	1.23^{B}	0.00^{B}
LF	3.30^{B}	1.95^{C}	0.69^{BC}	0.00^{B}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; *E.coli*: *Escherichia coli*.
Means followed by different letters are significantly different ($P < 0.05$) using HSD Tuckey mean separation test.

Table III. 3 - Results of log means CFU of *E. coli*/ml of slurry (44 °C) in the different sampling dates.

Treatment	**3 days**	**30 days**	**60 days**	**90 days**
LF+Bio+Acid+ *E.coli*	5.14^{A}	4.18^{A}	3.62^{A}	3.12^{A}
LF+Bio+Acid+Benefic+ *E.coli*	4.89^{A}	3.93^{AB}	3.53^{A}	2.40^{A}
LF+Benefic+ *E.coli*	4.99^{A}	3.04^{B}	0.10^{F}	0.04^{B}
LF+Acid+Benefic+ *E.coli*	4.96^{A}	3.60^{AB}	2.48^{BC}	0.54^{B}
LF+Acid+ *E.coli*	4.90^{A}	4.17^{A}	2.72^{AB}	0.16^{B}
LF+ *E.coli*	5.02^{A}	3.10^{B}	1.19^{DE}	0.00^{B}
LF+Bio+Benefic+ *E.coli*	4.42^{A}	3.03^{B}	1.45^{CD}	0.04^{B}
LF+Bio+ *E.coli*	4.43^{A}	3.10^{B}	0.35^{EF}	0.00^{B}
LF	3.33^{B}	1.80^{C}	0.79^{DEF}	0.00^{B}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; *E.coli* : *Escherichia coli.*
Means followed by different letters are significantly different ($P < 0.05$) using HSD Tuckey mean separation test.

In the last years, there was an intensification of animal and agriculture in the world. For this intensification to be sustainable, the use of natural resources and ecosystem services need to be tenable (Rizzo and Giuffré, 2018). One of the major problems of intensive animal production is the quantity of animal effluents produced in small areas. Manure represents one of the most valuable organic fertilizers, however it could contain a large number of microorganisms that can have pathogenic potential as *E. coli* (Ferens and Hovde, 2011; Nolan et al., 2018).

The liquid fraction contain a significant part of the total bacteria of slurry (>80%), including the pathogen indicator organisms such as *E. coli* (Popovic et al., 2011). The understanding of microbial survival in manure, and in the soil represents a way to minimizing borne pathogenic risk to environment, animals and humans (Hodgson et al., 2016; Hruby et al., 2018). In the study of Ottosen et al. (2009) is was described that slurry acidification is capable to reduce the microbial activity, however in the work of Zhang et al. (2011) was referred that in some conditions, acidification have a weaker effect on pathogenic bacteria.

Escherichia coli is capable to survive much longer in soil than in the manure representing a major risk to contaminate water or vegetable (Islam et al., 2004; Hruby et al., 2018). Studies refereed also that *E. coli* survives longer than other slurry pathogens on storage processes (Biswas et al., 2016). According to Hodgson *et al.* (2016), a significant increase of *E. coli* was observed when slurry was applied to grassland via shallow injection compared to surface application and the decay rates were significantly higher for slurry applied to grassland in spring relative to summer and autumn (Hodgson et al., 2016). Some experiments showed, that the survival rate of *E. coli* in soil was related to the local pH; moreover, *E. coli* O157:H7 owns systems for survival at low pH (Sang et al., 2000; Van Elsas et al., 2011).

Other studies suggest as well that the temperature could be one of the most important factors conditioning bacteria survival in the slurry, having high temperatures the most effective effect on the pathogens elimination (Chadwick et al., 2011). With the temperature used in the present study (storage at 20 °C), it was predictable that a CFU/ml bacteria reduction happened.

To minimize slurry environmental effects, some treatments can be considered, like mechanical separation, acidification or additives addition (Chadwick et al., 2011; Owusu-Twum et al., 2017). The treatments used in our study are commonly used in farms because they are beneficial to the soil and to minimize *greenhouse gases*, for

example acidification, used to reduce ammonia emissions when slurry is applied to soil (Kai et al., 2008).

However, according to our study acidification is not efficient to eliminate *E. coli.* For the three treatments used in our study, such as Biochar and beneficial microorganisms, there are no scientific literature testing bacteria survival. Additional studies focused on the evaluation of different pathogens survival correlated with different slurry treatments are needed, as well studies that evaluate pathogen survival on treated slurry applied to soil.

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Chapter IV

Enterococcus spp. Survival During Storage of Treated Cattle Slurry

IV. *Enterococcus spp.* survival during storage of treated cattle slurry

Chapter submitted as an original paper in a SCI journal, Q2 Impact factor: 1.275:

Soares A.S., C. Miranda, C.A. Teixeira, J. Coutinho, H. Trindade, A.C. Coelho, (2019). *Enterococcus* spp. survival during storage of treated cattle slurry. *Animal Production Science, Manuscript ID AN19455*

IV. Abstract

The presence and abundance of *Enterococcus* in slurry´s is a known fact. When the slurry is used to fertilize fields, bacteria and antibiotic resistance genes presents in this slurry can be transmitted to the environment. Based on this virulence factors production and there high antibiotic resistance *Enterococcus* spp. are considered emerging pathogens. The aim of this study was to evaluate the presence and survival of *Enterococcus* spp. on the liquid fraction of cattle-slurry, during the storage process with the appliance of different treatments. These treatments have been investigated to reduce the Greenhouse Gas (GHG) emissions but the effect of them on slurry microorganisms is acknowledged. For the determination of the presence of *Enterococcus* spp. in slurry, Slanetz and Bartley Medium (Oxoid®) was used. The number of microorganisms was expressed as colony forming units (CFU) per gram of slurry. The different treatments applied to slurry according to the different sampling dates (30, 60 and 90 days) showed statistically significant differences for the CFU/ml ($P < 0.001$). In the literature, the treatment of slurry with acid has the propose to maintain low the GHG emissions, however, in this study the treatments containing added acid have the highest bacterial growth during the three months of storage.

Keywords: Slurry storage process, *Enterococcus*, survival analysis, cattle

IV.1 Introduction

The intestinal microbiota of animals and humans is constituted by a multiple set of bacteria. Most of these microorganisms are beneficial to intestinal motility. The *Enterococci* have long been known as commensals of the gastrointestinal tract and as one of the present bacteria in the environment (Fisher and Phillips, 2009; van Schaik and Willems, 2010; John and Carvalho, 2011). These Gram-positive bacteria can growth under a variety of hostile conditions. *Enterococcus* spp. are facultative anaerobic organisms which can survive in high salt concentrations and survive in pH extremes (as low as 4.8 and as high as 9.6 (Huycke et al., 1998; Fisher and Phillips, 2009; van Schaik and Willems, 2010).

For many years, *Enterococcus* species were believed to be harmless to humans and unimportant medically. Although these bacteria do not produce toxins, have virulence factors in the form of aggregation substances, so they can cause disease based in the way they adhere to the host tissues. Based on this virulence factors production and there high antibiotic resistance *Enterococcus* are now considered emerging pathogens (Vu and Carvalho, 2011; Aslam et al., 2012; Guerrero-Ramos et al., 2016; Ahmed and Baptiste, 2017). In the last years, *Enterococci* gain medical importance in nosocomial, iatrogenic and in bacteraemia infections has grown, they are the cause of urinary tract infections, endocarditis, meningitis, wound infections, abdominal and pelvic infections (Vu and Carvalho, 2011; Ahmed and Baptiste, 2017).

Based on their abundance in faeces of mammals and their long survival in the environment *Enterococci* are commonly used as faecal indicators in water management and in studies that focused on these microorganisms as indicators of the source of faecal pollution. The presence and abundance of *Enterococcus* in slurry´s is a known fact (Manero et al., 2002). When slurry, resultant from animal production is used to fertilize fields, bacteria and antibiotic resistance genes presents in the slurry can be transmitted to the environment (Sengeløv et al., 2001; Wallace et al., 2018).

Animal production is responsible for important gaseous emissions including the emission of greenhouse gases (GHG) that are considered one of the main causes of climate change (Oenema et al., 2009). One of the most prominent source of greenhouse gas and NH_3 emissions, in the livestock production, is the manure management including animal housing, handling, storage, and application to the soil (Mohankumar Sajeev et al., 2017).

Manure treatments are the most common strategy to ensure lower emissions. Application of additives to slurry, like acids and biochar, has been used with the aim to affect chemical and biological composition of the effluent, reducing the gaseous emissions and enhancing degradation of organic material (Owusu-Twum et al., 2017b). Acidification is used to maintain the pH of manure low (< 6), inhibiting the activity of urease-production bacteria, inhibiting methanogenesis and decreasing the activity of nitrifying bacteria (Fangueiro et al., 2015; Mohankumar Sajeev et al., 2017; Owusu-Twum et al., 2017a). Application of biochar as slurry treatment is essentially based on its capacity to mitigate N_2O fluxes, one of the most powerful GHG, and sequestering carbon when slurry is applied to soil (Bruun et al., 2011). To minimize slurry environmental effects, additives addition can also be considered (Chadwick et al., 2011).

The aim of this work was to evaluate the presence and survival of *Enterococcus* spp. during the storage process of cattle slurry liquid fraction treated by acidification, additive addiction and biochar.

IV.2 Material and Methods

Whole slurry (WS) was collected from a dairy farm in Vila Real (Portugal) and subjected to solid-liquid separation by sieving (0.5 mm) to obtain two fractions, a liquid (LF) and a solid fraction (SF). The LF was preserved at 4 ºC in plastic barrels until required. This fraction was analysed for the presence of *Escherichia coli*, total coliforms and *Salmonella* sp. prior to inoculation.

The liquid fraction obtained was placed in 2L Kilner jars. Each jar was filled with 1L of LF and seven treatments were considered in this study. One control containing untreated liquid slurry was used and maintained in the same conditions as the jars with the treated slurry. All the treatments were stored at 20 ºC +/- 0.5 ºC using an incubation chamber with temperature control.

In the treatments with Biochar (Piroeco Bioenergy, S.L®), this was added 4.5% in slurries and it was derived from wood shavings (2mm diameter) pyrolised in a muffle furnace at 900 °C. The treatments with acid were performed by addition of concentrated sulphuric acid (H_2SO_4, 96%) at a rate of 4.5 ml kg-1slurry (acidification of the liquid fractions to pH 5.5). Continuous stirring and pH measurement were performed during the acidification process. The adding of beneficial microorganisms (Bio buster, Bio-

Systems Europe®, England) was performed following the manufacturer's instructions. In the combined treatments Biochar, beneficial microorganisms and concentrated sulphuric acid were added as previously described, being acidification made after the addition of the other treatments. Three replicates of each treatment and control were considered for microbiological analysis.

Samples for the microbiological analysis were collected before the storage and at 3, 30, 60 and 90 days of storage. From the collected initial LF and all jars, 2 ml were placed in a sterile flask and immediately transported in a cooled (4 °C) transport box to the laboratory of Medical Microbiology, UTAD

The screening for the presence/absence of *Salmonella* sp. was determined only from the liquid fraction of the cattle-slurry before the addition of treatments, according to standard procedures (ISO 6579:2002). In addition, the samples were incubated in MacConkey agar (VWR®), for total coliforms, in which the pure culture of *E. coli* produced lactose fermenting colonies and pure culture of *Salmonella* sp. produced colourless colonies.

