Hygiene Aspects of the Biogas Process with Emphasis on Spore-Forming Bacteria

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Abstract

Biogas is a renewable source of energy which can be obtained from processing of biowaste. The digested residues can be used as fertiliser. Biowaste intended for biogas production contains pathogenic micro-organisms. A pre-pasteurisation step at 70°C for 60 min before anaerobic digestion reduces non spore-forming bacteria such as *Salmonella* spp. To maintain the standard of the digested residues it must be handled in a strictly hygienic manner to avoid recontamination and re-growth of bacteria. The risk of contamination is particularly high when digested residues are transported in the same vehicles as the raw material. However, heat treatment at 70°C for 60 min will not reduce spore-forming bacteria such as *Bacillus* spp. and *Clostridium* spp. Spore-forming bacteria, including those that cause serious diseases, can be present in substrate intended for biogas production.

The number of species and the quantity of *Bacillus* spp. and *Clostridium* spp. in manure, slaughterhouse waste and in samples from different stages during the biogas process were investigated. The number of species of clostridia seemed to decrease following digestion, likewise the quantity. However, *Bacillus* spp. seemed to pass unaffected through the biogas process.

In laboratory-scale experiments the effects on clostridia during pasteurisation and digestion were investigated. Pathogenic clostridia were inoculated in substrates from homogenisation tanks and digester tanks. The inoculated clostridia remained after pasteurisation, but the impacts of digestion differ between different species.

Culture followed by identification of *C. chauvoei* by PCR in samples from cattle died from blackleg, is faster and safer than culture followed by biochemical identification of *C. chauvoei*. However, for environmental samples the PCR method is not practically applicable for detection of *C. chauvoei*.

To avoid spreading of diseases via biogas plants when digested residues are spread on arable land, a pasteurisation stage at 70°C for 60 min before anaerobic digestion gives adequate reduction of most non spore-forming bacteria, such as salmonella. However, caution should be exercised before digested residues are spread in areas without endemic problems of pathogenic spore-forming bacteria. In Sweden, official recommendation is that digested residues from biogas plants only should be applied on arable land, and not on grasslands for animal pasture.

Keywords: *Bacillus* spp., biogas plants, *Clostridium* spp., digested residues, Sweden, environment, hygiene safety, PCR, 16S rRNA sequencing.

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Samanfattning

Metan bildas när biologiskt material bryts ner utan närvaro av syre. I biogasanläggningar utnyttjas detta för produktion av biogas, en blandning av gaser med bland annat metan. Biogas är en miljövänlig och förnyelsebar energikälla. Rötresten kan användas som gödsel och ersätter konstgödning. Med det biologiska avfallet följer även smittämnen (patogena mikroorganismer) som kan orsaka sjukdomar hos djur och människor. Pastörisering i 70°C under 60 min före anaerob rötning minskar halten av salmonella. Efter rötningen måste rötresten hanteras på ett hygieniskt säkert sätt för att undvika att rötresten återsmittas, till exempel om transportbilarna inte är tillräckligt rengjorda.

Sporbildande bakterier, exempelvis *Clostridium* spp. och *Bacillus* spp., påverkas dock inte av pastöriseringen. Många *Clostridium* spp. och *Bacillus* spp. är ofarliga miljöbakterier, vissa är till och med nödvändiga tarmbakterier hos djur och människor. Det finns dock några sporbildande bakterier som orsakar fruktade sjukdomar hos både djur och människor, till exempel mjältbrand, botulism och stelkramp. Andra orsakar svåra sjukdomar bara hos djur, till exempel frasbrand och svindysenteri.

I det här arbetet har fem olika delstudier genomförts. I den första delstudien togs prover i olika processteg från biogasanläggningar. Proverna analyserades avseende olika patogena bakterier, bland annat *Salmonella* spp. och *Escherichia coli* O157, samt sporbildande bakterier (*Clostridium* spp. och *Bacillus* spp.). Resultatet visade att de icke sporbildande bakterierna avdödades under pastöriseringen, men under återtransport till gårdarna återsmittades rötresten med salmonella. De sporbildande bakterierna påverkades inte av biogasprocessen. Även i delstudie 2, som var en simulering av pastöriseringssteget, blev resultatet att 70°C under 60 min är ett effektivt sätt att avdöda *Salmonella* spp.

Syftet med delstudie 3 var att kartlägga vilka sporbildande bakterier som normal förekommer i gödsel, slakteriavfall och i substrat taget från olika steg av biogasprocessen. En del patogena klostridier påvisades i gödsel, slakteriavfall, före och efter pastörisering, men inte efter rötning. Det verkar som om både antalet arter och det totala antalet av *Clostridium* spp. minskar efter rötningen, men antalet arter och totalantalet av *Bacillus* spp. passerar opåverkade.

I delstudie 4 simulerades pastörisering och rötning för olika tillsatta patogena klostridier. Resultatet visade att de tillsatta bakterierna överlever pastöriseringen, men effekten av rötningen var olika från art till art.

Frasbrand hos nötkreatur orsakas av *Clostridium chauvoei*. Diagnosen ställs genom odling av vävnadsprov. I delstudie 5 jämfördes olika analysmetoder med varandra. Odling följd av identifiering med PCR gav säkrast diagnos. I studien undersöktes även jord- och gödselprover samt prover från olika steg i biogasprocessen.

För att undvika smittspridning via biogasanläggningar och rötrester, bör biologiskt avfall pastöriseras före anaerob rötning. Detta leder till att bakterier som till exempel salmonella dör, men att sporbildande bakterier kan finnas kvar. Om patogena sporbildande bakterier finns i avfallet till biogasanläggningar, och de överlever pastöriseringen, finns det en risk att patogena sporbildande bakterier sprids via rötresten till åkermark. I Sverige rekommenderas inte spridning av rötrest på bete, utan bara på odlingsmark.

Det högsta är inte att aldrig falla utan att resa sig efter varje fall

Kinesiskt ordspråk

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Bagge E, Sahlström L, Albihn A. (2006). The effect of hygienic treatment on the microbial flora of biowaste in biogas plants. *Water Research* 39, 4879-4886.

II Sahlström L, Bagge E, Emmoth E, Holmqvist A, Danielsson-Tham M-L, Albihn A. (2008). A laboratory study of survival of selected micro-organisms after heat treatment of biowaste used in biogas plants. *Bioresource Technology* 99, 7859-7865.

III Bagge E, Persson M, Johansson K-E. Diversity of spore forming bacteria in cattle manure, slaughterhouse waste and biogas plants. In manuscript.

IV Bagge E, Albihn A, Båverud V, Johansson K-E. Survival of pathogenic spore-forming bacteria after pasteurisation and during digestion in biogas plants - a laboratory study. In manuscript.

V Bagge E., Sternberg-Lewerin S., Johansson K.-E. (2009). Detection and identification of *Clostridium chauvoei* in clinical cases bovine faeces, and substrates from a biogas plant isolates by PCR. *Acta Veterinaria Scandinavica* 51:8.

Abbreviations

ABP	Animal by-products
BGP	Biogas plant
CCUG	Culture Collection, University of Gothenburg
cfu	Colony forming units
DNA	Deoxyribonucleic acid
EC	European Commission
EHEC	Enterohaemorrhagic *Escherichia coli*
FAA	Fastidious anaerobic agar plates
NMKL	Nordic Committee on Food Analysis
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
rRNA	Ribosomal ribonucleic acid
SJV	Swedish Board of Agriculture
SMI	Swedish Institute for Infectious Disease Control
SRM	Specific risk material
SVA	National Veterinary Institute
TSC	Tryptose sulphite cycloserine agar
VFA	Volatile fatty acids
VRG	Violet red bile agar
VTEC	Verotoxinogenic *Escherichia coli*

Key definitions

Animal by-products: Defined in EEC regulation EC no. 1774/2002 and EC no. 208/2006. For example: manure, blood, lipids, food waste, carcasses and other slaughterhouse waste.

Arable land: Land that can be used for growing crops.

Biogas: Gas produced during anaerobic degradation of biological material, mainly consisting of methane (CH_4) and carbon dioxide (CO_2).

Biogas plant: Large-scale biogas plant where different kinds of biowaste, except sewage sludge from waste water treatment plants, are used for production of biogas.

Biowaste: Source separated biodegradable wastes from households, restaurants, food industry, medical industry, slaughterhouse waste (animal by-products) and manure and slurry from pig and dairy farms.

C/N ratio: The balance between carbon and nitrogen. A C/N ratio between 16 and 19 is optimum for the performance of methanogenic micro-organisms.

Coliforms: Bacteria belonging to the family *Enterobacteriaceae*.

Draff: Waste product after production of ethanol.

Mesophilic digestion: Digestion at approximately 35-37°C. The common retention time is 25-30 days for mesophilic digestion.

Pathogen: Infectious micro-organism that causes disease in plants, animals and/or humans.

Retention time: The period of time which the biowaste is processed in the digester. Depending on digester volume and substrate feeding, the retention time can be calculated. Other factors that effect the retention time are access to substrate and type of substrate.

Slurry: Animal faeces and urine mixed together.

Specific risk material: SRM, *e.g.* brain, spleen, eyes and intestines from ruminants, which may contain prions.

Stabilisation of waste during digestion: Reduction of organic matter in biowaste, nutrients are mineralised to ammonia and phosphate.

Thermophilic digestion: Digestion at approximately 55°C. The normal retention time is 10-12 days for thermophilic digestion.

1 Introduction

1.1 Use of biogas and digested residues

With global concerns about energy shortages and greenhouse gas emissions through combustion of fossil fuels, more work is needed to expand the production of renewable energy (McCarty, 2001). Biogas production is an expanding field, especially in Europe, as a response to reports of global warming since biogas is renewable as well as carbon dioxide neutral (McCarty, 2001; Gijzen, 2002; Enocksson *et al.*, 2002).

Anaerobic digestion is an excellent way to convert crops and unpleasant biowaste into useful products. In full-scale commercial biogas plants (BGP) in Sweden, different kinds of biowaste such as food waste, manure and slaughterhouse waste are mixed and fermented anaerobically to produce biogas. In addition to the BGPs, waste water treatment plants produce biogas during processing of sewage sludge by anaerobic digestion. The valuable part of the gas, methane (CH_4), is used as fuel for both stationary and vehicular engines. In other European countries biogas is used for electricity production or heating. In developing countries without electricity, simple digesters can produce biogas for cooking and illumination. The residues from the digestion are used as fertiliser on arable land.