For the determination of the presence of *Enterococcus* spp. in slurry, Slanetz and Bartley Medium (Oxoid®) was used. The plates were incubated under aerobic conditions at 37 °C for 24/48 hours.

The number of microorganisms was expressed as colony forming units (CFU) per gram of slurry.

Isolates were characterized phenotypically by Gram staining, oxidase and catalase tests. In addition, presence of hemolysis and API 20 Strep identification system (bioMérieux®, Marcy l'Etoile, France) was also performed, according to the manufacturer's instructions.

Whole slurry and fractions obtained were analyzed in terms of dry mater (DM) content, pH value, electrical conductivity (EC), nitrogen (N), NH_4^+ content, NO_3^- content, carbon (C), sulphur (S), potassium (K) and phosphorus (P). These screenings were also performed in three replicates of each treatment and controls after 90 days of slurry storage. The methods used were previously described (Soares et al., 2019).

Data from *Enterococcus* plate count were log10 transformed and subjected to ANOVA complete randomized design and using a split plot layout, with treatment type as main plot factor and dates as the subplot factor. Slurry data was also subjected to ANOVA considering a complete randomized design with a single factor (treatment types). When factors and their interaction were statistically significant ($P < 0.05$) means

were separated by the post hoc comparisons Tukey test ($P < 0.05$). All these analyses were performed using Statistix 10 (Analitycal® Software version 10.0 (2005), Tallahassee, FL).

IV.3 Results and Discussion

In the analysis made to LF of slurry before treatments and storage, there was no evidence of the presence of *Salmonella* sp, for *E. coli*, a count of 60 000 CFU/ml and for *Enterococcus* a count of 5 000 CFU/ml were obtained.

Enterococcus are in general a robust bacteria that have the capacity to survive under extreme conditions (Cools et al., 2001b), the results obtained in this study corroborate previous findings. The different treatments applied to slurry according to the different sampling dates (30, 60 and 90 days) showed statistically significant differences for the CFU/ml ($P < 0.001$) (Table III.1).

Table VI.1 - Values of *Enterococcus* plate counts means (CFU/ml) at 0, 3, 30, 60 and 90 days of storage of the cattle slurry liquid fraction subjected to the different treatments. For the same day of storage, means followed by different letters are significant.

Treatment	**3 days**	**30 days**	**60 days**	**90 days**
LF+Bio+Acid+*E.coli*	5.19^{A}	5.42^{ABC}	5.87^{A}	4.77^{A}
LF+Bio+Acid+Benefic+ *E.coli*	5.77^{A}	5.69^{AB}	6.39^{A}	5.70^{A}
LF+Benefic+ *E.coli*	4.31^{A}	3.87^{BC}	1.61^{B}	1.84^{B}
LF+Acid+Benefic+ *E.coli*	5.06^{A}	6.19^{A}	6.20^{A}	4.68^{A}
LF+Acid+ *E.coli*	4.69^{A}	5.29^{ABC}	6.42^{A}	5.62^{A}
LF+ *E.coli*	4.26^{A}	3.76^{BC}	2.13^{B}	0.79^{B}
LF+Bio+Benefic+ *E.coli*	4.80^{A}	3.76^{BC}	1.83^{B}	1.54^{B}
LF+Bio+ *E.coli*	4.10^{A}	3.87^{BC}	2.13^{B}	1.20^{B}
LF	4.32^{A}	3.53^{C}	3.36^{B}	1.98^{B}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; *E.coli* : *Escherichia coli*.

Slurry acidification led to an increase of the means of *Enterococcus* plate counts in 60 and 90 days of storage (Figure VI.1; Table VI.1). The treatments applied to slurry are based on their efficacy for reducing the nutrient loss and pathogen loading, they have suffered a devolvement during last years (Wallace et al., 2018). Based on the already known presence of intestinal bacteria in slurry including *Enterococcus* (Manero et al., 2002), and on is capacity to transmit to the environment antibiotic resistance genes (Aslam et al., 2012; Guerrero-Ramos et al., 2016), as well on our results, this study shows the importance of evaluate correctly the conditions and the treatments applied to slurry before land application. Extensive surveillance, of the bacteria presents in animal slurry must be done to avoid public health concerns.

Before the beginning of the experiment, the main physic-chemical characteristics of whole slurry, liquid fraction and solid fraction, were espectively: 8.48, 4.55 and 13.69% of DM; a pH value similar in the three materials ≈8; an electrical conductivity of 4.73, 3.46 and 2.12 mS/cm; a total N of 2.70, 1.79 and 2.79 g N L^{-1}; 213.1, 159.0 and 77.7 mg $NH4^{+}$ L^{-1}; an $NO3^{-}$content below the detectable level (< 0.01); a carbon content of 397.5, 351.2 and 392.2 g kg^{-1}; 5.33, 8.44 and 3.73 g P kg^{-1}; 34.2, 59.1 and 18.5 g K kg^{-1}; 3.7, 5.7 and 3.6 g $kg^{-1}$of (S).

At the end of the storage period, the same parameters have been evaluated for each treatment and controls. The different treatments applied to slurry showed statistically significant differences for the parameters analysed (DM; pH; EC; N; NH4+; C; P; K and S) ($P < 0.005$) as demonstrated in Soares et al., (2019).

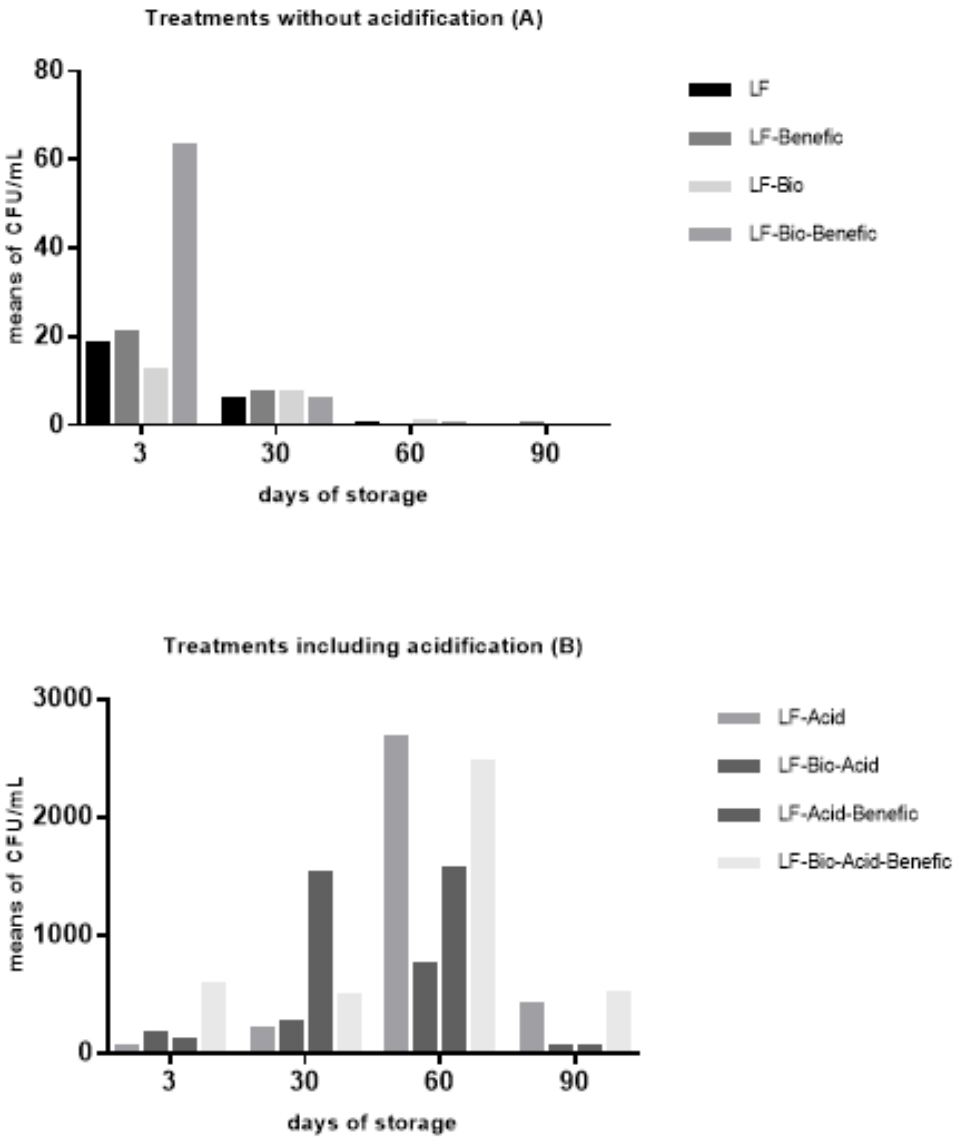

Figure VI. 1 - Graphic representation of the means of 10^3* CUF/ml for at 3, 30, 60, 90 days of slurry storage for the treatments without acidification and for treatments including acidification.

The treatment of slurry with sulphuric acid has the propose to reduce the NH_3 and GHG emissions (Owusu-Twum et al., 2017a). However, in our study the treatments containing acidification have the highest plate counts during the three months of storage, comparing to the treatments without acidification (Figure III.1). These results are difficult to explain. Probably, these treatments may be benefit to improve the

nutrient value of the slurry and for the control of emissions, but they are not benefit to eliminate intestinal microorganisms. Additional studies focused on the evaluation of different bacteria survival correlated with different slurry treatments are needed, as well studies that evaluate the survival of bacteria's on slurry applied to soil.

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Chapter V

Evaluation of *Salmonella enterica* Presence/Absence in Treated Cow Slurry During Storage

V. Evaluation of *Salmonella enterica* presence/absence in treated cow slurry during storage

Chapter submitted as an original paper in a SCI journal, Q2 Impact factor: 1.751:

Soares A.S., C. Miranda, H. Trindade, A.C. Coelho, (2019). Evaluation of *Salmonell*a sp. presence/absence in treated cow slurry during storage. *Research in Veterinary Science,* Manuscript ID RVSC_28

V. Abstract

The presence of *Salmonella* sp. on livestock is associated with a negative economic impact, both on the commercialization of live animals and their products. Infection occurs for long periods and this bacteria is shed by infected animals faeces, contaminating environment, food and water. *Salmonella* sp. in the slurry is a serious concern in public and animal health, also constituting a risk in the environment, and it is urgent to control this microorganism. The aim of this study was to evaluate the presence/absence of *Salmonella* sp. in treated cow slurry during storage at 4 °C and 20 °C. Samples for the microbiological analysis were collected before the storage and at days 3, 8, 15 30, 60, 80 and 90 after storage in all the treatments considered (Biochar, Acid (Sulphuric acid) and Acid + Biochar). The screening for the presence/absence of *Salmonella* sp. was determined from the liquid fraction of the cattle-slurry, based on standard procedures ISO 6579:2002. In the screening performed at the third day after storage, all samples showed positive results to the presence of *Salmonella* (at both temperatures). At day 15, there were already differences between the two temperatures, at 20 °C all samples Acid + Biochar were negative and at 4 °C all samples in all treatments were positive. At day 30, for the samples stored at 20 °C there was a decrease of presence of *Salmonella* sp., having only the presence of the pathogen in two samples of the acid treatment, situation that was maintained until day 90 after storage. All samples stored at 4 °C were positive until de the end of the study, being this temperate the less efficient to eliminate *Salmonella*. The results suggest that the highest temperature (20 °C) was more effective on the elimination of *Salmonella* sp., the treatment containing Sulphuric acid was the less efficient to eliminate the pathogen when stored at 20 °C.