1.1.1 Advantages

Production of methane

The anaerobic digestion process generates biogas (Zinder, 1984; van Lier *et al.*, 2001; Zábranská *et al.*, 2003; Hartmann and Ahring, 2006) by degradation of biowaste from which methane can be extracted for use as a renewable fuel. When methane is combusted the only residues are carbon dioxide (CO_2) and water. Methane produced in BGPs is renewable and

therefore the biogas is carbon dioxide-neutral, unlike fossil fuels (*e.g.* natural gas).

Greenhouse gas emissions

The greenhouse gas emissions from biowaste are reduced through anaerobic digestion. The emissions of methane can be reduced by at least 50%, especially in a temperate climate, if the slurry is treated by digestion instead of storage only (van Lier *et al.*, 2001; Steinfeld *et al.*, 2006). The global warming potential of methane is 23 times higher than that of carbon dioxide (Steinfeld *et al.*, 2006). In addition, the methane is collected and used as fuel when manure or slurry is digested. Therefore the role of livestock in the greenhouse gas production is decreased (Enocksson *et al.*, 2002). This leads to a lower gas emission of animal farming assuming that the transport distances of manure/digested residues to/from BGPs are not too long. When digested residues are used as fertiliser instead of manure or slurry, the greenhouse gas emissions are lowered (Steinfeld *et al.*, 2006).

Fertiliser

The digested residues from BGPs are an excellent fertiliser, rich in plant nutrients and humus, a soil improver (McCarty, 2001; Hartmann and Ahring, 2006). Digested residues also give better utilisation of plant nutrients than manure or slurry. This is due to the fact that the organic materials are mineralised, and nutrients, such as ammonia and phosphate, are released, but not removed during anaerobic digestion (Gijzen, 2002). The higher amount of ammonia may cause greater ammonia emissions, but this can be avoided with the right spreading technique (Rodhe *et al.*, 2006). Spreading of digested residues to agricultural land reduces the need for artificial fertilisers, which is essential in sustainable farming. Futhermore, phosphorus is a limited natural resource (Enocksson *et al.*, 2002).

Chemicals

Hazardous chemicals in biowaste are in some cases neutralised during anaerobic digestion. More specifically, chlorinated compounds are decomposed by bacteria in the absence of oxygen (Zinder, 1984; Christiansen, 1995; van Lier *et al.*, 2001). For example polychlorinated biphenyls (PCB) are converted to less harmful forms (McCarty, 2001).

1.1.2 Disadvantages

Cost

Modern highly automated full-scale BGPs are technically complex and hence an expensive investment. The operation of a full-scale BGP needs large volumes and a continuous supply of energy-rich substrates for processing. Shortages of local substrate sources may lead to expensive long-distance transport of substrates and long return trips of digested residues from BGPs.

Odour

Both incoming biowaste to BGPs and digested residues have an unpleasant odour. However, following digestion the odour noticeably decreases because degradable compounds in biowaste are stabilised, but the smell from BGPs may still be unpleasant for neighbours in the surrounding area. This is important when planning BGPs close to neighbouring villages.

Explosiveness

With the right mixture of oxygen, methane is explosive. It is not possible to build either BGPs or gas filling stations without the authorities' approval and many safety restrictions exist concerning handling the gas (Sprängämnesinspektionen, 1995; Räddningsverket, 2008). Biogas powered cars are designed to minimize the risk of explosion and safety is highly prioritised by manufacturers.

Filling stations

In Sweden, most biogas stations for vehicles are located in the south-west of the country. On the west coast natural gas is available as back-up if there is a lack of biogas. However, the numbers of filling stations are too few compared with the demand from privately owned biogas cars, especially in northern Sweden. In the past, biogas powered cars also had a shorter range than petrol powered cars, but new models have increasing ranges.

Contaminants

Biowaste may contain heavy metals, organic pollutants (van Lier *et al.*, 2001) and pathogenic micro-organisms. The contents of such harmful components must be minimized when digested residues are used as fertiliser. At most full-scale BGPs in Sweden, only source-separated biowaste is accepted in order to minimize the introduction of undesirable contamination. Pathogenic micro-organisms in the biowaste should be treated in a

hygienically acceptable way to avoid the risk of spreading diseases to animals, humans and plants through digested residue fertiliser.

1.2 The biogas process

Biowaste

Biowaste can consist of food waste separated at source, waste from food industries, draff, smuggled liquor (in Sweden) and waste from pharmaceutical industries are the most common substrates in the biogas process. Some BGPs also receive animal by-products (ABP) such as manure, cattle and pig slurry, blood and lipids from slaughterhouses. In Sweden none of the full-scale commercial BGPs use sewage sludge as substrates. Biowaste is generally transported to the BGPs by tanker lorries, but pipe-lines are also used.

Homogenisation tank

Incoming, non-homogeneous biowaste is minced and then mixed in the homogenisation tank. The particle size is not allowed to exceed 12 mm since particle size has significant influence of the degradation rate due to the fact that the total surface area increases with decreasing particles size (Hartmann and Ahring, 2006).

Pasteurisation stage

Most BGPs in Sweden have a separate batch-wise pasteurisation stage at 70°C for 60 min. When a BGP receives animal by-products, pasteurisation is compulsory in EU-countries. To ensure the effect of the pasteurisation, the process is monitored continuously. The monitoring system issues fault alarm if time and/or temperature differ from expected values.

Anaerobic digestion

After pasteurisation, biowaste is fermented anaerobically. Digestion stabilises the biowaste and reduces the emissions of methane to the atmosphere. The temperatures in the anaerobic digester is either mesophilic or thermophilic.

- Mesophilic anaerobic digestion at 35-37°C with a common retention time of 25 to 30 days.
- Thermophilic anaerobic digestion at 53-55°C with a normal retention time of 10 to 12 days.

Anaerobic digestion at thermophilic temperatures is more sensitive to toxic compounds (van Lier *et al.*, 2001; Hartmann and Ahring, 2006). In some systems it can take from months up to one year before a mesophilic digester flora has adapted to thermophilic temperatures (Zábranská *et al.*, 2000; Ahring *et al.*, 2002). After adaptation, thermophilic and mesophilic digestion may be equally stable against variations, such as substrate pH, carbon/nitrogen balance (C/N ratio), *etc.* (Zábranská *et al.*, 2000, van Lier *et al.*, 2001). However, the thermophilic process is faster than processes at lower temperature, and therefore changes in the stability also take place faster (Varel *et al.*, 1977; Zinder, 1986). In addition, it has been shown that the higher the temperature, the more toxic the effect of ammonia.

Thermophilic anaerobic digestion needs more energy for operation (Zinder, 1984), but the methane yield is higher (Ahring *et al.*, 1992; Zábranská *et al.*, 2000) and the retention time is shorter than in mesophilic digestion (Zinder, 1984; van Lier *et al.*, 2001; Hartmann and Ahring, 2006). The increase in gas yield in thermophilic digestion is sufficient to compensate for the increase in energy consumption for heating the digester (Zinder, 1984; Zábranská *et al.*, 2000; van Lier *et al.*, 2001; Hartmann and Ahring, 2006).

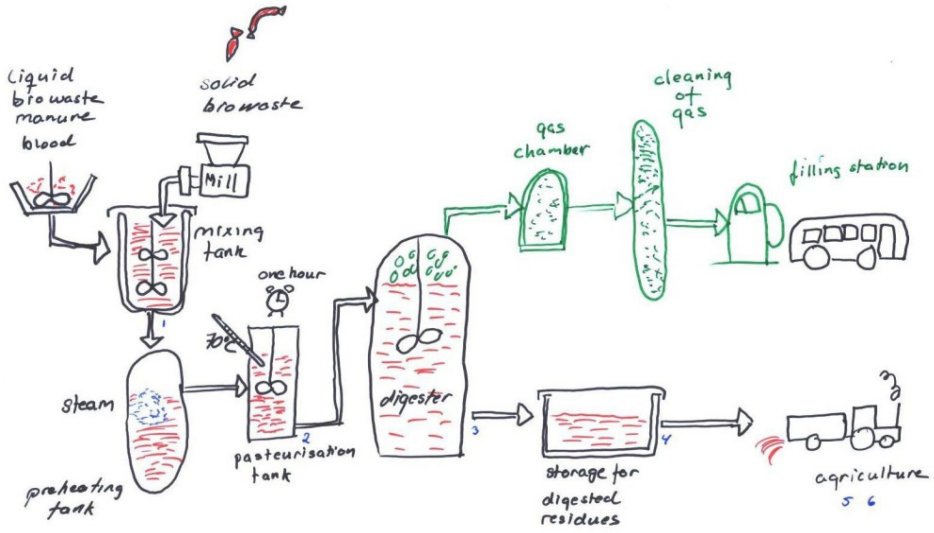

Figure 1. Schematic picture over the flow of biowaste through a BGP. The locations of sampling in the first study (Paper I) are marked with blue numerals. Red represents biowaste and residues and green biogas. (Illustration: E. Bagge)

To increase the biochemical reaction activity and to avoid sedimentation, digesters are equipped with mixing systems, *e.g.* continuous stirring systems or re-circulation of the gas in the tank through the digester substrate. To optimise the effect of the digestion process, chemical parameters such as pH, volatile fatty acids, ammonium-nitrogen (NH_4^+-N), gas yield and gas composition are continuously monitored

Storage

Following digestion the residues are stored at the BGP before being transported to farms. Digested residues are stored on the farms in underground wells until used as fertiliser.

1.3 EU-regulations

The compulsory treatment method for animal by-products is heating to 70ºC for 60 min or equivalent before anaerobic digestion, to reduce the risk of pathogen spread if manure and animal by-products are present in the substrate. This is regulated by European Commission Regulations EC no. 1774/2002 and no. 208/2006. The latter allows treatments other than pasteurisation at 70ºC for 60 min, but with equivalent effect regarding the reduction in pathogens. Such alternative treatments must be approved by each EU Member State. The pasteurisation stage is not necessary if animal by-products are excluded from the substrate.

Animal by-products are separated into three categories according to EC-regulations, depending on the expected occurrence of pathogens in the material (Table 1).

Category 1 includes Specific Risk Material (SRM, *e.g.* brain, spleen, intestines and eyes from ruminants), which may contain prions. Prions can *e.g.* cause bovine spongiform encephalopathy (BSE, mad cow disease). Entire bodies of dead ruminants containing SRM are also included in category 1. Category 1 materials must be incinerated or used for technical purposes (Stig Widell, SJV, personal communication, Mars 2009). The use of SRM is not permitted for biogas production.

Category 2 includes animal by-products from animals other than those included in category 1 or those being slaughtered for human consumption, *e.g.* dead pigs, horses and poultry. Manure and digestive tract contents are included in category 2. Category 2 materials must be sterilised at 133°C at 3 bars for 20 min before it is allowed to be used in BGPs, with the exception of manure, which is permitted for use in BGPs after pasteurisation

In category 3, only animal by-product materials from healthy animals approved for human consumption are included. Category 3 material requires maximum particle size of 12 mm and pasteurisation at 70°C for 60 min or treatment of equivalent hygienically effect, before anaerobic digestion in BGPs.

Table 1. *Summary in brief of categorisation and treatment requirements of animal by-products (ABP) according to EC no. 1774/2002.*

Animal by-products category	Examples of included material	Treatment requirements	Accepted use after treatment
Category 1	SRM Animals with suspected infection of BSE Entire bodies of cattle Pet animal, zoo animal	Incineration	Technical use
Category 2	Dead pigs, poultry and horses and their ABP. Parts of from ruminants, except SRM Digestive tract content Manure	Sterilisation at 133°C for 20 min, 3 bar pressure (exceptions for manure exist)	Substrates for biogas production or use for composting. Technical use
Category 3	ABP from animals approved for human consumption Blood	Pasteurisation at 70°C for 60 min or equivalent	Substrates for biogas production or use for composting. Pet food

SRM, Specific Risk Material. BSE, bovine spongiform encephalopathy

1.4 Anaerobic digestion

Biogas produced in BGPs consists of methane, carbon dioxide, dihydrogen sulphide (H_2S) and ammonia (NH_3). Biogas also contains water and particles. If the gas production occurs under controlled conditions in closed vessels, the gas can be collected and used directly for heating, cooking or illumination (van Lier *et al.*, 2001; Gijzen, 2002). To be useful in engines or generators, the gas has to be upgraded to natural gas quality. Upgraded biogas is obtained by drying and removing pollutants from methane. Biogas, upgraded or not, can also supply the local municipal gas grid.

The use of bacterial fermentation and anaerobic respiration under anaerobic conditions for treatment of biowaste and waste water is nothing new. Simple constructions of digesters have been used to treat manure and agricultural waste for a long time in Asia and in other parts of the world

with temperate climates (Gijzen, 2002). In addition to the gain of useful energy, a stabilisation of the waste occurs (Zábranská *et al.*, 2000; Hartmann and Ahring, 2006). Modern technology regarding anaerobic digestion of sewage sludge in waste water treatment plants, was introduced in the 1860s in France (McCarty, 2001). However, more efficient and advanced technology was developed in the 1970s. In the 1980s, various types of waste, including manure, food waste and organic household waste, were introduced to anaerobic digestion (McCarty, 2001; Gijzen, 2002).

There are a number of micro-organisms that are involved in the process of anaerobic digestion and the essential process is microbial degradation of organic matter. Protein, lipids and carbohydrates are degraded to carbon dioxide and methane in four stages: hydrolysis, fermentation, anaerobic oxidation and methanogenesis. In all stages, various kinds of micro-organisms are involved in the degradation process (McInerney and Bryant, 1981; Zinder, 1984).

In the hydrolysis stage, complex organic materials (lipids, proteins and polysaccharides) are degraded to mono- and oligomers (amino acids, peptides and sugar) (Schönborn *et al.*, 1986). This degradation is performed by enzymes produced by hydrolytic and fermentative bacteria. During fermentation, in the absence of oxygen, mono- and oligomers are degraded to alcohols, long-chain fatty acids and organic acids (McInerney and Bryant, 1981; Zinder, 1984; Schönborn *et al.*, 1986).

During anaerobic oxidation, acetogenic bacteria produce acetate, carbon dioxide and hydrogen (H_2) from the fermented products. From these compounds methane is produced by methane-forming *Archaea* (methanogens, *e.g. Methanosarcina* spp. and *Methanosaeta* spp.). These processes are sensitive to changes of compounds in the substrate or other environmental factors (*e.g.* pH, C/N ratio, metal ions and presence of toxic compounds). Therefore, when feeding digesters it is of the utmost importance to have the right proportions of proteins, lipids, carbohydrates (*e.g.* sugar, cellulose and starch), and to avoid lignin. Lignin is not degraded and disturbs the digestion due to foam formation. Micro-organisms involved in acidogenic and methanogenic reactions have a slow rate of multiplication and this may limit the digestion capacity (Zinder, 1984). A balance between acidogenic and methanogenic reactions must be maintained to prevent acidification (Gijzen, 2002). Organisms with different metabolic properties, but depending on each other for their existence, *e.g.* acidogenic bacteria and methanogenic bacteria, are known as syntrophic organisms. In general, a higher biodiversity of species of micro-organisms are active in mesophilic

digestion than in thermophilic digestion. Therefore the thermophilic process is less tolerant to changes, than the mesophilic process.

1.5 Micro-organisms

The biowaste added to digesters contains various types of micro-organisms. Some of the micro-organisms in the biowaste are pathogenic bacteria (Ilsøe, 1993; Larsen *et al.*, 1994; Gibbs *et al.*, 1995, Larsen, 1995; Bendixen, 1996), fungi, parasites (Bendixen, 1996; Chauret *et al.*, 1999) and viruses (Bendixen, 1996; Aitken *et al.*, 2005). As a consequence, animals and humans can be infected with pathogenic micro-organisms from insufficiently treated biowaste. Heating at 70°C for 60 min reduces indicator bacteria and *Samonella* spp. (Bendixen, 1996; Papers I and II). However, some spore-forming bacteria (Larsen *et al.*, 1994; Larsen, 1995; Papers I and II), heat-resistant viruses (Haas *et al.*, 1995; Kim *et al.*, 2000; Paper II) and prions (Huang *et al.*, 2007) are not reduced and can persist unaffected.

Treatments of biowaste with high levels of ammonia have some sanitation effect since the high pH inhibits the growth of bacterial pathogens (Bujoczek *et al.*, 2002; Zábranská *et al.*, 2003; Ottosson *et al.*, 2008). However, an elevated ammonia concentration also inhibits the biogas process (the methanogenic bacteria) and increases the risk of ammonia emissions when digeted residues are spread as fertiliser.

The digester harbours many various kinds of micro-organisms participating in digestion, especially bacteria, fungi and protozoa.

1.5.1 Indicator bacteria

Searching for each possible pathogenic micro-organism is impractical (Schroeder and Wuertz, 2003). Many pathogens require time-intensive tests and are difficult to quantify due to the need for enrichment steps during analysis and detection. Analysis of pathogens can be expensive and difficult to manage due to unacceptable bio-security risks. Therefore, culture of indicator bacteria can be used as a model instead of pathogens (Aitken *et al.*, 2005). Optimal indicator bacteria would normally occur in the same substrate as the pathogens of interest and be easy and cheap to analyse (Toranzos and McFeters, 1997) and easy to quantify. An increase in indicator bacteria is assumed to reflect an increase in pathogens. Common indicator bacteria are *Escherichia coli* and *Enterococcus* spp. (Berg and Berman, 1980; Toranzos and McFeters, 1997; Moce-Llivina *et al.*, 2003; Zábranská *et al.*, 2003). Such indicator bacteria are usually non-pathogenic and occurring in large quantities in the intestinal tract of humans and animals. *Enterococcus*

spp. are the most suitable indicator bacteria in thermophilic treatments of biowaste (Larsen *et al.*, 1994; De Luca, 1998). *Clostridium perfringens* or *Bacillus* spp. are used as indicators for spore-forming bacteria (Larsen *et al.*, 1994).

1.5.2 Spore-forming bacteria

Spore-forming bacteria (*e.g.* *Bacillus* spp. and *Clostridium* spp.) grow as vegetative cells under favourable conditions. When growth conditions are poor or nutrient deprivation occurs (Labbé and Remi-Shih, 1997), the bacteria can sporulate and persist as very resistant dormant spores (Mitscherlich and Marth, 1984; Gyles and Thoen, 1993). When the growth conditions become more favourable, the spores germinate to vegetative cells. The spores are tolerant to heat, disinfectants and desiccation. *Clostridium* spp. only grows under anaerobic conditions (Gyles and Thoen, 1993; Quinn *et al.*, 1994c; Songer and Post, 2005d).

Normal intestinal flora and faeces from animal species contain various kinds of spore-forming bacteria (Timoney *et al.*, 1988; Gyles and Thoen, 1993). Most of them are harmless and necessary inhabitants of the gut. Some spores pass unaffected through the digestive systems of animals. During grazing there is a risk that spores contaminate pasture.

Clostridium spp. (Larsen *et al.*, 1994; Chauret *et al.*, 1999; Aitken *et al.*, 2005; Papers I and II) and *Bacillus* spp. (Larsen *et al.*, 1994; Paper I) are commonly found in manure from cattle and hence in substrate from BGPs. These bacteria are regularly detected after pasteurisation (Larsen *et al.*, 1994; Papers I and II). Less is known about the effect of digestion on various pathogenic spore-forming bacteria. The quantities of spore-forming fungi do not decrease during digestion (Schnürer and Schnürer, 2006).

Pathogenic spore-forming bacteria of special concern for animal health are *Bacillus anthracis*, *Clostridium botulinum*, *Clostridium chauvoei*, *Clostridium haemolyticum*, *C. perfringens*, *Clostridium septicum*, *Clostridium sordellii* and *Clostridium tetani*. All these bacteria are lethal or cause serious clinical diseases in farm animals which can lead to extensive economic losses for farmers. These bacteria can occur in biowaste to be used in BGPs. Hence it is important to investigate the risk of spreading spore-forming bacteria from BGPs due to their survival in the biogas process. Many of these pathogenic bacteria are soil bacteria (Timoney *et al.*, 1988; Gyles and Thoen, 1993; Munang'andu *et al.*, 1996; Hang'ombe *et al.*, 2000; del Mar Gamboa *et al.*, 2005). In soil, spores can persist for many years (Mitscherlich and Marth, 1984; Gyles and Thoen, 1993). It is important to acquire knowledge about the influence of the digestion process on various pathogenic spore-forming

bacteria in order to mitigate the risk of spreading diseases through digested residues from BGPs.

1.6 Pathogenic bacteria of concern in biogas production

1.6.1 Spore-forming bacteria

Bacillus spp.