V.1 Introduction

Salmonella sp. is a microorganism belonging to the Enterobateriaceae family, with a wide distribution in nature, with the man and animals being its main reservoirs. It is considered one of the main agents causing food outbreaks in developed countries. Birds and cattle are considered the main disseminators of this pathogen (Shinohara et al., 2008). The type of pathogens and levels of contamination of animal effluents vary with heath status, age and diet of the animals as well with the physical and chemical characteristics of the proper effluent. The survival time of this microorganism in the animal manures depends not only on the characteristics of microorganisms itself but also on the composition of manure (Hutchison et al., 2005; Spiehs and Goyal, 2007;Manyi-Loh et al., 2016).

Salmonellosis is considered the most widespread zoonosis in the world. As the transmission cycle of *Salmonella* involves practically all vertebrates, their control poses a challenge to public health in both developed and developing countries. Most *Salmonella* serovars associated with pathologies in animals are the strains: *S.* Dublin (bovines), *S.* Choleraesuis and *S.* Typhisuis (swine), *S.* Abortusequi (equines), *S.* Pullorum and *S.* Gallinarum (birds). However, since *Salmonella* is a zoonotic agent, some of its strains cause undifferentiated infection between humans and other animal species (Maurer, 2007).

Being *Salmonella* sp. the agent of an infection associated with production animals, it have a negative economic impact on the commercialization of these animals and their products, because when an animal present in a herd is considered positive, normally, the entire herd is treated as positive (Warnick et al., 2003; Magwedere et al., 2015) . As this infection have long periods of latency, it is possible that occurs release of bacterial cells from faeces of the infected animals, contaminating environment, food and water (Wakchaure and Ganguly, 2015; Tohidi et al., 2017).

Recently, slurry has achieved the greatest importance among the natural fertilizers. However, incorrect practices of management creates a potential microbiological risk for environmental and public health (Owusu-Twum et al., 2017b; Nolan et al., 2018). This effluent is a reservoir of a vast number of microorganisms as faecal streptococci, *Escherichia coli* and *Salmonella* sp. It is also possible the isolation or detection of other pathogenic microorganisms such as multiple virus, *Brucella*, *Mycobacterium*, *Leptospira*, *Chlamydia*, *Campylobacter* as well parasites (Guan and

Holley, 2003; Bornay-Llinares et al., 2006; Olszewska and Skowron, 2013a; Nolan et al., 2018).

Considering the variety of microorganisms present in slurry and their survival time, temperature and conditions of storage assume a particular importance (Guan and Holley, 2003). In the study of Diao et al. (2015), *S*. Typhimurium was survived in dairy compost with various particle sizes after a 30 days of storage under greenhouse condition (Diao et al., 2015). Chen et al. (2018) have observed that dairy compost can provide long-term survival conditions for *Salmonella enterica*, including survival for more than 168 days, at 5 ºC with high inoculum levels (Chen et al., 2018).

In the study of Biswas et al. (2016) it was showed a clear relation between the temperatures of storage and the *Salmonella* survival.

There were not many studies evaluating pathogenic survival time in treated animal effluents. The aim of this study was to evaluate the presence/absence of *Salmonella* sp. in treated cow slurry during storage at 4 °C and 20 °C.

V.2 Material and Methods

In this study, a liquid fraction of slurry were collected from a dairy farm (in November of 2017, in Vila Real, Portugal) and obtained from a mechanical separator.

The liquid fraction obtained was dived liter per liter and distributed 1 L in each Kilner jar. Three treatments and one control were considered, as well two different temperatures of storage 4 ºC and 20 ºC. The three treatments were Biochar, Acid (Sulphuric Acid) and Acid + Biochar. The control was constituted by 1 L of liquid fration of slurry and 1 ml of a turbidity of 0.5 McFarland solution of *Salmonella* sp. (~1 × 10^8 colony forming units (CFU)/ml). The treatment with Biochar is constituted by 1 L of liquid fraction of slurry and 1 ml of a turbidity of solution of *Salmonella enterica*. at 0.5 McFarland (~1 × 10^8 CFU/ml) plus 3-5% of Biochar (Piroeco Bioenergy, S.L®) approximately (it was derived from wood shavings (2 mm diameter) pyrolised in a muffle furnace at 900 °C). In the treatments with acidification, an addition of concentrated Sulphuric acid (H_2SO_4, 96%) at a rate of 4.5 ml kg^{-1} was performed until the liquid fractions reach pH 5.5. In the combined treatments, the Biochar and the acid, where added together in rates explained above. Four replicates of the treatments and control were considered for each one of the temperatures (Figure IV.1)

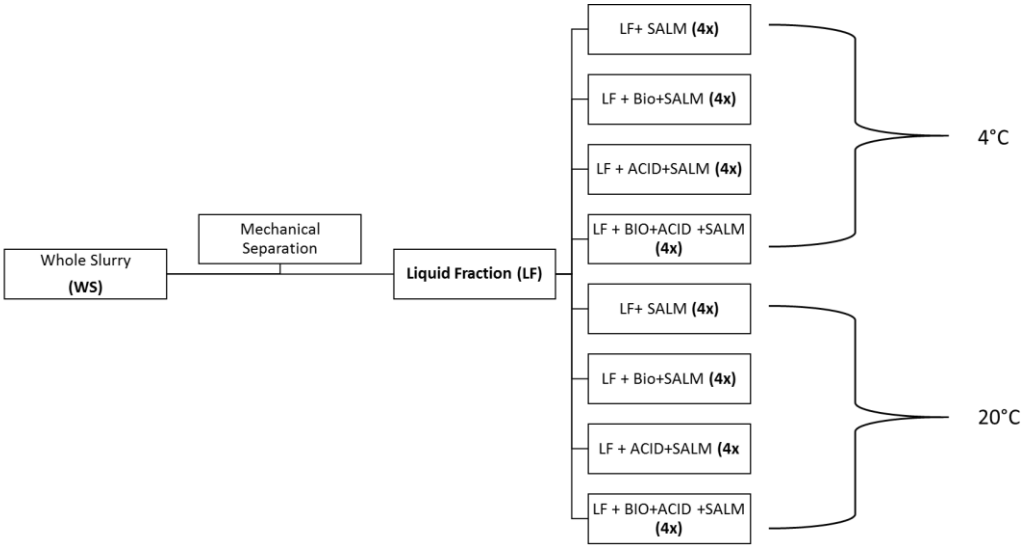


Figure V. 1 - Summary of the treatments applied to cattle-slurry.

LF- liquid fraction of slurry*; Salm- Salmonella enterica.;* Bio- Biochar; Acid- Sulphuric acid

To inoculate liquid slurry with *Salmonella enterica*., an isolated colony from the laboratory of medical microbiology was used, which it was previously characterized as *Salmonella enterica* by traditional microbiological methods. The bacteria concentration in agar plates was calculated to create suspensions in sterile saline solution (1:10, w/v) with concentration 0.5 in McFarland scale (10^8 CFU/ml), using DensiCHEK® Plus 21255-P1ML1 instrument and 1 ml of the culture was inoculated in each treatment.

Samples for the microbiological analysis were collected before the storage (to ensure that no *Salmonella* sp. were found in the input material), at days 3, 8, 15 30, 60, 80 and 90 after storage. The screening for the presence/absence of *Salmonella* sp. was determined from the liquid fraction of the cattle-slurry, based on standard procedures ISO 6579:2002, as stated by the type of sample, in other to the reliability of the results obtained. When the source of sample is faecal, is prudent to do successive stages, including a pre-enrichment in non-selective liquid medium followed by enrichment in selective liquid media, after that plating out and identification using at least two selective solid media and in the end the confirmation of identity (Mooijman, 2016).

First, the sample was inoculated in Buffered peptone water (BPW-pre-enrichment in non-selective liquid medium) at room temperature, following the incubation in Modified semi-solid Rappaport-Vassiliadis (MSRV medium, Scharlau® - Enrichment on selective semi-solid medium) contained 20 mg/l of novobiocin (OXOID®) at 42 °C (Olszewska and Skowron, 2013a). From the culture obtained, selective solid media are inoculated: Chromagar Salmonella agar®; Xylose lysine

deoxycholate agar (XLD agar)® and Salmonella-Sighella agar (SS agar®) to verify the growth of *Salmonella* sp. In addition, the samples were incubated in MacConkey agar (VWR®), which the pure culture *Salmonella* sp. produced colourless colonies. Isolates were characterized phenotypically by Gram staining and oxidase test was performed. In addition, API 20E identification system (bioMérieux®, Marcy l'Etoile, France) was also performed, according to the manufacturer's instructions (Standard, 2002).

The pH values and the weight of the different jars where verified fortnightly. The results of laboratorial detection of *Salmonella* sp. is based on a criteria of presence/absence in the samples (Standard, 2002).

V.3 Results and Discussion

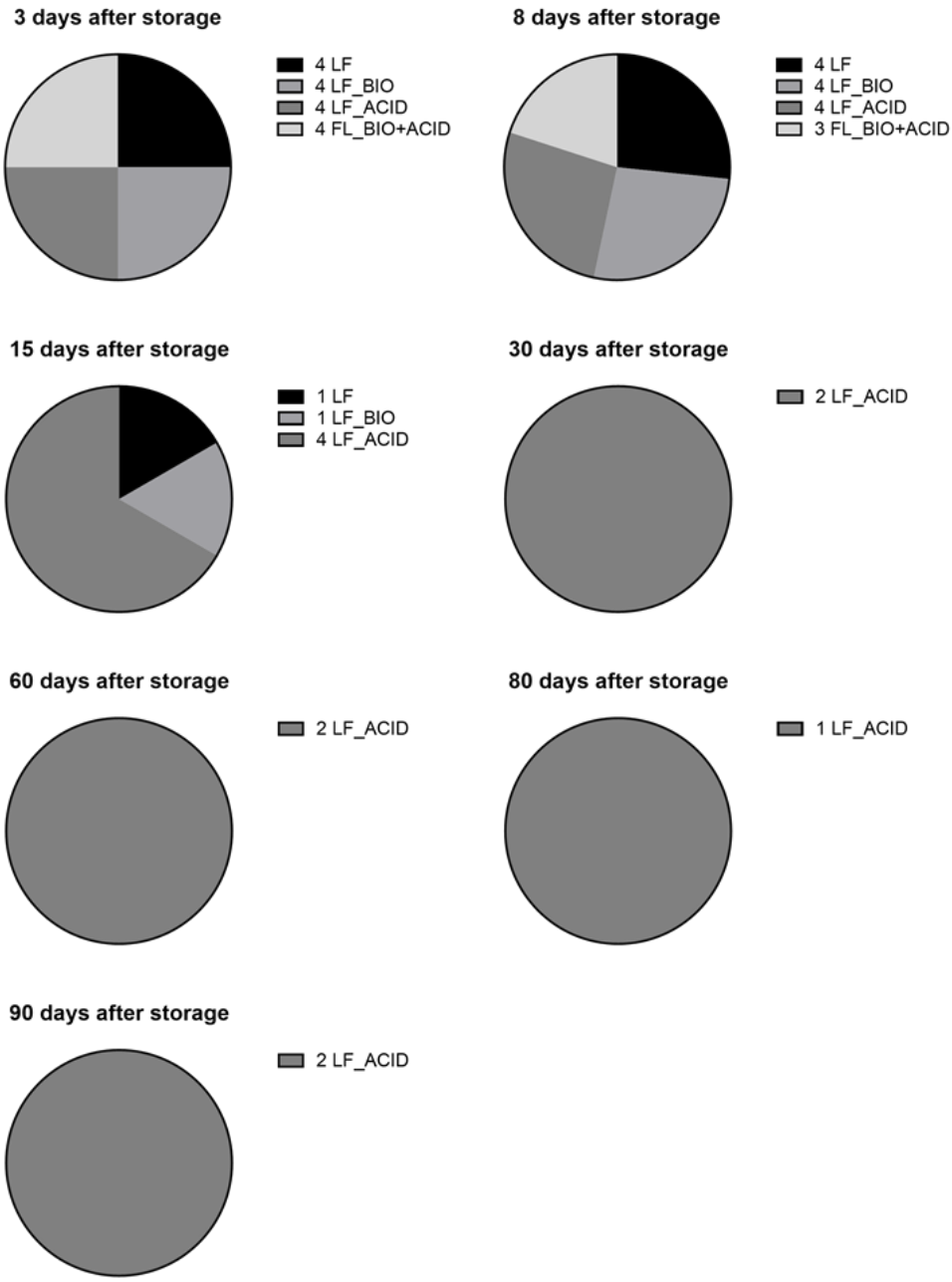
In the microbiological analysis made before the storage, no microorganisms of the *Salmonella* sp. were found in the input material. In the screening made 3rd day after storage, all the samples give positive results to the presence of *Salmonella* for both temperatures. At the 3rd screening (day 15) after storage, there were already differences between the two temperatures. At 20 °C all samples Acid + Biochar were negative, and at 4 °C all samples in the three treatments were positive. At day 30, the presence of *Salmonella* was only observed in the two samples stored at 20 °C with the Acid treatment. Although this situation was maintained until day 90 after storage, all samples stored at 4 ºC where positive for *Salmonella* presence until the end of the study (Table IV.1).