Bacillus spp. form a group of large, Gram-positive spore-forming rods, which grow during aerobic or facultative anaerobic conditions and are catalase-positive. Most of them are motile, except *B. anthracis* (Quinn *et al.*, 1994b). The spores can persist for decades in soil (Mitscherlich and Marth, 1984). Apart from *B. anthracis* (see below), most *Bacillus* spp. are harmless saprophytes, that are found in the general environment. However, *Bacillus cereus* causes food poisoning in humans (Quinn *et al.*, 1994b) and mastitis in cattle (Schiefer *et al.*, 1976; Turnbull *et al.*, 1979; Radostits *et al.*, 2000; Songer and Post, 2005a). *Bacillus licheniformis* and *Bacillus pumilus* can cause mastitis in cattle, but do so rarely (Nieminen *et al.*, 2007). *Bacillus licheniformis* can cause abortions in cattle (Quinn *et al.*, 1994b; Songer and Post, 2005a). *Bacillus cereus, B. licheniformis, B. pumilus* and *Bacillus subtilis* can be considered opportunistic pathogens and can cause disease in humans under particular circumstances (Sliman *et al.*, 1987; Banerjee *et al.*, 1988; Ozkocaman *et al.*, 2006). *Bacillus* spp. infections in humans are mostly found in combination with conditions such as wound infections, trauma (Turnbull *et al.*, 1979; Åkesson *et al.*, 1991), pneumonia (Ozkocaman *et al.*, 2006) or acquired immune deficiency syndrome (Sliman *et al.*, 1987). Non-anthrax *Bacillus* spp. infections can affect cancer patients (Sliman *et al.*, 1987; Banerjee *et al.*, 1988; Ozkocaman *et al.*, 2006).

The spores of *Bacillus* spp. seem to pass unaffected through the biogas process (Olsen and Larsen, 1987; Bendixen, 1993; Larsen *et al.*, 1994; Papers I and III). Since most *Bacillus* spp. are harmless for humans and animals, these bacteria are not a hygiene problem in connection with BGPs and digested residues.

Bacillus anthracis

Bacillus anthracis, which causes anthrax, is found in soil and water world-wide. During the Second World War, British research with biological weapons was carried out on the island of Gruinard of the west-coast of Scotland. An airborne cloud laden with *B. anthracis* spores was spread by an explosion, causing long-term contamination of the island (Willis, 2002).

Sanitation of the island was carried out more than once because of the resistant spores of *B. anthracis* in the soil. In 1990, the island was declared safe (Willis, 2002).

Anthrax is known as a per-acute, life-threatening, dreaded disease, particularly the pulmonary form, which can occur after inhalation of *B. anthracis* spores (Songer and Post, 2005a; Bravata *et al.*, 2007). Ruminants are susceptible to *B. anthracis* and frequently suffer a per-acute course of events and sudden death (Quinn *et al.*, 1994b). In humans, skin infections from wounds causing cutaneous anthrax are most common (Songer and Post, 2005a). Rawhide workers are a risk group for anthrax (Semple, 1973). A third variant of anthrax is the intestinal form, which arises after ingestion of spores (Songer and Post, 2005a; Bravata *et al.*, 2007).

Methods of detection for *B. anthracis* are culture and PCR. *Bacillus anthracis* produces a unique capsule, which can be visualized in blood smears stained with Giemsa and methylene blue (Quinn *et al.*, 1994b).

Clostridium spp.

Clostridium spp. comprise a large group of Gram-positive spore-forming rods, which grow under anaerobic conditions, are fermentative, oxidase-negative and catalase-negative (Quinn *et al.*, 1994c). Most of the clostridia are harmless and can be found in the environment and in the gut flora, but some can cause diseases. The pathogenic clostridia are divided into four groups:

- Neurotoxic clostridia (*C. botulinum, C. tetani*)
- Histiotoxic clostridia (*C. chauvoei, C. septicum, C. haemolyticum, C. sordellii*)
- Enterotoxaemic clostridia (*C. perfringens*)
- Clostridia associated with antibiotic treatment (*C. difficile* and *C. spiroforme*)

Clostridium botulinum

Spores and vegetative cells of *C. botulinum* can be found in soil, water, decaying vegetation at the edges of ponds and lakes (Huss, 1980; del Mar Gamboa *et al.*, 2005; Songer and Post, 2005d) and in manure from cattle and pigs (Dahlenborg *et al.*, 2001; Dahlenborg *et al.*, 2003). *Clostridium botulinum*, both bacteria and toxins, have been found in composted biowaste in one investigation (Böhnel and Lube, 2000). Toxin production can occur in the environment under the right conditions (Notermans *et al.*, 1979; Timoney *et al.*, 1988; Böhnel and Lube, 2000).

The disease caused by *C. botulinum* is not an infection, but an intoxication. Most commonly, botulism arises from ingestion of food, feed or water contaminated with pre-formed toxins from *C. botulinum* (Timoney *et al.*, 1988; Deprez, 2006) or contaminated forage, *e.g.* carrion in silage. The toxins act by blocking nerve function and this leads to flaccid paralysis of respiratory muscles and other skeletal muscles. The toxins are heat labile and are destroyed during heating or cooking of food (Licciardello *et al.*, 1967).

Clostridium botulinum is able to grow during acid conditions, from pH 4.5, in food or feed (Margosch *et al.*, 2006). Ingestion of spores of *C. botulinum* does not appear to produce toxins in the alimentary tract in general (Timoney *et al.*, 1988). Spores ingested by infants can cause infant botulism or intestinal botulism, after colonisation of the organisms in the gut and subsequent release of toxins (Timoney *et al.*, 1988; Songer and Post, 2005d). A common cause of botulism in infants, is ingestion of *C. botulinum* spores in honey (Nevas *et al.*, 2002). In shaker foal syndrome, spores are ingested and the toxins are produced in the gut (Deprez, 2006). Toxins can be produced in necrotic wounds infected with *C. botulinum* and cause wound botulism, which has been described in horses and humans (Peck and Duchesnes, 2006).

The toxins of *C. botulinum* are some of the most potent known toxins. There are seven various neurotoxin groups: BoNT types A-G (Deprez, 2006). The toxin groups have species specificity, *i.e.* different hosts are affected by different toxins (Timoney *et al.*, 1988; Quinn *et al.*, 1994c). Horses are sensitive to toxins from BoNT type A, B and C, cows to B, C and D, birds to C and E, fish to E (Huss, 1980; Desprez, 2006), and humans are sensitive to A, B, E and F (Peck and Duchesnes, 2006).

Clostridium chauvoei

In cattle and sheep, *C. chauvoei* causes a disease called blackleg. Young, fast-growing animals on pasture are especially sensitive (Songer, 1997). Animals other than ruminants are rarely infected (Timoney *et al.*, 1988; Quinn *et al.*, 1994c). The infection route appears to be oral during grazing or when eating spore contaminated silage or hay. The bacteria produce several toxins, and neuraminidase may play a significant role (Useh *et al.*, 2003), but all pathogenicity mechanisms are not clearly understood. One effect is widespread skeletal muscle damage, and the plasma enzymes associated with muscle damage increase markedly (Pemberton *et al.*, 1974). Clinical symptoms of blackleg are fever and swollen muscular tissues with entrapped gas. The mortality is high and sudden death without clinical signs occurs

(Timoney *et al.*, 1988; Sternberg *et al.*, 1999; Songer and Post, 2005d). Infection by *C. chauvoei* appears rarely as myocarditis in calves (Uzal *et al.*, 2003b). In areas where blackleg is endemic, affected farms usually have costly vaccination routines, in Sweden as well as world-wide.

The quantity of *C. chauvoei* is high in most muscle samples from cattle that died from blackleg. The standard method for detection of *C. chauvoei* is based on culture and biochemical identification.

Clostridium chauvoei is commonly present in soil and faeces (Smith and Holdeman, 1968; Timoney *et al.*, 1988; Gyles and Thoen, 1993; Hang'ombe *et al.*, 2000; del Mar Gamboa *et al.*, 2005). Once a pasture has become heavily contaminated, the disease usually occurs year after year in susceptible animals (Timoney *et al.*, 1988; Songer, 1997). Infections are most common during the summer on permanent pastures and in wetlands (Sternberg *et al.*, 1999). Outbreaks of blackleg seem to increase after heavy rain-fall (Useh *et al.*, 2006).

Clostridium haemolyticum

Clostridium haemolyticum causes haemoglobinuria, or redwater, in cattle. The disease is a frequent complication of liver damage caused by the liver fluke (*Fasciola hepatica*) or other causes (Gyles and Thoen, 1993; Songer, 1997). A strongly haemolytic beta-toxin produced by the bacteria causes cell lysis (Gyles and Thoen, 1993). The bacterium is a common pathogen in *e.g.* the Rocky Mountains, USA (Timoney *et al.* 1988) but not in Sweden. *Clostridium haemolyticum* can be found in soil (del Mar Gamboa *et al.*, 2005), but has a predilection for alkaline water. Most occurrences of redwater are associated whith grazing cattle on pasture in swampy areas with pH greater than 8.0.

For optimal growth, *C. haemolyticum* needs strict anaerobic conditions and tryptophane (Timoney *et al.*, 1988). The colonies produce haemolysis on blood agar plates.

Clostridium perfringens

Clostridium perfringens is usually found in the intestine of animals and humans (Songer and Post, 2005d). Manure and slurry brought into BGPs commonly contain *C. perfringens* and it is well known that these bacteria pass unaffected through the biogas process (Bendixen, 1993; Larsen *et al.*, 1994; Larsen, 1995; Papers I-III).

Most *C. perfringens* are harmless, but can under certain circumstances be pathogenic (Gyles and Thoen, 1993). *Clostridium perfringens* can be subtyped into five toxin types, A, B, C, D and E, based on the production of four

major lethal toxins (Gyles and Thoen, 1993; Quinn *et al.*, 1994c; Songer and Post, 2005d; Songer, 2006b) (Table *2*).

Table 2. *The major toxins of Clostridium perfringens*. (*Modified from Niilo, 1980*)

Clostridium perfringens	Major toxin			
type	*alfa*	*beta*	*epsilon*	*iota*
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	++

++ Predominat toxin fraction. + Smaller quantities of toxin. – Not produced

- *Clostridium perfringens* type A causes food poisoning in humans, wound infection in horses (Gyles and Thoen, 1993), abomasitis in calves and lambs (Songer and Miskimins, 2005), and necrotic enteritis in poultry (Johansson, 2006).
- *Clostridium perfringens* type B causes lamb dysentery (Quinn *et al.*, 1994c; Songer and Post, 2005d).
- *Clostridium perfringens* type C causes severe haemorrhagic enterotoxaemia with diarrhea and dysentery in newborn piglets. Older piglets develop a chronic form of enteritis. *Clostridium perfringens* type C can also cause disease in lambs and calves (Timoney *et al.*, 1988; Gyles and Thoen, 1993; Songer, 2006a).
- *Clostridium perfringens* type D is associated with pulpy kidney disease in young sheep (Wierup and Sandstedt, 1983; Quinn *et al.*, 1994c). Furthermore it can cause enterotoxaemia in calves (Niilo, 1980).
- *Clostridium perfringens* type E causes enterotoxaemia in calves and lambs (Quinn *et al.*, 1994c; Songer, 2006b).

The standard method for detection of *C. perfringens* is based on culture, biochemical identification and toxin typing by PCR (Engström *et al.*, 2003).

Clostridium septicum

Clostridium septicum is common in soil and in the intestinal tract of many animals (Timoney *et al.*, 1988; Gyles and Thoen, 1993; Munang'andu *et al.*, 1996). *Clostridium septicum* causes malignant edema in connection with infected wounds in many animal species (Songer, 1997; Songer and Post, 2005d). The clinical symptoms of malignant edema are fever and anorexia,

often followed by death within 24 h. These symptoms are similar to those of blackleg caused by *C. chauvoei*, but unlike blackleg the muscular tissue contains little or no gas (Timoney *et al.*, 1988).

The bacteria can cause braxy or bradsot in lambs and dairy calves (Gyles and Thoen, 1993; Songer and Post, 2005d; Songer, 2006a). Braxy is an infection of the abomasum, possibly after damage of the abomasal epithelium, but the invasion mechanisms are not clearly understood. After ingestion of coarse fodder or frozen feed, the risk of braxy increases (Songer, 2006a).

Clostridium sordellii

Clostridium sordellii is a common intestinal tract inhabitant of many animal species but it is also found in soil (Gyles and Thoen, 1993; del Mar Gamboa *et al.*, 2005; Songer, 2006b). Cattle, sheep and horses can get gas gangrene from *C. sordellii* if wounds are infected (Quinn *et al.*, 1994c). *Clostridium sordellii* can also cause fatal postoperative infections (Songer and Post, 2005d). Sometimes *C. sordellii* can cause myositis, liver disease and large edemas in subcutaneous tissues or muscular fascia (Songer, 1997). In extremely infected animals, subendocardial haemorrhages and septicaemia can be shown *post mortem* (Gyles and Thoen, 1993; Songer, 2006b). Numerous toxic substances are produced by the bacteria, and it is assumed that these toxins are involved in the pathogenesis (Songer and Post, 2005d).

Clostridium tetani

Clostridium tetani is a soil bacterium, but can also be found in manure (Timoney *et al.*, 1988; del Mar Gamboa *et al.*, 2005; Deprez, 2006).

Clostridium tetani produces three different toxins: the neurotoxin tetanoplasmin, haemolysin and peripherally active non-spasmogenic toxin. Tetanoplasmin causes the characteristic clinical features of tetanus, a spastic paralysis (Timoney *et al.*, 1988; Quinn *et al.*, 1994c). The bacteria multiply and subsequently produce toxin as soon as the local oxygen level is sufficiently low (Songer, 1997; Deprez, 2006). Horses are particularly susceptible to tetanus toxin (Songer and Post, 2005d; Deprez, 2006), but most animals can be infected. Usually the disease starts in deep and narrow penetration wounds (*e.g.* nail wounds), unclean wounds with necrotic tissues (castration wounds) or infection via the umbilicus. In Europe today the disease is uncommon due to a highly effective vaccine for horses and humans.

Clostridium tetani grows slowly with small colonies and produces haemolysis on blood agar plates. There are no good routine diagnostic

methods available for identification of the toxin, and the diagnosis of tetanus is almost entirely based on the typical clinical symptoms.

Clostridium tyrobutyricum

Clostridium tyrobutyricum is a non-pathogenic clostridium, but is of economic concern in the dairy industry since C. *tyrobutyricum* produces butyric acid in cheese. This results in the so-called late blowing of hard cheese, which causes production problems for cheese producers (Dasgupta and Hull, 1989; Thylin, 2000). Spores of *C. tyrobutyricum* can be detected in manure and are not reduced by pasteurisation or digestion (Dasgupta and Hull, 1989; Jo *et al.*, 2008). The occurrence of C. *tyrobutyricum* in digested residues from BGPs may contaminate the silage when digested residues are used as fertiliser. Neither anaerobic digestion (Jo *et al.*, 2008) nor ensiling inactivates *C. tyrobutyricum* (Johansson *et al.*, 2005). Thus silage may be a source of C. *tyrobutyricum* for cows. Spores consumed with fodder result in excretions in the dung and subsequently into the milk.

1.6.2 Non spore-forming bacteria

Salmonella spp.

Salmonella spp. are important world-wide zoonotic bacteria with multi-resistant strains (Romani *et al.*, 2008). They are one of the most common pathogens in manure, slurry (Larsen *et al.*, 1994; Larsen, 1995) and sewage sludge (Gibbs *et al.*, 1995; Jepsen *et al.*, 1997; Ward *et al.*, 1999).

Salmonella spp. can persist from months to years in the environment under favourable conditions and can grow in temperatures between 6 and 47°C (Mitscherlich and Marth, 1984; Nicholson *et al.*, 2005). All serotypes of *Salmonella* spp. are potentially pathogenic to humans, which make *Salmonella* spp. important in biowaste disposal.

Pasteurisation at 70°C (Papers I and II) or thermophilic digestion at 55°C (Bendixen, 1993; Larsen *et al.*, 1994; Larsen, 1995; Zábranská *et al.*, 2003; Aitken *et al.*, 2005) reduces salmonella in biowaste. After mesophilic digestion only some decline in numbers of salmonella can be shown (Kearney *et al.*, 1993b; Larsen *et al.*, 1994; Horan *et al.*, 2004; Smith *et al.*, 2005). Hence, mesophilic digestion is not a sufficient treatment of biowaste since the remaining numbers of *Salmonella* spp. or other bacterial pathogens are sufficient to sustain re-growth. (Gibbs *et al.*, 1997; Gerba and Smith, 2005; Nicholson *et al.*, 2005).

Salmonella spp. can cause enteritis in animals but pneumonia, abortions, poor growth and sudden death can also occur (Morter *et al.*, 1989; Trueman

et al., 1996; McDonough *et al.*, 1999). In Swedish livestock the frequency of *Salmonella* spp. is low and to maintain this situation and avoid costly outbreaks of salmonella, all farmers are associated with strict control programmes. In humans, *Salmonella* spp. most commonly cause food-borne enteritis, with diarrhea, nausea, and abdominal pain (Schroeder and Wuertz, 2003). Complications of salmonella infection in humans include arthritis, heart failure and even death (Gokhale *et al.*, 1992). After infection both animals and humans can subclinically shed *Salmonella* spp. for more than one year (Linton *et al.*, 1985; Stenström, 1996; Stege *et al.*, 2000; Huston *et al.*, 2002).

Salmonella spp. belong to the family of *Enterobacteriaceae,* which are motile, Gram-negative rods. The detection methods used in routine laboratories are enrichment in broth, selective agar plates and PCR.

Verotoxinogenic *Escherichia coli O157*

Verotoxinogenic *Escherichia coli* O157 (VTEC O157) and other serotypes of VTEC are found in cattle manure (Kudva and Hovde, 1998; Albihn *et al.*, 2003). Survival of VTEC O157 in bovine faeces varies depending on temperature and initial quantity of bacteria (Wang *et al.*, 1996). VTEC O157 can persist and produce toxins for months to years in manure and is able to multiply at 22°C (Wang *et al.*, 1996; Nicholson *et al.*, 2005). In ovine manure VTEC O157 persists for several months (Kudva and Hovde, 1998). The long-term survival of VTEC O157 in manure illustrates the need for appropriate farm waste management to prevent environmental contamination by these bacteria and transmission of infective agents to animals and humans (Kudva and Hovde, 1998; Gerba and Smith, 2005). Mesophilic anaerobic digestion reduces the numbers of *E. coli,* but does not eliminate them (Larsen, 1995; Horan *et al.*, 2004).

In humans, VTEC O157, also known as enterohaemorrhagic *E. coli* (EHEC), can cause enteric disease characterised by haemorrhagic diarrhea and abdominal pain. Serious complications may follow, such as haemolytic uraemic syndrome (HUS), a life-threatening renal failure mainly affecting children and elderly people (Schroeder and Wuertz, 2003). VTEC O157 has become an important water- and food-borne infection in humans, where faeces are the primary source of contamination of food products, *e.g.* consumption of raw or undercooked food (Doyle, 1991). The verotoxin produced by VTEC O157 inhibits protein synthesis and causes damage in endothelial cells, which is important in haemorrhagic colitis and HUS (Griffin and Tauxe, 1991; Bielaszewska and Karch, 2005). In ruminants, symptoms are uncommon.

Healthy cattle and other ruminants sporadically harbour VTEC O157 in their gastrointestinal tracts and are thus reservoirs of this human pathogen (Kudva and Hovde, 1998; Eriksson *et al.*, 2005). In a Swedish study performed in 1998-2000, 9% of the herds were infected by VTEC O157 (Eriksson *et al.*, 2005). The study revealed regional differences with the highest prevalence (23%) found in south-west Sweden (county of Halland), whereas VTEC O157 was not detected at all in northern Sweden.

Escherichia coli is a Gram-negative rod-shaped bacterium belonging to the family of *Enterobacteriaceae*. The detection methods used in routine laboratories are enrichment in broth, culture on selective agar plates, immuno-magnetic-absorbent assays and latex agglutination test.

Campylobacter spp.

Campylobacter jejuni and *C. coli* are often present in slurry (Kearney *et al.*, 1993a, Sahlström *et al.*, 2004) and raw sewage sludge (Steltzer *et al.*, 1991; Stampi *et al.*, 1998/99). *Campylobacter* spp. have been isolated in manure from poultry (Hansson, 2007), cattle and pigs (Larsen, 1995). Furthermore, *Campylobacter* spp. have also been isolated from water samples, which were probably contaminated with faeces (Gallay *et al.*, 2006). In some studies *Campylobacter* spp. seemed to be sensitive to mesophilic anaerobic digestion and were reduced (Steltzer *et al.*, 1991; Stampi *et al.*, 1998/99). However, in other studies *Campylobacter* spp. have not been reduced during mesophilic digestion (Kearney *et al.*, 1993a; Kearney *et al.*, 1993b; Horan *et al.*, 2004). In slurry *Campylobacter* spp. can persist up to three months and after land application up to one month (Nicholson *et al.*, 2005).

Campylobacter spp. occur as commensals in the intestinal tract of many animal species, especially in birds. *Campylobacter jejuni* can cause abortions in ruminants, but this is rare (Quinn *et al.*, 1994d; Songer and Post, 2005c). In humans, thermophilic *Campylobacter* spp. can cause diarrhea, nausea, vomiting, fever and abdominal pain. The infection is usually self-limiting in a couple of days. Post infection complications of campylobacteriosis occur, including arthritis and Guillain-Barré syndrome, an autoimmune disease affecting the peripheral nervous system (Taboada *et al.*, 2007). Campylobacteriosis is the most commonly reported water borne bacterial gastroenteritis throughout the world. It is also a food-borne infection associated with insufficiently cooked poultry or contamination by raw poultry meat (Berndtson, 1996; Schroeder and Wuertz, 2003).