Table V. 1 - Survival of *Salmonella enterica*. during storage at 4 °C and 20 °C for each four replicates.

Sampling dates	**LF+ SALM 4 ºC**	**LF+ SALM 20 ºC**	**LF+BIO+ SALM 4 ºC**	**LF+BIO+ SALM 20 ºC**	**LF+ACID+SALM 4 ºC**	**LF+ACID+SALM 20 ºC**	**LF+BIO+ ACID+ SALM 4 ºC**	**LF+BIO+ ACID+ SALM 20 ºC**
0	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
3	4/4	4/4	4/5	4/4	4/4	4/4	4/4	4/4
8	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4
15	4/4	1/4	4/4	1/4	4/4	4/4	4/4	0/4
30	4/4	0/4	4/4	0/4	4/4	2/4	4/4	0/4
60	4/4	0/4	4/4	0/4	4/4	2/4	4/4	0/4
80	4/4	0/4	4/4	0/4	4/4	2/4	4/4	0/4
90	4/4	0/4	4/4	0/4	4/4	2/4	4/4	0/4

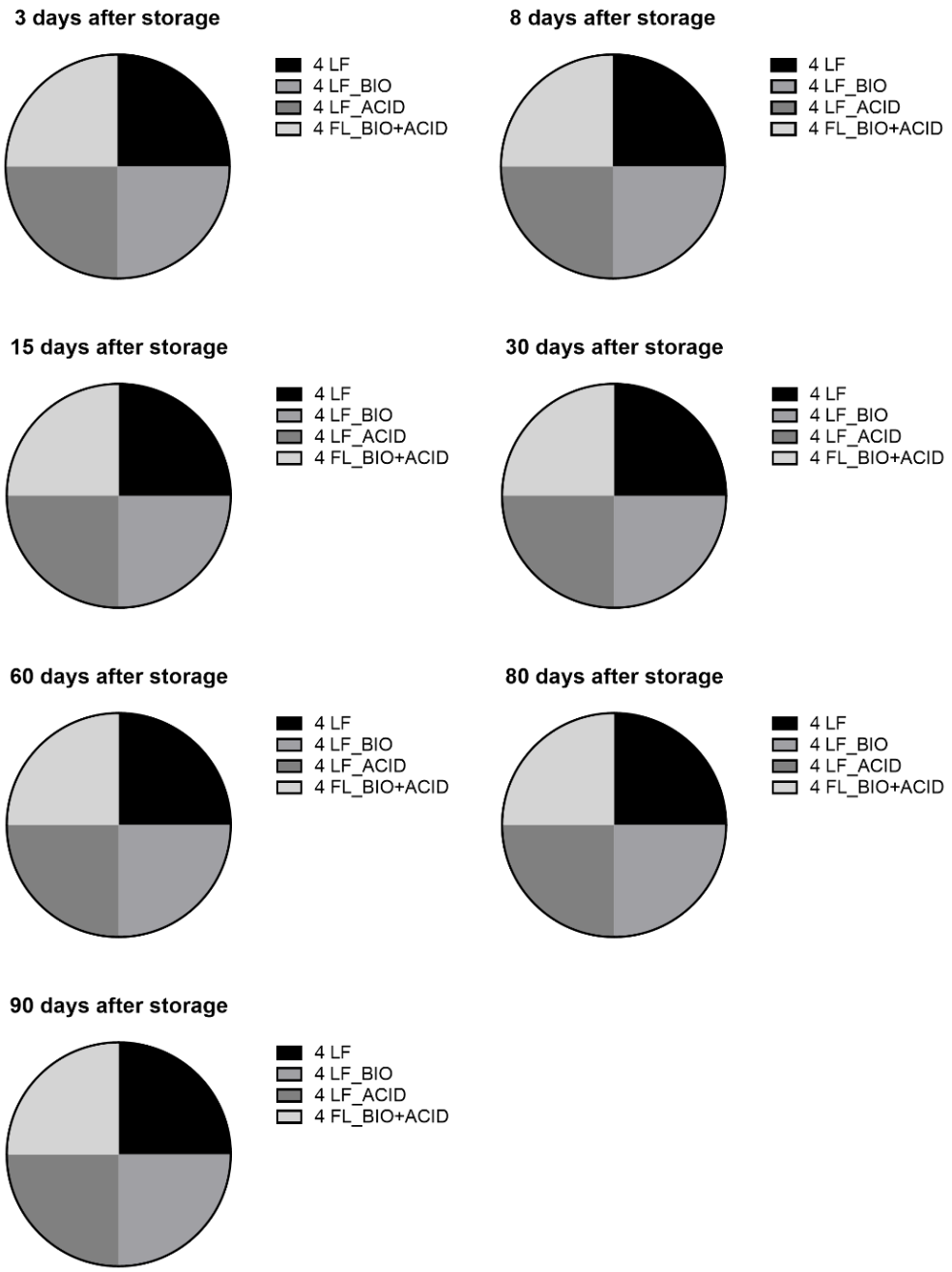
Subtitle: LF- liquid fraction of slurry; Salm- *Salmonella enterica*.; Bio- Biochar; Acid- Sulphuric acid.

Two of the four replicates remained positive until the last harvest for samples stored at 20 ºC treated with Sulphuric Acid. In this study, differences between the two temperatures were evident for *Salmonella enterica*. (Figure V.1 and V.2).



LF- liquid fraction of slurry; Salm- Salmonella enterica.; Bio- Biochar; Acid- Sulphuric acid.

Figure V. 2 - Graphical representation of the survival of *Salmonella enterica*. during storage at 20 °C.



LF - liquid fraction of slurry; Salm- *Salmonella enterica*; Bio- Biochar; Acid- Sulphuric acid.

Figure V. 3 - Graphical representation of the survival of Salmonella sp. during storage at 4 °C.

In last decades, several studies have been done and showed that *Salmonella* sp. survival is influenced by storage temperature, suspended solids content and pH value of the slurry (Guan and Holley, 2003;Arrus et al., 2006; Côté et al., 2006; Biswas et al., 2016; Olszewska and Skowron, 2013a). In the liquid fraction of slurry selected to our study, no presence of *Salmonella* sp. were detected, however, a study conducted by Arrus et al. (2006) showed that about the *Salmonella* survival, there were no differences between *Salmonella* serovars naturally presented and cultures inoculated in *in vitro* studies. Previous studies, Arrus et al (2006) and Guan and Holley (2003), identified animal wastes as a way to contamine soil, because they may contain pathogens of public health concern, such as *Escherichia coli* O157:H7 and *Salmonella* sp. justifing the importance of our study.

The results of the present study suggest a more efficient elimination of *Salmonella* sp. in the samples stored at 20 °C compared to the samples stored at 4 °C. These results corroborate studies that refer that storage conditions used, as temperature, influences the survival results. In which, high temperatures presents better results in the elimination of *Salmonella* sp. (Plachá et al., 2001; Côté et al., 2006; Olszewska and Skowron, 2013a;). In the study of Paluszak et al. (2016), in the process of anaerobic digestion of slurry occurring at 49-51.5 °C in 13 hours after the digestion starts, there was a complete elimination of *Salmonella* sp., and this process gave guaranties that the slurry was microbiological safe for the environment (Paluszak et al., 2016). Other studies suggest that naturally contamination of slurry with pathogens like *Salmonella* sp. is less common in Summer (Kearney et al., 1993). Biswas et al. (2016) found a clear relation between high temperatures of storage and a decrease in the capacity of *Salmonella* survival which corroborate our results . However, other studies in seasonal temperatures profiles in slurry storage suggest contradictory results and presented that winter temperatures can allow easier contamination on soil application, and that may explain the results observed at 4 °C (Arrus et al., 2006). A study in pig slurry suggests that the storage of slurry in adequate conditions as separated, aerated and stored during 2 months becomed microbiologically safe to be applied to soil (Mannion et al., 2007).

The treatment of slurry with Sulphuric acid has the propose to reduce the greenhouse gases emissions, and improve the nutrient value of the slurry (Fangueiro et al., 2015; Owusu-Twum et al., 2017b). Our results suggest that the treatments containing acidification (and stored at 20 ºC) seem to favour the survival of the

pathogen studied, comparing to the treatments that not have acidification suggesting that these treatments were not efficient in the elimination of *Salmonella* sp. Despite difficulties concerning laboratory studies with on-farm conditions, our results reinforce that slurry can represent a source of transmission of *Salmonella* sp. and provide knowledge on the *Salmonella* sp. survival time in treated slurry. In addition, these results have potential to be used to calculate the environment, and public health risk of these dairy effluents.

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Chapter VI

Occurrence of Bad Water Quality in Dairy Farms in Portugal

VI. Occurrence of bad water quality in dairy cattle farms in Portugal

Chapter submitted as an original paper in a journal:

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VI. Abstract

Microbial water quality is a major concern in the world, since the ingestion of water contaminated with microorganism poses a risk for human and animal health. Poor water quality is supposed to contribute to the contamination of milk and to poor milk quality.

The aim of this study was to evaluate drinking water microbiological quality of dairy cattle farms.

The study was carried out to determine the occurrence of coliforms and enterococcus species from drinking water samples obtained from selected dairy cattle farms located in four different areas of Portugal (North, Central, South and Islands). A questionnaire was applied to collected relevant information regarding farm characteristics. In this study, a convenience sample of 32 dairy cattle farms was subject to screening of water quality. The number of animals in study was 8086 cattle, with a mean herd size of 253 of which 51.7% were cows. Coliform and *Enterococcu*s species were identified by performing the membrane filtration method.

The occurrence of bad water quality was recorded at 19 (59.4%) cattle farms. Bacteria isolated included *Escherichia coli* (20.0%), *Enterococcus faecalis* (25.0%), total coliforms (65.6%) and faecal coliforms (43.8%).