Campylobacter spp. are Gram-negative spirally curved rods. The bacteria are motile by a unipolar flagellum or bipolar flagella, and move rotating like a corkscrew. Most of the *Campylobacter* spp. grow at microaerophilic

conditions, and four *Campylobacter* spp. (*C. jejuni, C. coli, C. lari* and *C. upsaliensis*) are often referred to as thermophilic campylobacter, as they exhibit optimal growth at a temperature of 41 to 42°C (Quinn *et al.*, 1994d).

Listeria monocytogenes

An ubiquitous pathogen found in soil, silage, faeces and sewage sludge is *L. monocytogenes* (Donald *et al.*, 1995; De Luca *et al.*, 1998; Johansson *et al.*, 2005). *Listeria monocytogenes* is common in uncultivated fields, plant-soil environments and decaying vegetation (Weiz and Seeliger, 1975). The bacteria can persist and even grow between 1° and 45°C in digested residues (Junttila *et al.*, 1988). Several weeks after sewage sludge is spread on land; the reduction in number of *L. monocytogenes* is reported to be negligible (Watkins and Sleath, 1981). In slurry *L. monocytogenes* can persist up to six months (Nicholson *et al.*, 2005) and in composts for several weeks (Lemunier *et al.*, 2005). For that reason, *L. monocytogenes* is a pathogen that should be considered a potential health risk when spreading digested residues from BGPs and sewage sludge from waste water treatment plants (Weis and Seeliger, 1975; De Luca *et al.*, 1998; Gerba *et al.*, 2002). During mesophilic digestion the number of *L. monocytogenes* declines slowly, but the organism is not completely eliminated (Horan *et al.*, 2004). *Listeria monocytogenes* is vulnerable in environments with low oxygen concentration, which is the case during anaerobic digestion or in silage (Kearney *et al.*, 1993b; Donald *et al.*, 1995; De Luca *et al.*, 1998). In a laboratory-scaled study *L. monocytogenes* could be detected in silage at day 7, but not after 60 days (Johansson *et al.*, 2005). However, *L. monocytogenes* is tolerant to acid environments (Cotter and Hill, 2003) and a risk factor for infection of *L. monocytogenes* for dairy cattle is silage (Vilar *et al.*, 2007).

In both ruminants and humans *L. monocytogenes* causes spontaneous abortions, septicaemia, eye infection and meningitis (Ito *et al.*, 2008; Yildiz *et al.*, 2007). For animals, ingestion of *L. monocytogenes* seems to be the most common route of infection (Songer and Post, 2005b; Quinn *et al.*, 1994a). In humans *L. monocytogenes* is a food-borne pathogen and the clinical manifestations are usually flu-like (Yildiz *et al.*, 2007).

Listeria monocytogenes is a Gram-positive, rod-shaped, motile bacterium, which can grow at low temperatures (*e.g.* in food in a refrigerator, +4°C) (Junttila *et al.*, 1988).

1.7 Phylogenetic classification of spore-forming bacteria

Bacteria are classified into different taxonomic categories: phylum, class, order, family, genus, species and subspecies, where phylum is the highest rank and subspecies is the lowest. Subspecies has not been defined for all bacteria. Bacteria within the phyla *Actionbacteria* and *Cyanobacteria* are classified in a slightly different way. Two examples of classification of bacteria are given in Table *3*. Both *Bacillus* spp. and *Clostridium* spp. have a Gram-positive structure of the cell wall, but phylogenetic analysis based on 16S rRNA gene sequence has revealed divergence into two phylogenetic lineages (Stackebrandt and Rainey, 1997)

Table 3. *Examples of classification of bacteria.*

Taxonomic rank	Classification of *Bacillus subtilus*	Classification of *Clostridium chauvoei*
Phylum	*Firmicutes*	*Firmicutes*
Class	*Bacilli*	*Clostridia*
Order	*Bacillales*	*Clostridiales*
Family	*Bacillaceae*	*Clostridiaceae*
Genus	*Bacillus*	*Clostridium*
Species	*Bacillus subtilis*	*Clostridium chauvoei*
Subspecies	*Bacillus subtilis* subsp. *subtilis*	not defined

The phylogeny of the genera *Bacillus* and *Clostridium* (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999) is based on 16S rRNA gene sequences. The fragments of the 16S rRNA genes were generated by PCR with universal primers and the sequence data were compared by phylogenetic methods based on neighbour-joining. Highly variable positions in genes are more useful for purposes of close relationships, while mutations in more conserved regions reflect long-term events in evolution (Stackebrandt and Rainey, 1997). The 16S rRNA gene contains well-defined segments of different evolutionary variability. There are conserved, semi-conserved and variable regions. This molecule can therefore be used to study phylogenetic relations between both closely related and very distantly related organisms.

The genus *Bacillus* has been taxonomically reorganised and new families in the order *Bacillales* are described (Collins *et al.*, 1994). New genera such as *Paenibacillus* spp. and *Lysinobacillus* spp. have also been introduced. *Bacillus anthracis, B. cereus* and *B. thuringiensis* are difficult to distinguish by phylogenetic analysis based on 16S rRNA sequencing due to high sequence similarity of these bacteria (Sacchi *et al.*, 2002). Many bacterial species have multiple 16S rRNA operons, and the homologous constituting genes are

not necessarily identical within one strain (Stackebrandt and Rainey, 1997). These sequence differences are known as polymorphism. Members of the genera *Bacillus* and *Clostridium* have unusually large numbers of rRNA operons. *Bacillus cereus* and *C. difficile* for instance have 13 and 11 operons, respectively.

Phylogenetic analysis based on 16S rRNA sequencing of the genus *Clostridium* has shown this genus to be a heterogeneous group (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999). The genus *Clostridium* can be divided into different clusters. Some of these clusters contain species other than clostridia (Collins *et al.*, 1994). The type species of the genus, *Clostridium butyricum,* belongs to cluster I and nearly half of the clostridia belong to this cluster (Collins *et al.*, 1994; Stackebrandt and Rainey, 1997). Most of the pathogenic members of the genus *Clostridium* are included in cluster I, for instance *C. botulinum, C. chauvoei, C. haemolyticum, C. perfringens, C. septicum* and *C. tetani* (Stackebrandt *et al.*, 1999). *Clostridium sordellii* can be found in cluster XI.

Figure 2. Biogas plant at Linköping during a visit when the study in paper I was performed. (Photo: E. Bagge, Mars 2000)

2 Aims

The main aims of this thesis were to study the hygiene quality of digested residues from full-scale biogas plants and to establish whether or not digested residues can be regarded as hygienically safe for use as an agricultural fertiliser when the risks of spreading animal diseases are considered.

The specific aims were to:

- Establish whether zoonotic bacterial pathogens and spore-forming bacterial pathogens of animal concern are sufficiently reduced when biowaste is processed in biogas plants.
- Determine whether there is any risk for re-contamination of digested residues under storage and transportation.
- Investigate under laboratory-scale conditions whether heat treatment at 55°C under similar conditions as the pasteurisation step at 70°C is sufficient for replacing pasteurisation at 70°C.
- Investigate the spore-forming bacterial flora occurring in manure, slaughterhouse waste and in different phases of the biogas process.
- Study the fate of pathogenic species of clostridia through pasteurisation and digestion under laboratory-scale conditions.
- Establish the prevalence of *Clostridium chauvoei* in heavily contaminated samples such as faeces, soil, biogas substrate and digested residues from a biogas plant.
- Apply a method based on PCR for detecting *Clostridium chauvoei* in clinical samples
- Determine whether digested residues can be safely used as a fertiliser.

3 Considerations on Materials and Methods

To meet the aims of this thesis, studies leading to five individual papers were performed. Paper I was a screening study of zoonotic bacteriological pathogens and spore-forming bacteria from the different processing stages of full-scale BGPs and from farm storage wells. The objectives were to investigate whether pathogens in biowaste are reduced and whether digested residues intended for use as fertiliser, can be regarded as hygienically safe after treatment, storage and transport. In Paper II the objective was to investigate the pasteurisation stage in laboratory conditions with references to the same bacteria as in Paper I. The results in Papers I and II showed that spore-forming bacteria were not reduced. Therefore Paper III was a screening study to identify the spore-forming bacteria occuring in biowaste and in the biogas process. Pathogenic spore-forming bacteria were found. The objective of Paper IV was under laboratory conditions to investigate the fate of spore-forming bacteriological pathogens of animal concern during digestion in order to determine whether they are sufficiently reduced and whether digested residues can be regarded as safe for use as a fertiliser. One spore-forming bacterium of special interest from an endemic and economic point of view is *C. chauvoei*. There is a risk that digested residues may spread *C. chauvoei* to other regions. In Paper V, the prevalence of *C. chauvoei* in heavily contaminated samples such as faeces, soil, biogas substrate and digested residues was investigated. A PCR-based method was used for detecting *C. chauvoei* in environmental samples and in muscle tissue samples from cattle that died from blackleg.

All details of materials and methods are described in the different papers (I-V), apart from the pilot study, which was published as a report in Swedish with an English summary (Ekvall *et al.*, 2005).

3.1 Non spore-forming pathogenic bacteria in the biogas process (Papers I and II)

In order to investigate the hygiene quality of the biogas process, four Swedish commercial BGPs were studied in Paper I, while in Paper II the pasteurisation stage was investigated in a laboratory study. The BGPs studied here process animal by-products such as slaughterhouse waste, manure and slurry from pig- and dairy farms, and biowaste from food industries, households and restaurants. The different kinds of biowaste were mixed, pasteurised and thereafter digested. In Paper I, the samples from each BGP were taken before and after pasteurisation, after anaerobic digestion, in the storage for digested residues and from storage wells on two different farms (*Figure 1*). Three of the BGPs in the study have mesophilic digestion at 37°C and one has thermophilic digestion at 55°C. In Paper II, the substrate came from the homogenisation tank from one BGP and in the laboratory study samplings were made before, during and after pasteurisation.

The samples described in Papers I and II were analysed for pathogenic bacteria (*Salmonella* spp., *Listeria monocytogenes*, *E. coli* O157, *Campylobacter* spp.), indicator bacteria (coliforms, *Enterococcus* spp. and *E. coli*) and spore-forming bacteria (*Clostridium* spp. and *Bacillus* spp.). Indicator bacteria and spore-forming bacteria were analysed quantitatively and pathogenic bacteria qualitatively. In Paper I, salmonella serotypes found on more than one sampling occasion or sampling place were typed by pulsed-field gel electrophoresis (PFGE), as described by Palmgren *et al.* (2006). Four pathogens, the same as those detected in Paper I, were inoculated in homogenisation tank substrate before the trial in Paper II.