Farms that did not routinely performed disinfection had worst water quality (70.8%) when compared with herds that performed disinfection (25.0%) (P = 0.022).

This research revealed the importance of screening the quality of drinking water in cattle, which could contribute to improve animal, human and environmental health in a One Heath approach.

Keywords: *Escherichia coli*, *Enterococcus*, Water, Contamination, Portugal

VI.1 Introduction

Livestock animals' beverage water is from different sources: town water, water well or rivers. Depending on the hygienic status of those sources public health problems may occur (Mohammed, 2014). Usually coliform bacteria shed in the faeces of healthy livestock, including dairy cattle. The poor hygienic condition of animals, contaminated water, unsanitary milking practices and manage conditions may increase coliform count. Microbial water quality is a major issue for animal, human and environment health (Pachepsky et al., 2018). Water must be free of pathogenic, dangerous agents and adequate to provide the nutritional requirements in animal production (Nazemi et al., 2017). The consume of water contaminated with excreta containing pathogenic microorganism carriage a risk to both humans and animals because it can increase the incidence of zoonotic diseases (Mohammed, 2014). *Escherichia* (*E. coli)* is used as the principal indicator for drinking water pollution monitoring and it is considered a superior indicator. This bacteria is found in all mammal faeces and it was chosen as a biological indicator based on its capacity of survival in drinking water for 4 to 12 weeks (Edberg et al., 2000). This has been the primary indicator of faecal contamination in water quality monitoring for many decades, *E. coli* is a member of faecal coliforms that contaminate drinking water derived from human and animal faecal waste. During rainfalls, these coliforms can be washed into creeks, rivers, streams, lakes or ground water. Untreated drinking water coming from these sources contains coliforms including *E. coli* (Odonkor and Addo, 2018). Due to their ubiquity in human and animal faeces and persistence in the environment, *Enterococcus* has been adopted as indicators of faecal pollution in water due to their ubiquity and persistence in the environment (Gilmore et al., 2014).

Because of that, the consumption of water from a contaminated water supply by production animals, can introduce a high number of microorganism in to this animal group but also can create a "multiplier" effect through the food chain (Olkowski, 2009). To preserve animal health and productivity, it is important to identify and correct water problems and mandatory that dairy farmers adopt good farming practices, such as use of clean and pure water (Lardner et al., 2005; Lemma D et al., 2018). Potable water should too be used to wash hands, udders and utensils before milking, guarantying the milk safety (Vanitha et al., 2018). The parameters typically subjected to assessment in water are physical properties, chemical constituents and the presence of microbiological agents (Beede, 2012).

This study aims to assess water quality in dairy cattle Portuguese farms and factors associated with the occurrence of microbiological contamination in water.

VI.2 Material and Methods

Study Population

A convenience sample of 32 dairy cattle farms were included in the study. The number of animals in study was 8086, with a mean herd size of 253 animals per farm. Inclusion criteria were geographic locations in Portugal, milk production on farm and dairy breed.

The study was conducted between March and August of 2018, in four different areas of Portugal (North, Central, South and Islands). Each farm was visited once by 1 of 2 trained assessors. A structured questionnaire was developed to assess the water quality in cattle farms. This questionnaire was developed after a review of the literature and it was previously pre-tested. The questionnaire was designed with multiple-choice questions to survey factors associated with water quality. During the visit, the questionnaire was completed by the farmer, mediated by the assessor and all information was collected by face-to-face interviews. The questionnaire comprised of 26 questions (21 multiple-choice questions and 5 open questions). It was divided into two sections. One section consisted of 5 questions that covered the demographics of the farmers being surveyed, such as gender, age, place of residence, educational level, and the another section consisted in 21 questions about characteristics of the cattle farm, as well as number of animals per herd, number of adult cows, production type, water source, use of disinfectants and pasture area. On average, the farmers spend around 15-20 minutes to complete the survey. In this study a farm was considered with bad water quality if coliform and/or *E. coli* and/or *Enterococcus* colony forming units (CFU) were higher than zero.

Microbiological Analysis

In total 32 water samples (one per farm) were collected under aseptic conditions. Samples were collected from hole, well, mine and tape water. All water sampling and preservation procedures were performed according to Standard Methods for the examination of water and wastewater (APHA, 1995; APHA, 1998). Sampling for bacteriological analysis was done aseptically with care in 500 ml sterile bottles,

ensuring no external contamination of samples. All samples were transported to the laboratory of Medical Microbiology, UTAD, Portugal within 24 hours.

The membrane filter technique test was conducted according to Standard Methods to detect coliforms and enterococci. An appropriate volume of sample (100 ml) was filtered through a 0.45-µm pore size nitrocellulose filter of 47 mm of diameter (Millipore Corporation, Bedford®, MA, USA) to retain the bacteria present in the sample. One filter was then placed on HiCrome™ Coliform Agar (Sigma-Aldrich) plate and incubated at 37 ± 0.5 ºC for 24 ± 2h and the salmon to red colonies were counted as total coliforms. Another filter was plated in the same culture media and incubated at 44 ± 0.5 ºC for 24 ± 2h and dark blue to violet coloured coliform colonies were counted as *Escherichia coli*. To confirm *Escherichia coli* identification a drop of Kovac′s reagent has been added on the dark blue to violet colonies, the formation of cherry-red colour has indicated the positive reaction. Positive coliform colonies were subcultured on MacConkey agar for confirmation of *Escherichia coli* and other enteric coliforms. The filters have been placed on triplicate plates.

All the strains isolated were identified by the IMVIC method (indole, methyl red, Voges- Proskauer, citrate) and Triple Sugar Iron. Bacteria not identifiable by these methods were examined using the API 20E® System (BioMerieux®, Marcyl'Etoile, France).

Kanamycin Aesculin Azide agar® (KEA) (Sigma-Aldrich) media was used for the recovery of faecal streptococci/enterococci. A 100 ml sample was filtered through a 0.45-µm pore size nitrocellulose filter of 47 mm of diameter. The filter was then placed on KEA agar plate and incubated at 36 ± 1°C, 24-48 h. The filters will place on triplicate plates. Typical colony appearance was translucent to whitish colonies surrounded by dark brown to black halos. Strains were further confirmed by using API 20 NE® System.

Statistical analysis

Data from farm visit questionnaires were coded and a database established in Excel® for Windows. Statistical analysis were carried out employing a Pearson Chi square test and Fisher exact test to compare values relatively to categorical variables. Analyses were performed with SPSS® 22.0 software for Windows (SPSS Inc. Chicago, IL, USA), considering 0.05 as the level of significance (P). Several continuous variables were transformed into categories, as number of animals in the herd.

VI.3 Results

In this study, a total of 32 cattle farms and 8086 cattle, in which 51.7% were cows were subject to screening of water quality. The mean herd size for all farms visited was 253, ranging from 51 to 127951. All cattle included in the study were of the Holstein Friesians breed.

The occurrence of cattle farms with bad water quality was detected in 19 out 32 (59.4 %.) studied farms.

Bacteria isolated included *Escherichia coli* (20.0%) in six farms, *Enterococcus faecalis* (25.0%), total coliforms (65.6%) and faecal coliforms (43.8%). No *Salmonella* sp. were recorded.

Table VI.1 summarizes the proportion of bad water quality farms, according to characteristics of the herd. Bad water quality was mostly found in the North of Portugal (60.0%) but differences were not statistically significant (P = 0.892). The quality of water collected in Spring (50.0%) or in Summer (66.7%) was not significantly different (P = 0.341).

In regards the constitution, bad water quality in cattle farms with equal or more than 204 animals (68.8%) was higher than in herds with less animals (50.0%) though differences were not statistically significant (P = 0.279). The number of females in the herd was not significant (P = 0.719) with 62.5% of the herds with equal or more than 199 with bad water quality and 56.3% in herds with less than 199. The number of males and the number of heifers were not significant (P = 0.926 and P = 0.821, respectively).

The occurrence of bad water quality was higher in herds with milk production type (84.2%) then in herds with mixed production type (15.8%) though without statistic differences (P = 0.604).

The occurrence among herds with the intensive husbandry was higher (59.3%) but not statistically significant (P=0.933). The herds with their own pasture area showed a similar frequency of bad water quality (60.0%) compared with herds that used pastures with other owners (59.3%). Differences were not statistically significant (P = 0.975).

Bad water quality was most frequent in farms where the water comes from a hole (62.5%) or well (58.3%) compared with other water sources but the differences were not statistically significant (P = 0.560).

Table VI.1 - Proportion of bad water quality in cattle farms according to characteristics of the herds in Portugal.

Variables	Positive farms with bad water quality (n; %) (n=19)	Occurrence (%)	P
Region			0.892
North	15 (78.9%)	60.0	
Other	4 (21.1%)	57.1	
Season			0.341
Spring	7 (36,8%)	50.0	
Summer	12 (63.2%)	66.7	
Number of heads in the herd			0.279
Less than 204 heads	8 (42.1%)	50.0	
Equal or more than 204 heads	11 (57.9%)	68.8	
Number of adult cows in the herd			0.719
Less than 199	9 (47.4%)	56.3	
Equal or more than 199	10 (52.6%)	62.5	
Number of males in the herd			0.926
0-2	7 (63.2%)	58.3	
Equal or more than 3	12 (36.8%)	60.0	
Number of heifers in the herd			0.821
Less than 40	8 (42.1%)	57.1	
Equal or more than 40	11 (57.9%)	61.1	
Production type			0.604
Milk	16 (84.2%)	61.5	
Mixed	3 (15.8%)	50.0	
Husbandry			0.933
Intensive	16 (84.2%)	59.3	
Extensive/semi-intensive	3 (15.8%)	15.8	
Pasture area			0.975
Own pastures	3 (15.8%)	60.0	
Others pastures	16 (84.2%)	59.3	

Water Source			0.560
Hole	10 (52.6%)	62.5	
Well	7 (36.8%)	58.3	
Tap water	1 (5.3%)	1/2	
Other	1 (5.3%)	1/2	
Effective periodic disinfection			0.022
Yes	2 (10.5%)	25.0	
No	17 (89.5%)	70.8	

In this study, herds that did not use per routine disinfectants showed worst water quality (70.8%) when compared with herds that use disinfectants (25.0%). Differences were statistically significant (P = 0.022).

Table VI.2 presents the proportion of bad water quality herds according to characteristics of the farmer in Portugal. The two herds with where the responsible for the husbandry were women were both positive. In farms were the farmer had an age equal or less than 50 years (63.6%) and when the farmer had higher education (73.7%) but none of these differences were statistically significant.

In this study, 94.7% of farmers had training in husbandry (94.7%). The occurrence of bad water quality in farmers with training was 58.1% (P = 0.302).

Herds with farmers with exclusive dedication to the herd showed lower occurrence of bad water quality (57.7%) but the differences were not statistically significant (P = 0.684).

Table V.2 - Proportion of bad water quality in cattle farms according to characteristics of the farmer in Portugal.

Variables	Positive farms with bad water quality (n; %) (n=19)	Occurrence (%)	P
Responsible for the husbandry			0.140
Man	17 (89.5%)	56.7	
Woman	2 (10.5%)	2/2	
Age of the farmer			0.467
31-50 years	14 (73.7%)	63.6	
More than 50 years	5 (26.3%)	50.0	
Education level			0.364
Basic	5 (26.3%)	5/8	
Secondary	8 (42.1%)	53.3	
Higher education	6 (31.6%)	66.7	
Training in husbandry			0.302
Yes	18 (94.7%)	58.1	
No	1 (5.3%)	1/1	
Farmers with exclusive dedication			0.684
Yes	15 (78.9%)	57.7	
No	4 (21.1%)	66.7	

VI.4 Discussion

Knowledge is one of the cornerstone of surveillance and monitoring programmes, since it is decisive on whether to apply control measures or not, and delivers data for the evaluation of the effectiveness of these measures and it is the basis for modification (Mousing et al., 1997).

This is the first epidemiological study that describes the quality of water in dairy farms in Portugal. The aim of this study was to characterize the water quality by herd size, species, constitution of the herd and type of production, using only animals and herds with this information properly registered. Bacterial contamination can invade groundwater by several routes and slurry and manure can be a potential source

(Mohammed, 2016). Comparing our data with those of other studies (Hancock et al., 1994; Zhao et al., 1995), we observed the occurrence of *E. coli* and other pathogenic microorganism contamination in drinking water of dairy cattle . The findings of this study are also in accordance with other studies (Din et al., 2014; Mohammed, 2016) that identified coliform bacterial species as *E. coli* in drinking water. *Escherichia coli* is the bacteria usually established to be the main contaminant in dairy farms (Fairbrother and Nadeau, 2006; LeJeune and Wetzel, 2007).

Poor water quality is supposed to contribute to the contamination of milk and to poor milk quality. And the presence of *E. coli* in the various water sources may spell health hazards such as diarrhoeal diseases and problems of antibiotic resistance since *E. coli* its known to be resistant to a wide range of antibiotics (Adesiyun et al., 1997; Costa et al., 2008; Gonçalves et al., 2012; Odonkor and Addo, 2018).

In the present study, *Enterococcus* and coliforms were also isolated in drinking water in cattle farms. Coliforms, including total and faecal coliforms are the primary method of assessing contamination *Enterococcus* spp. in the European Union are indicator of faecal water contamination of human or animal origin (The Council of the European Union, 1998).

Water is one of the most important vehicles for the transmission of infectious diseases and faecal contamination of water sources occur mainly in developing and underdeveloped countries (Hout, 2012). Being the most of zoonotic outbreaks caused by water contamination with faeces from infected animals or humans (Lobna and Metawea, 2013).

The bacterial load/population in the studied water samples of different dairy farms in Portugal was higher than standard bacterial concentration level by World Health Organization (WHO). Water quality behave as an important role for the farmers in animal performance, nutrition and health, however, the microbiological quality does not always reach accepted standards. Moreover, water quality is not regularly measured in some dairy farms (Davies-Colley et al., 2004). While the quality of water samples taken directly from the source is frequently assessed, the quality of water at the end points of the distribution system, where cattle really drink the water, is assessed less often. However, there may be large differences in quality between source and end points (van Eenige et al., 2013).

Animal production drink contaminated water with enteric bacteria could serve as a common source of exposure to potential pathogens to cattle. That could result in an

infection of a big number of animals during a brief period of time. The period of time in which water troughs serve as reservoirs for enteric microorganisms and the frequency that waterborne transmission of these pathogens happens from water to cattle, is not until now fully known (LeJeune and Wetzel, 2007). Poor water quality is often defined in terms such as “unsatisfactory” or “unsafe”, but little is known about the effects of coliform bacteria in water on animal health or rumen microflora. Having cattle drinking water contaminated by microorganisms may in the end be a public health hazard, beyond contaminated milk or the contaminated drinking water source itself (Eenige Van et al., 2013). Concerning that high-producing cows drink about 120 litres water per day, depending on dry matter intake from feed, milk production level, ambient temperature and sodium intake, water is considered the most important “nutrient” for cattle (LeJeune et al., 2001). Cows are particularly susceptible to different disorders when ingest contaminated water, because they are often in a negative energy balance, they can develop ketosis or rumen acidosis, and when subjected to various stressors (e.g. social stress, housing change stress, hormonal stress) they may develop other health (mastitis) and reproduction disorders (LeJeune et al., 2001). Concerning all of this data, the quality of water on dairy farms could be reflect herd health and productivity, and even public health and food safety (van Eenige et al., 2013).

Previous studies have highlighted the role of disinfectants in the water quality (Mohammed, 2016; Rossi-Fedele et al., 2010). In this study the only factor associated with bad water quality was the absence of effective periodic disinfection. Microbial pathogens can grow in biofilms on conduit walls in case of water stagnation and warmer situations (Wingender and Flemming, 2011; Ashbolt, 2015). The use of correct disinfectants is required for inactivation of pathogenic microorganisms during water treatment (Ding et al., 2019).

In the present study was not possible to ascertain the origin of faecal contamination of water, but it is possible that it is due to contamination of groundwater by manure and slurry, which enhances the need for studies to reduce microorganisms in manure and slurry.

The limitations and potential sources of bias in this study must be considered when weighing the results. This survey did not adequately capture the farms of Portugal since is a convenience sample. Study farms were selected on a voluntary basis. Despite these limitations, our study indicates that a high percentage of cows drink water with bad quality.

VI.5 Conclusions

This study assessed the water quality in 32 Portuguese farms. Based on the research findings 59.4% of the studied herds had bad water quality. *Escherichia coli* was recovered from 20.0% water samples. The information could be used as a starting point to design control measures associated with the use of disinfectants that are needed in order to help decrease bad water quality in farms and to improve animal and environment health, and productivity.

VI.6 Acknowledgements

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Chapter VII

Concluding remarks

Concluding remarks

This thesis reports additions to the knowledge of animal effluents management, in particular for pathogenic and emerging bacteria survival those effluents. Being this thesis based in a One Health perspective, it also contains a screening about water quality in dairy farms. Based on the work developed in this thesis, the follow conclusions can be draw:

Bacterial survival testing

- In our study testing *E. coli* capacity of survival in dairy cow slurry treated using methods commonly used in farms because of their benefit to the soil and to minimize greenhouse gases emissions. Acidification was not efficient to eliminate *E. coli*, being the treatments containing Sulphuric acid the ones that demonstrated the highest means for CFU/ml in the end of the storage process (90 days).

- *Enterococcus* sp. is considered a robust bacterial genus that have the capacity to survive under extreme conditions. The treatments containing acidification of the slurry led to an increase of the means of for CFU/ml of *Enterococcus* plate counts in 60 and 90 days of storage.

- Our study shows a clear relation between high temperatures (20 ºC) of storage and a decrease in the capacity of *Salmonella* survival. In contrast, in of all of treatments stored at the low temperature (4º C) the pathogen presence has been maintained until the end of the storage process (90 days). For the treatments that combine acidification and storage at 20 ºC there was a favorable to the survival of the pathogen studied.

Screening of water quality in dairy farms

- In this study the subject was to screening the water quality, in dairy cattle farms. All cattle included in the study were of the Holstein Friesians breed. The occurrence of cattle farms with bad water quality was detected in 59.4% studied farms. The bacteria isolated included *Escherichia coli*, *Enterococcus faecalis*, total coliforms and faecal

coliforms. Farms without effective period of disinfection showed worst water quality. Despite its limitations, our study indicates that a high percentage of cows drink water with bad quality.

Future perspectives

This thesis provides an extend in the knowledge of bacterial capacity of survival in dairy cow effluents. Establishes a knowledge base for this capacity of survival when those effluents are treated with methodology's used to reduce greenhouse gases emissions. However, further investigation is required to provide conclusions about which is the best treatment in an environmental perspective (lowing GHG) but also in a public health perspective by lowing bacterial survival and propagation:

- New studies focusing on different bacterial agents;
- New studies testing different slurry treatments and combinations between them;
- Since the treatments including acid have given the worsts results for bacteria survival in the three studies made for these thesis, it is important to continue to evaluate how acidifying of slurry can affect different bacterial survival;
- It is important to do studies testing bacterial survival after the treated effluent was applied to soil, in other to understand the environmental contaminations;

Also, like our epidemiological survey indicates that high levels of contamination in the water used for cow watering, concerning all of the public health implications, it is important to do more studies with a lager sample size of farms included.

According to our study acidification is not efficient to eliminate *E. coli.* For the three treatments used in our study, such as Biochar and beneficial microorganisms, there are no scientific literature testing bacteria survival. Additional studies focused on the evaluation of different pathogens survival correlated with different slurry treatments are needed, as well studies that evaluate pathogen survival on treated slurry applied to soil.

In conclusion, new insights presented in this thesis may be used as direction in further research, in the field of strategies for management of animal effluents, microbial

pathogens survival, their propagation in environment having in consideration a One Health perspective.

Appendix

Appendix A

List of international publications:

List of international poster presentations

- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC. (2019) Pathogens in animal effluents and their different capacity of survival. *1st international conference of the European College of Veterinary Microbiology. Athens, Greece, 26-27 September*.

- C Miranda, **AS Soares**, AC Coelho, H Trindade and CA Teixeira.(2018) Environmental impacts of sulphuric acid and added biochar on cattle slurry – a life cycle assessment. *69th EAAP Annual meeting in Dubrovnik 27-31 August*.

- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC.(2017) Impact of cattle slurry treatment on survival of *Escherichia coli* during storage. *7th International Congress of Energy and Environment Engineering and Management (CIIEM7), University of Las Palmas de Gran Canaria, Spain. 17-19 July*.

- Miranda C, **Soares AS**, Teixeira CA, Coelho AC, Trindade H. (2017) Impact of different treatments of cattle slurry liquid fraction on greenhouse gases emissions during storage. *7th International Congress of Energy and Environment Engineering and Management (CIIEM7), University of Las Palmas de Gran Canaria, Spain. 17-19 July*.

List of international oral presentations

- **AS Soares,** C Miranda, H Trindade and AC Coelho.(2018) Does *Salmonella* sp. survive in cow slurry during storage*? 69th EAAP Annual meeting in Dubrovnik 27-31August*.

Appendix B

List of national publications:

List of national poster presentations

- **Soares AS**, Miranda C, Pinto S, Trindade H, Coelho AC. (2019) Microbiological quality of drinking water from dairy farms in Portugal/Qualidade microbiológica da água de abeberamento em explorações leiteiras em Portugal. *XII Jornadas de Biologia, Semana da Ciência e Cidadania, , UTAD, Vila Real, 25 of February to 1 March*.
- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC. (2017) *Enterococcus* spp. survival on treated cow slurry during storage. *Encontro Ciência'17, Centro de Congressos de Lisboa. 3-5 July*.

List of national oral presentations

- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC. (2018) Evaluation of the survival of Salmonella gender in dairy cow slurry storage process/Avaliação da sobrevivência do genro Salmonella no processo de armazenamento de chorume de vacas leiteiras frísias. *III Jornadas Interact - II SASGEO - Simpósio Internacional de Águas, Solos Geotecnologias, Universidade de Trás-os-Montes e Alto Douro 17-18 May*.
- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC. (2017) Evaluation of the survival of *E. coli* and *Enterococcus* spp. during the storage of cattle slurry under different treatments,*2as Jornadas do projeto Interact, 12-13 July*.
- Miranda C, **Soares AS**, Teixeira CA, Coelho AC, Trindade H. (2017) "Emissions of methane, nitrous oxide and carbon dioxide during storage of cattle slurry subjected to different treatments",*2as Jornadas do projeto Interact, 12-13 July*.
- **Soares AS**, Miranda C, Teixeira CA, Trindade H, Coelho AC. (2016)Task 1 – Impact of different treatments on *Escherichia coli* and on the greenhouse gases emissions during storage of cattle slurry,*1as Jornadas do projeto Interact, 16 November*.