3.2 Hygiene in transportation vehicles (pilot study)

In Paper I, recontamination of the digestion residue after transportation was observed. The same vehicles were used for both transportation of biowaste to the BGP and transportation of digested residues to the farms. The transport tankers were cleansed and disinfected between inward- and outward-transportations. The observations reported in Paper I resulted in a pilot study on the efficiency of cleansing of transportation vehicles. The cleansing process must be effective for prevention of recontamination, both on the exterior and in the interior of the vehicles. The objective of the pilot study was to develop a method for assessment of cleansing efficiency. This study is presented in Ekvall *et al.* (2005).

Three tanker lorries were included in this pilot study. These were used for transport of manure and slaughterhouse waste into the BGPs and then

for transport of digested residues to farms. The samples were taken on one occasion per tanker lorry after routine cleansing and disinfection, after emptying of biowaste and before filling with digested residues. The inside of the tanks was cleansed by high pressure flushing with hot water and then rinsed with disinfectant: 0.2% lye (NaOH) or Virkon S®. The outside of the vehicles was cleansed by high pressure flushing with hot water only. The samples were taken both from the inside and outside of the tanker lorries (*Figure 4*). The different sampling methods were used and compared.

Compresses: The compresses were rubbed over approximately 1 dm^2 of surface. For sampling inside the tank, a 2 m long steel stick was used for the compresses (*Figure 3*). Immediately following sampling, the compresses were placed in bottles with 10 mL peptone saline solution.

Contact plates: Doublesided contact plates (SVA, Uppsala, Sweden) have one side with violet red bile agar (VRG) for coliforms and the other side with tryptone glucose yeast extract agar (TGE) for total viable counts. Both sides of the contact plates were pressed against the inside of the manhole hatch, at the top and against the bottom of the tank.

Physiological saline: Physiological saline (1L/sample) was poured into the tank from the manhole hatch and then part of it was collected through the bottom valve. Following collection, the physiological saline sample was divided into two tubes.

Compresses, contact plates and the tubes with physiological saline were promptly transported to the laboratory (< 24 h). At the laboratory the compresses and one of the two tube samples of the physiological saline were analysed quantitatively for coliforms on VRG and incubated at 37°C for 24 h. The second tube with physiological saline sample was centrifuged and the pellet was enriched in 5 mL serum broth (SVA, Uppsala, Sweden) overnight before culture on VRG for coliforms. The contact plates were incubated at 37°C for 24 h and the colonies were counted. The results for all sampling methods were expressed as colony forming units per square centimetre (cfu/cm^2).

Figure 3. The author in front of one of the tanker lorries included in the pilot study. For sampling inside the tank, a 2 m long steel stick was used for the compresses.
(Photo: Nils-Gunnar Ericsson, November 2004)

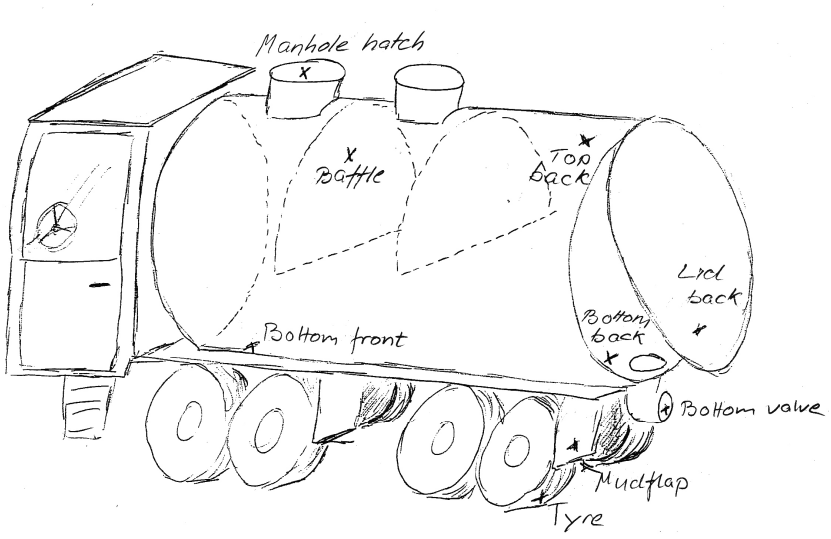

Figure 4. Schematic picture of a tanker lorry. The locations of sampling in the pilot study are marked with X. (Illustration: E. Bagge)

3.3 Screening of spore-forming bacteria (Paper III)

Clostridium spp. and *Bacillus* spp. normally occur in different kinds of biowaste and the biogas process can influence the type and quantity. To trace and compare the type and quantity of spore-forming bacteria from substrates through the biogas process, samples were taken from cattle manure, slaughterhouse waste and BGPs at the different phases of the biogas process.

Manure: Ninety-seven individual faecal samples were taken from cows in 10 dairy farms on one occasion per farm. The samples were collected from the floor behind each animal (healthy cows and heifers).

Slaughterhouse waste: Animal by-product samples intended for biogas production were taken for analysis once a day on ten occasions from two slaughterhouses.

Biogas plants: Two BGPs sent one sample each week on 10 occasions, from tanks before pasteurisation, after pasteurisation and after digestion.

In order to reduce the outgrowth of contamination flora, all samples were heated before culture. The samples were analysed quantitatively for *Clostridium* spp. and *Bacillus* spp. Various types of *Clostridium* spp. and *Bacillus* spp. were counted and subcultured. *Clostridium* spp. were cultured on Tryptose Sulphite Cycloserine agar (TSC), but TSC promotes *C. perfringens* and therefore, the samples were also cultured on Fastidious Anaerobic Agar (FAA) plates. *Bacillus* spp. were cultured on horse blood agar plates. *Clostridium perfringens* and *B. cereus* were identified by conventional biochemical methods. Other species of suspected *Clostridium* spp. or *Bacillus* spp. strains were identified by biochemical methods, but when the biochemical methods gave an ambiguous result, the strain was identified by 16S rDNA sequencing.

3.4 Preparation of clostridial strains (Papers IV and V)

Five clostridia (*C. chauvoei, C. haemolyticum, C. perfringens* type C, *C. septicum* and *C. sordellii*) were cultured in enrichment broth. Some of these clostridia grow slowly and therefore four different enrichment broths were tested for all five clostridia before the pasteurisation and digestion trials were

initiated. The enrichment broths tested were Tryptone Glucose Yeast (TGY) broth, cattle meat broth (SVA, Uppsala, Sweden), serum broth and Fastidious Anaerobic Broth (FAB). The best enrichment broth was found to be TGY. Following incubation, the inoculated broths were left under anaerobic conditions at room temperature (approximately 20°C) for 5 days to promote spore formation (Båverud *et al.*, 2003), except *C. perfringens* type C, which needs 5 days at room temperature and 7 days at room temperature in aerobic conditions. The concentrations were obtained by 10-fold dilution series for each clostridium.

3.5 Pasteurisation of spore-forming bacteria (Papers I, II and IV)

Spore-forming bacteria are known to be heat tolerant and will most likely pass unaffected through the pasteurisation stage at 70°C for 60 min. In order to investigate any decrease in quantity during pasteurisation, *Clostridium* spp. and *Bacillus* spp. were both quantitatively analysed before and after pasteurisation in Paper I. In samples before pasteurisation, the blood agar plates for detection of *Bacillus* spp. were frequently contaminated by *Proteus* spp., and therefore it was impossible to count *Bacillus* spp. colonies. In Paper II the analysis of *Bacillus* spp. was excluded, since the indigenous number of *Bacillus* spp. was unreliable. The indigenous numbers of *Clostridium* spp. were reliable and qualitatively analysed.

In Paper IV, *C. chauvoei, C. haemolyticum, C. perfringens* type C, *C. septicum* and *C. sordellii* were separately inoculated into homogenisation tank substrates and were then heated at 70°C for 60 min to simulate the pasteurisation stage. Before inoculation, the bacteria were prepared to encourage spore formation. The concentrations were established quantitatively before inoculation. In samples before and after the simulated pasteurisation, the inoculated clostridia were detected by PCR and specific primer pairs, after a culture step on FAA plates.

3.6 Digestion of samples with pathogenic clostridia (Paper IV)

Digester tank substrates from four full-scale commercial BGPs were collected and promptly delivered to SVA. The digester substrates were distributed into vials under flushing with N_2/CO_2 (80/20%) gas to obtain the appropriate atmosphere in the vials. Each species of the five pathogenic clostridia (*C. chauvoei, C. haemolyticum, C. perfringens* type C, *C. septicum* and *C. sordellii*) was separately inoculated into the vials. Inoculated vials were incubated at mesophilic (37°C) or thermophilic (55°C) conditions and

regularly interrupted 13 times over 20 days (thermophilic digestion) or 30 days (mesophilic digestion). On each interruption day, three inoculated vials and one uninoculated vial were taken. The inoculated clostridia were detected by PCR and specific primer pairs after a culture step on FAA and TSC plates.

3.7 Detection of *Clostridium chauvoei* in muscle samples and environmental samples (Paper V)

In this study, the suitability of using PCR for detecting *C. chauvoei* in muscle tissue taken at autopsy and in contaminated samples such as faeces, soil, biogas substrate and digested residues, was investigated.

Cooperating local veterinarians sent muscle tissue samples from cattle that had died from blackleg. At the laboratory, these samples were cultured on FAA plates. To determine the presence of *C. chauvoei*, the isolated bacteria were identified by biochemical methods and PCR. Pieces of muscle tissue and meat juice were also used directly for DNA preparation and analysed by PCR. Specimens were taken from the muscle tissues by swabs (Amies' medium with charcoal), which were left at room temperature for 1, 3 and 6 days, simulating a postal service. The swabs were cultured and growth of *C. chauvoei* was confirmed by PCR and a specific primer pair.

Faeces, soil and silage were taken from dairy farms from a region where blackleg is endemic. During the grazing period, faecal samples were taken from the floor behind the cows in the barn, or collected from cowpats on pasture outside. On the same occasion, soil samples were collected from the farm. Samples from the local BGP were taken weekly on 11 occasions at three processing stages; before pasteurisation, after pasteurisation and after digestion. To avoid outgrowth of the normal surrounding flora, the samples were heated before culture. The samples were cultured on FAA plates and, following DNA preparation, the occurrence of *C. chauvoei* was detected by PCR and a specific primer pair.

Before the trials mentioned above, two different kinds of PCR primer pairs were tested. Sasaki *et al.* (2000a) described a primer pair based on the spacer region 16S-23S rDNA and this primer pair was designated 23UPCH and IGSC4. Kojima *et al.* (2001) described another primer pair based on the flagellar gene of *C. chauvoei* and this primer pair was designated CCF516 and CCR516. The primer pair designated 23UPCH and IGSC4 was chosen for the studies.