Appendix C

Soares A.S., Miranda C., Teixeira C.A., Coutinho J., Trindade H., Coelho A.C., (2019). Impact of different treatments on *Escherichia coli* during storage of cattle slurry. *Journal of Environmental Management, 236, 323–327.*. Q1:4.865 (2018)

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Research article

Impact of different treatments on *Escherichia coli* during storage of cattle slurry

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ABSTRACT

There are different types of effluents from farming operations including untreated slurry (a mixture of manure, urine, spilt feed, and water), and treated slurry that normally is filtered to separate the solid fraction from the liquid fraction. With the amount of slurry applied on the soils as fertiliser every year, there are necessary to measure the leaching of microbial capable of transmitting infective agents that can be normally on slurry, because slurry can be a potential biohazard capable of transmitting infective agents. The aim of this study was to investigate the presence and survival of *Escherichia coli* (*E. coli*) on liquid fraction of dairy slurry, with the addition of different treatments during storage, such as addition of Biochar, beneficial microorganisms, sulphuric acid and the combinations of them. All the applied treatments to slurry show statistically significant differences ($P < 0.001$), according to the different sampling dates. Results showed that there are conditions and treatments that benefit the survival probability of *E. coli*, the treatments that include the acidification of slurry have the highest averages of CFU/ml (243288.3 at 37 °C; 136584 at 44 °C). These results can contribute to the improvement of the quality of slurry applied on soils, beneficiating agriculture but also public health.

1. Introduction

Livestock effluents have long been used as organic fertilizers. However, in recent years, the growth of concentration of animal production in certain areas has been responsible for the production of large volumes of livestock effluents that if where not managed correctly there can be significant risks that can effect humans, animals and the environment. The animal manures including poultry, cattle, and swine are the primary source of water resources contamination by water-borne pathogens, as well as *Escherichia coli* (*E. coli*) or *Salmonella* sp. (Collins et al., 2005; Cardoso et al., 2012). The concentration of fecal indicator microorganisms contained in animal manures are variable depending on the manure type, the storage conditions and environmental factors (Hodgson et al., 2016). Every year, one billion tones of animal manure and slurry are produced in the Europe, consequently, in the last years the management of slurry was become one of the central dairy activities in farms (Fangueiro et al., 2014). With the amount of slurry applied on the soils as fertiliser every year, there is a need of studies to measure the leaching of microbial agents normally presents in slurry (Hodgson et al., 2016; Krog et al., 2017). The contamination with gastrointestinal pathogenic bacteria is a serious public health concern, because exposure to these microorganisms can result in severe animal and human diseases (Kudva et al., 1998; Mead et al., 1999; Cardoso et al., 2012).

The aim of this study is to investigate the presence and pathogen survival of *E. coli* on liquid fraction of dairy slurry during the storage process for which they were applied different treatments such as addition of Biochar, beneficial microorganisms and acidification.

2. Material and methods

2.1. Slurry

The cattle slurry was collected from a dairy farm in Vila Real (Portugal). A hundred kg of whole slurry (WS) was subjected to solid-liquid separation by screeners to obtain two fractions, a liquid (LF) and a solid fraction (SF). The LF was preserved at 4 °C in plastic barrels until required. This fraction was analysed for the presence of *E. coli*, total coliforms and *Salmonella* sp., prior to inoculation to ensure that they were *Salmonella* free.

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2.2. Slurry treatments

The liquid fraction obtained was placed in 2 L Kilner jars. Each jar was filled with 1 L of LF and a total of seven treatments were considered in this study:

1. LF + Biochar + *E. coli*
2. LF + Sulphuric acid + *E. coli*
3. LF + Beneficial microorganisms + *E. coli*
4. LF + Biochar + Beneficial microorganisms + *E. coli*
5. LF + Biochar + Sulphuric acid + *E. coli*
6. LF + Sulphuric acid + Beneficial microorganisms + *E. coli*
7. LF + Biochar + Sulphuric acid + Beneficial microorganisms + *E. coli*

Two controls were used one control was constituted by untreated liquid slurry and the other containing *E. coli*. All controls were maintained in the same conditions as the jars with the treated slurry. Three replicates of each treatment and controls were considered for microbiological analysis.

In the treated slurries with Biochar (Piroeco Bioenergy, S.L.®), this was added 4.5% in slurries and it was derived from wood shavings (2 mm diameter) pyrolised in a muffle furnace at 900 °C. The acidification of the liquid fractions to pH 5.5, were performed by addition of concentrated sulphuric acid (H_2SO_4, 96%) at a rate of 4.5 ml kg-1 slurry. Continuous stirring and pH measurement were performed during the acidification process. The addition of beneficial microorganisms (Bio buster, Bio-Systems Europe, England) was performed following the manufacturer's instructions.

In the combined treatments Biochar, beneficial microorganisms and concentrated sulphuric acid added as previously described, being acidification made after the addition of the other treatments.

2.3. Slurry inoculation with E. coli

To inoculate liquid slurry with *E. coli*, an isolated colony was used. Which it was previously characterized as *E. coli* by microbiological methods, obtained from the microbial analysis applied to slurry before the treatments. For the determination of the presence *E. coli* in slurry, 1 ml of the sample were diluted in 9 ml of sterile saline solution (1:10, w/v). Each diluted sample was plated in Brilliance™ *E. coli*/coliform Selective Agar (OXOID®) by incorporation and incubated at 37 °C for 24 h and confirmed by API 20E (bioMérieux ®, Marcy l'Etoile, France).

Escherichia coli concentration in agar plates was calculated to create suspensions in sterile saline solution (1:10, w/v) with concentration 0.5 in McFarland standard scale (10^9 CFU/ml) using DensiCHEK Plus 21255-P1M1.1 instrument and then 1 ml of the culture was inoculated in each treatment. Using suspensions of such high concentrations allowed observation of the sanitization effectiveness of the selected methods under conditions of the extreme level of slurry contamination (Bauza-Kaszewska et al., 2015).

Escherichia. coli was inoculated in liquid fraction of dairy slurry after the addition of Biochar, sulphuric acid and beneficial microorganisms. *E. coli* suspension was introduced directly into the jars, except in control jar only with LF. The jars were kept at 20 °C during the storage.

2.4. Microbiological analysis

Samples for the microbiological analysis were collected before the storage and in different time intervals for the period of 90 days (0, 3, 30, 60 and 90 days).

From the obtained initial liquid fraction and for each treatment, 2 g were placed in a sterile flask and immediately transported in a cooled (4 °C) transport box to the microbiology laboratory.

The screening for the presence/absence of *Salmonella* sp. was determined only from the liquid fraction of the cattle slurry before the addition of treatments, according to standard procedures (ISO 6579:2002). First, the sample was inoculated in Buffered peptone water (BPW - pre-enrichment in non-selective liquid medium) at room temperature, following the incubation in Modified semi-solid Rappaport-Vassiliadis (MSRV medium, Scharlau® - Enrichment on selective semi-solid medium) contained 20 mg/l of novobiocin (OXOID®) at 42 °C (Olszewska and Skowron., 2013). From the culture obtained, selective solid media are inoculated: Xylose Lysine Desoxycholate Modified agar (VWR®), Hektoen Enteric agar (SIGMA-ALDRICH®) to verify the growth of *Salmonella* sp. In addition, the samples were incubated in MacConkey agar (VWR®), for total coliforms, which the pure culture of *E. coli* produced lactose fermenting colonies and pure culture of *Salmonella* sp. produced colourless colonies.

As previously described, the determination of the presence *E. coli* in slurry was performed using the Brilliance™ *E. coli*/coliform Selective Agar (OXOID®). The plates were incubated under aerobic conditions at 37 °C (total coliforms/*E. coli*) and 44 °C (*E.coli*), for 48 h.

The number of microorganisms was expressed as colony forming units (CFU) per millilitre of slurry.

Isolates were characterized phenotypically by Gram staining and biochemical characterization and species identification were performed using IMViC (Indole, Methyl red, Voges-Proskauer and Citrate), triple sugar iron agar, nitrate reduction, urease, oxidase and catalase tests. In addition, API 20E identification system (bioMérieux, Marcy l'Etoile, France) was also performed, according to the manufacturer's instructions.

2.5. Slurry analysis

Whole slurry and fractions obtained were analysed in terms of dry mater DM content, pH value, electrical conductivity (EC), nitrogen (N), NH_4 + content, NO_3^- content, carbon (C), sulphur (S), potassium (K) and phosphorus (P). The DM content was set by drying samples in an oven at 105 °C for 48 h. The pH value was analysed with a pH meter using fresh samples extracted with distilled water (1:5 w/v) and EC was measured in the same extract (filtered through Whatman No. 2 filter paper). The mineral N content was extracted with 0.01 M CaCl2 (1:10 w/v). The NH_4+ and NO_3- contents in the extracts were measured by segmented flow analysis (San Plus, Skalar, Breda, NL), based on the Barthelot reaction for NH_4+ and on the Griess-Ilosvay's reagent after the reduction to NO_2 in a cadmium column for the NO_3- (Houba et al., 1995). Total carbon was determined by dry combustion; there afterwards infrared detection was used with a Carbon-Nitrogen/Protein analyser (Primacs SN 22, Skalar, Breda; NL). Sulphur was determined after digestion with concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) followed by turbi-dimetric segmented flow analysis. Potassium content was quantified after hydrochloric acid (HCl) treatment of the ash through graphite furnace atomic absorption spectrophotometry (Unicam M Series). Phosphorous was determined using the ammonium vanadomolybdate method by molecular absorption spectrophotometry (Hitachi High-Tech Science Corporation, Japan 2000). These screenings were also performed in three replicates of each treatment and controls after 90 days of slurry storage.

2.6. Statistical analysis

Data from *E. coli* plate count after log10 transformed was subjected to ANOVA complete randomized design and using a split plot layout, with treatment type as main plot factor and dates as the subplot factor. Slurry data was also subjected to ANOVA considering a complete randomized design with a single factor (treatment types). When factors and their interaction were statistically significant ($P < 0.05$) means were separated by the post hoc comparisons Tukey test ($P < 0.05$). All these analysis were performed using Statistix 10 (Analitycal Software version 10.0 (2005), Tallahassee, FL).

Table 1
Main physical-chemical characteristics of the effluents at the end of the experiment (90 days of storage).

Treatments	DM %	PH	EC	NH_4^+ (mg L^{-1})	NO_3^- (mg L^{-1})	N total (g L^{-1})	C org (g kg^{-1})	S (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})
LF	5.14^{B}	8.82^{A}	3.10^{B}	45.19^{BC}	< dl	0.65^{B}	300.30^{B}	6.07^{C}	9.04^{A}	60.41^{AB}
LF + coli	5.76^{AB}	8.86^{A}	2.44^{B}	9.88^{C}	< dl	0.67^{B}	307.91^{B}	6.30^{C}	9.18^{A}	56.72^{ABC}
LF + Acid + Benefic + coli	6.00^{AB}	6.47^{B}	5.49^{A}	131.11^{AB}	< dl	1.90^{A}	263.82^{B}	52.88^{A}	5.83^{BC}	52.50^{ABC}
LF + Acid + coli	6.81^{AB}	6.50^{B}	4.12^{AB}	154.02^{A}	< dl	2.08^{A}	246.56^{B}	56.34^{A}	6.54^{B}	52.64^{ABC}
LF + Bio + Acid + Benefic + coli	10.65^{A}	6.71^{B}	5.65^{A}	131.59^{AB}	< dl	1.58^{A}	475.38^{A}	35.20^{B}	3.64^{D}	38.13^{BC}
LF + Bio + Acid + coli	9.33^{AB}	6.87^{B}	4.90^{A}	78.12^{ABC}	< dl	1.58^{A}	493.90^{A}	30.55^{B}	3.64^{D}	33.23^{C}
LF + Bio + Benefic + coli	7.32^{AB}	8.98^{A}	3.13^{B}	10.22^{C}	< dl	0.93^{B}	507.96^{A}	5.19^{C}	5.29^{BC}	51.97^{ABC}
LF + Bio + coli	8.72^{AB}	8.99^{A}	2.74^{B}	11.62^{C}	< dl	0.97^{B}	529.83^{A}	4.82^{C}	4.83^{CD}	45.54^{ABC}
LF + Benefic + coli	5.01^{B}	8.91^{A}	2.75^{B}	12.76^{C}	< dl	0.60^{B}	305.94^{B}	6.53^{C}	8.47^{A}	65.49^{A}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar. < dl: bellow detectable levels Values are the mean of three replicates; Means followed by different letters are significantly different ($P < 0.05$) using HSD Tuckey mean separation test.

3. Results and discussion

Before the beginning of the experiment, the main physic-chemical characteristics of whole slurry, liquid fraction and solid fraction, were respectively: 8.48, 4.55 and 13.69% of DM; a pH value similar in the three materials ≈8; an electrical conductivity of 4.73, 3.46 and 2.12 mS/cm; a total N of 2.70, 1.79 and 2.79 g N L^{-1}; 213.1, 159.0 and 77.7 mg NH_4^+ L^{-1}; an NO_3^-content below the detectable level (< 0.01); a carbon content of 397.5, 351.2 and 392.2 g kg^{-1}; 5.33, 8.44 and 3.73 g P kg^{-1}; 34.2, 59.1 and 18.5g K kg-1; 3.7, 5.7 and 3.6g $kg^{-1}$of (S).

At the end of the storage period, the same parameters have been evaluated for each treatment and controls. The different treatments applied to slurry showed statistically significant differences for the parameters analysed (DM; pH; EC; N; NH_4+; C; P; K and S) ($P < 0.005$) (Table 1). As expected the pH value was lower and S content was higher for the treatments where sulphuric acid was applied. Those treatments showed the highest means for NH_4+ and total N, data that corroborate other studies saying that slurry acidification can increase its fertiliser value (Fangueiro et al., 2014; Owusu-Twum et al., 2017; Kai et al., 2008). However, the highest P values were detected in the two controls of the experiment. In addition, the most conservative treatment for K was the treatment with beneficial microorganisms (65.5 gkg^{-1}), which showed to be significantly different from treatments combining Biochar + Acid (with or without Beneficial microorganisms).

In the analysis made to LF of slurry before treatments and storage, there was no evidence of the presence of *Salmonella* sp.; however, for *E. coli*, a count of 60 000 CFU/ml was obtained.

During the three months of cattle-storage, there was a marked decrease in CFU/ml for survival of *E. coli*, for all treatments (Figs. 1 and 2). In the different sampling dates, the different treatments applied to slurry revealed statistically significant differences for the CFU/ml

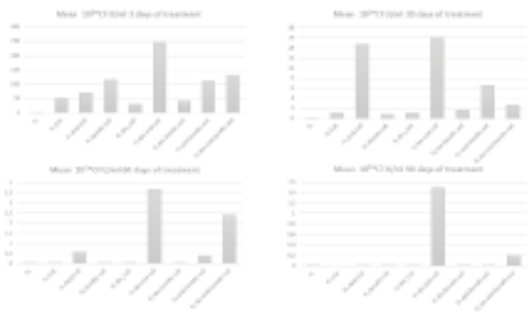

Fig. 1. Graphic representation of the means of 10^{3+} CUF/ml for *E. coli* at 3, 30, 60, 90 days of slurry storage, incubated at 37 °C. FL: Liquid fraction Acid: acidification; benefic: beneficial microorganisms; bio: Biochar.

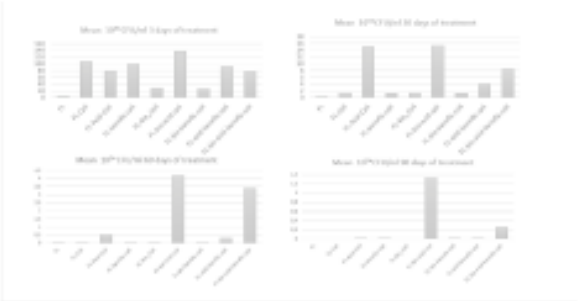

Fig. 2. Graphic representation of the means of 10^{3+} CUF/ml for *E. coli* at 3, 30, 60, 90 days of slurry storage, incubated at 44 °C. FL: Liquid fraction Acid: acidification; benefic: beneficial microorganisms; bio: Biochar.

Table 2
Results of log means CFU of *E. coli*/ml of slurry (37 °C) in the different sampling dates.

Treatment	3 days	30 days	60 days	90 days
LF + Bio + Acid + coli	5.39^{A}	4.20^{A}	3.56^{A}	3.17^{A}
LF + Bio + Acid + Benefic + coli	5.12^{A}	3.43^{AB}	3.38^{A}	2.31^{A}
LF + Benefic + coli	5.06^{A}	2.87^{BC}	0.64^{BC}	0.14^{B}
LF + Acid + Benefic + coli	5.04^{A}	3.82^{AB}	2.58^{A}	0.48^{B}
LF + Acid + coli	4.85^{A}	4.17^{A}	2.77^{A}	0.26^{B}
LF + coli	4.69^{A}	3.00^{BC}	0.04^{C}	0.00^{B}
LF + Bio + Benefic + coli	4.63^{A}	3.20^{AB}	0.70^{BC}	0.10^{B}
LF + Bio + coli	4.46^{A}	3.03^{BC}	1.23^{B}	0.00^{B}
LF	3.30^{B}	1.95^{C}	0.69^{BC}	0.00^{B}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; coli: *Escherichia coli*.
Means followed by different letters are significantly different ($P < 0.05$) using HSD Tuckey mean separation test.

($P < 0.001$) (Tables 2 and 3). However, these results showed that there are conditions and treatments that benefit the survival probability of *E. coli*. The samples incubated at 37 °C like LF treated with Biochar + Sulphuric acid demonstrated the highest means, 243 288 CFU/ml, 15 874 CFU/ml, 3654 CFU/ml and 1486 CFU/ml, at 3, 30, 60, 90 days of storage, respectively (Fig. 1). Similar results were obtained in the treatment with LF + Biochar + Sulphuric acid for the *E. coli* survival at 44 °C – 136 584 CFU/ml, 15 326 CFU/ml, 4175 CFU/ml and 1328 CFU/ml at 3, 30, 60, 90 days after the storage, respectively (Fig. 2). In this study, the treatments that include the acidification of slurry have the highest averages of CFU/ml, corroborating with studies that tested the survival and resistance of *E. coli* on acid mediums and in processes based on pH reduction (Conner and Kotrola, 1995; Lin et al., 1996; Presser et al., 1997; Nyambe et al., 2017).

In the last years, there was an intensification of animal and agriculture in the world. For this intensification to be sustainable, the use of

Table 3
Results of log means CFU of *E. coli*/ml of slurry (44 °C) in the different sampling dates.

Treatment	3 days	30 days	60 days	90 days
LF + Bio + Acid + coli	5.14^{A}	4.18^{A}	3.62^{A}	3.12^{A}
LF + Bio + Acid + Benefic + coli	4.89^{A}	3.93^{AB}	3.53^{A}	2.40^{A}
LF + Benefic + coli	4.99^{A}	3.04^{B}	0.10^{F}	0.04^{B}
LF + Acid + Benefic + coli	4.96^{A}	3.60^{AB}	2.48^{BC}	0.54^{B}
LF + Acid + coli	4.90^{A}	4.17^{A}	2.72^{AB}	0.16^{B}
LF + coli	5.02^{A}	3.10^{B}	1.19^{DE}	0.00^{B}
LF + Bio + Benefic + coli	4.42^{A}	3.03^{B}	1.45^{CD}	0.04^{B}
LF + Bio + coli	4.43^{A}	3.10^{B}	0.39^{EF}	0.00^{B}
LF	3.33^{B}	1.80^{C}	0.79^{DEF}	0.00^{B}

LF: Liquid fraction; Acid: acidification; Benefic: beneficial microorganisms; Bio: Biochar; coli: *Escherichia coli*.
Means followed by different letters are significantly different ($P < 0.05$) using HSD Tuckey mean separation test.

natural resources and ecosystem services need to be tenable (Rizzo and Giuffré., 2018). One of the major problems of intensive animal production is the quantity of animal effluents produced in small areas. Manure represents one of the most valuable organic fertilizers, however it could contain a large number of microorganisms that can have pathogenic potential as *E. coli* (Ferens and Hovde., 2011; Nolan et al., 2018).

The liquid fraction contain a significant part of the total bacteria of slurry (> 80%), including the pathogen indicator organisms such as *E. coli* (Popovic et al., 2011). The understanding of microbial survival in manure, and in the soil represents a way to minimizing borne pathogenic risk to environment, animals and humans (Hodgson et al., 2016; Hruby et al., 2018). In the study of Ottosen et al. (2009) is was described that slurry acidification is capable to reduce the microbial activity, however in the work of Zhang et al. (2011) was referred that in some conditions, acidification have a weaker effect on pathogenic bacteria.

Escherichia coli is capable to survive much longer in soil than in the manure representing a major risk to contaminate water or vegetables (Islam et al., 2004; Hruby et al., 2018). Studies refereed also that *E. coli* survives longer than other slurry pathogens on storage processes (Biswas et al., 2016). According to Hodgson et al. (2016), a significant increase of *E. coli* was observed when slurry was applied to grassland via shallow injection compared to surface application and the decay rates were significantly higher for slurry applied to grassland in spring relative to summer and autumn (Hodgson et al., 2016). Some experiments showed, that the survival rate of *E. coli* in soil was related to the local pH; moreover, *E. coli* O157:H7 owns systems for survival at low pH (Van Elsas et al., 2011).

Other studies suggest as well that the temperature could be one of the most important factors conditioning bacteria survival in the slurry, having high temperatures the most effective effect on the pathogens elimination (Chadwick et al., 2011). With the temperature used in the present study (storage at 20 °C), it was predictable that a CFU/ml bacteria reduction happened.

To minimize slurry environmental effects, some treatments can be considered, like mechanical separation, acidification or additives addition (Chadwick et al., 2011; Owusu-Twum et al., 2017). The treatments used in our study are commonly used in farms because they are beneficial to the soil and to minimize greenhouse gases, for example acidification, used to reduce ammonia emissions when slurry is applied to soil (Kai et al., 2008).

However, according to our study acidification is not efficient to eliminate *E. coli*. For the three treatments used in our study, such as Biochar and beneficial microorganisms, there are no scientific literature testing bacteria survival. Additional studies focused on the evaluation of different pathogens survival correlated with different slurry treatments are needed, as well studies that evaluate pathogen survival on treated slurry applied to soil.

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Appendix D

List of courses / seminars offered

- **Soares AS** (2019) The importance of animal effluents in public health/A importância dos Efluentes Animais em Saúde Pública, Seminário de Saúde Pública, (1 hour).