3.8 Methods for bacterial analysis

3.8.1 Quantitative methods (Papers I-V)

For investigation of *Clostridium* spp. in samples, 1 mL of the sample was mixed with melted TSC, incubated anaerobically and counted. In Paper III, *Clostridium* spp. were cultured on FAA plates in addition to TSC. Suspected colonies of *Clostridium* spp. were subcultured on horse blood agar. Following anaerobic incubation the isolated bacteria were Gram-stained. *Clostridium* spp. were identified as Gram-positive rods. The identification of clostridia was based on:

- Fermentation of glucose, maltose, lactose, sucrose, starch, mannitol and fructose.
- Production of lecithinase, tryptophanase and urease.
- Hydrolysis of aesculin.

Suspected colonies of *C. perfringens* were subcultured on both egg yolk agar and horse blood agar, and incubated under anaerobic conditions.

For detection of *Bacillus* spp., samples were cultured by spreading 0.1 mL of the sample on horse blood agar and incubating the plates. Suspected colonies of *Bacillus* spp. were counted and subcultured onto horse blood agar, catalase tested and Gram stained. *Bacillus* spp. were identified as catalase-positive, Gram-positive rods. *Bacillus cereus* was confirmed by culture on Mossel Cereus Selective agar (MCS) plates. Characterisation of other strains of *Bacillus* spp. was based on:

- The Voges-Proskauer (VP) test.
- Production of lecithinase and tryptophanase.
- Fermentation of glucose, arabinose, mannitol and citrate.
- Reduction of nitrate.

Samples for investigation of *Enterococcus* spp. were cultured by spreading 0.1 mL of the sample on Slanetz-Bartley agar (SlaBa). Deep red colonies suspected to represent *Enterococcus* spp. were counted after incubation.

Samples for investigation of coliform bacteria were analysed by mixing 1 mL of the sample with melted VRG and then incubating at 37°C. Suspected colonies were then counted and further analysed by culture in brilliant green bile lactose broth (BGB), which was incubated at 37°C. For investigation of thermotolerant coliforms and *E. coli,* the samples were mixed with melted VRG and incubated at 45°C. Suspected colonies were

then counted and further analysed by culture in lactose tryptone lauryl sulphate broth (LTLSB), also incubated at 45°C. Kovacs' indole reagent was added to the broth after incubation, for detection of *E. coli*.

For quantitative analysis of bacteria, 10-fold dilution series were made in peptone saline solution, based on Nordic Committee on Food Analysis (NMKL, 91:3:2001). The detection level was 10 cfu/mL for coliform bacteria and *Clostridium* spp. on TSC. These methods were performed by mixing 1 mL of the diluted sample in melted agar. For *Enterococcus* spp., *Bacillus* spp. and *Clostridium* spp. on FAA, the detection level was 100 cfu/mL. These methods were performed by spreading 0.1 mL of the diluted sample over the surface of the matching agar plates and incubating.

3.8.2 Qualitative methods (Papers I-II)

Samples for investigation of *Salmonella* spp. were pre-enriched in buffered peptone water (BPW). Thereafter, an aliquot (approximately 0.1 mL) was inoculated in Rappaport-Vassiliadis soy broth (RVS) followed by incubation. One loopful (approximately 5 μL of bacteria collected with a 10 μL loop) from the selective enrichment broth was streaked onto xylose-lysine-desoxycholate agar (XLD) and brilliant green-phenol red agar (BGA) followed by incubation. Suspected colonies were tested biochemically by indole/β-galactosidase test, culture on triple sugar iron agar, fermentation of mannitol, sucrose and malonate, and production of urease, sorbose phosphate and lysine decarboxylase. Serotyping of *Salmonella* spp. was performed by agglutination according to the Kauffmann-White scheme (WHO 1997).

Samples for investigation of *Listeria monocytogenes* were pre-enriched in Listeria enrichment broth (LEB). After incubation, an aliquot was streaked onto Listeria-selective agar (Oxford agar) and incubated. Suspected colonies were subcultured and confirmed by fermentation of rhamnose, glucose, lactose, maltose, sucrose and xylose. Other biochemical tests such as hydrolysis of aesculine, motility, catalase test and Gram-staining were also performed.

For investigation of *Campylobacter* spp. in samples, two swabs were taken and pre-enriched in Preston broth and incubated under microaerophilic conditions by Campygen. One loopful was then streaked onto Preston agar, which was incubated under microaerophilic conditions. Identification of *Campylobacter* spp. was based on colony morphology, microscopic appearance and phenotypic characteristics such as motility tests by phase-contrast microscopy, production of oxidase, catalase test and the hippurate hydrolysis reaction.

Samples for investigation of *Escherichia coli* O157 were incubated in peptone water for 6 to 8 h as pre-enrichment. The samples were then analysed by immuno-magnetic-absorbent assay with immunomagnetic beads (Dynabeads anti-*E. coli* O157, Dynal, Oslo, Norway) to isolate VTEC O157, followed by incubation on sorbitol-MacConkey agar with cefixime and potassium tellurite. Suspected colonies were further analysed by latex agglutination. The suspected colonies were streaked onto horse blood agar plates and confirmed by PCR (Hoorfar *et al.*, 2000) and API 20e for identification of *Enterobacteriaceae*.

3.9 Detection level of clostridia by PCR in biowaste (Papers IV and V)

In order to determine the detection levels for the PCR methods, spiked samples of cattle manure, soil, silage and substrate from BGPs (before and after pasteurisation and digested residues), were analysed.

Five clostridia (*C. chauvoei, C. haemolyticum, C. perfringens* type C, *C. septicum* and *C. sordellii*) were cultured in TGY and after incubation, 10-fold dilution series were made in peptone saline solution. One millilitre from the different dilution steps was thoroughly mixed in bottles with the substrates from the homogenisation tanks and digester tank. For each substrate, at least four different dilution steps in succession were analysed. The step selection was based on bacteria and substrate. For simulating the pasteurisation stage, bottles with homogenisation tank substrate were heated at 70°C for 60 min. The samples in the bottles before pasteurisation and from the digester were thoroughly agitated and then left to allow the content to settle. One loopful from each bottle was cultured on FAA plates and after incubation the inoculated strains were confirmed by PCR and specific primer pairs. The detection levels were obtained by observing positive and negative PCR results from the different dilutions of each bacterial species. The detection levels were established by repeating the trial twice.

Cattle manure, soil and silage were thoroughly mixed with one mL from the different dilution steps with *C. chauvoei* in bottles and then left to allow the content to settle. One loopful was cultured on FAA plates and after incubation confirmed by PCR and a specific primer pair for *C. chauvoei*. The detection level was obtained by observing positive and negative PCR results from the different dilutions of *C. chauvoei*. The detection level was established by repeating the trial twice.

3.10 PCR and sequencing (Papers III-V)

3.10.1 DNA preparation

Four different DNA preparation methods were compared for detection of clostridia in samples heavily contaminated by the indigenous flora. Five gram of soil, manure or biogas samples were mixed with a known quantity of *C. chauvoei*. The selected DNA preparation methods were phenol-chloroform extraction, boiled lysate and two commercial kits, for which the manufacturers` recommendations were followed. The most reliable method was boiled lysate with prior culture on FAA plates.

In Papers III, IV and V, colony material from agar plates was prepared as boiled lysate before PCR analyses. In Paper V muscle tissues and meat juice were directly prepared as boiled lysate without culture steps. The colony material or muscle tissues were suspended in phosphate-buffered saline and centrifuged. The supernatant was discarded and the pellet was washed again by the same procedure. Thereafter, the bacterial cells were lysed by boiling the suspension before storage at -20°C until further analysis. This lysate was used as template in PCR.

3.10.2 PCR

In Paper III, strains of *Clostridium* spp. and strains of *Bacillus* spp. were used for producing PCR products (amplicons) by amplification with universal primers. On all occasions, *Mycoplasma capricolum* subsp. *capricolum* (Calif. Kid^T), which also belongs to the phylum *Firmicutes*, was used as a positive control simultaneously with the samples.

In Paper IV *C. perfringens* type C was identified by PCR performed according to Engström *et al.* (2003) in order to establish the presence of *alfa, beta, epsilon* or *iota* toxin genes. For detection and identification of *C. chauvoei, C. haemolyticum* and *C. septicum*, specific PCR primer pairs complementary to the spacer region of the 16S-23S rRNA gene were used as described by Sasaki *et al.* (2000a; 2002). The method for *C. chauvoei* was also used in Paper V. For detection and identification of *C. sordellii*, a specific primer pair based on the 16S rRNA gene sequences was used as described by Kikuchi *et al.* (2002). On all occasions where PCR was used, positive and negative controls were always analysed at the same time as the samples. The amplicons were analysed by electrophoresis in agarose gels which were stained with ethidium bromide. The PCR-products were visualized under UV-light (*Figure 5*).

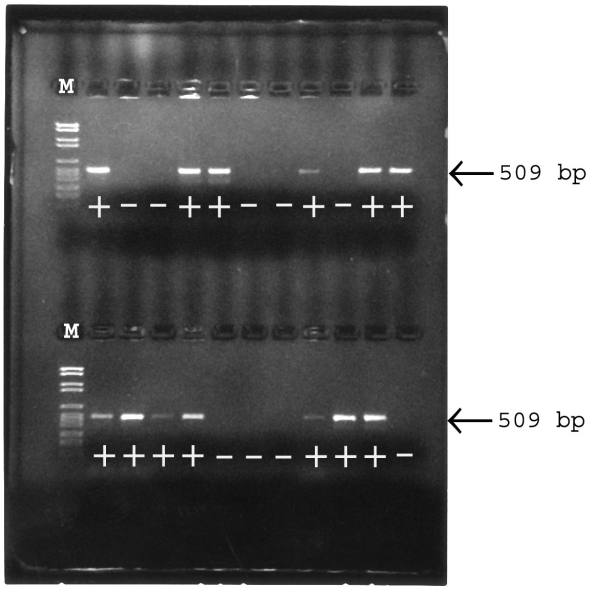

Figure 5. Example of PCR-products visualized under UV-light. This picture shows PCR-products from different methods of *Clostridium chauvoei* in Paper V. The picture shows positive and negative samples from clinical cases of blackleg. They were analysed by culture followed by PCR and direct PCR without preculture. Positive and negative controls were applied in the three last lanes in each row. The size of the PCR product is 509 base pairs (bp) (Sasaki *et al.* 2000a). M = Molecular size marker, (DNA Molecular Weight Marker VI, Roche Diagnostic GmbH, Mannheim, Germany).

3.10.3 16S rRNA sequencing

In Paper III, 50 unknown strains of *Clostridium* spp. and 51 strains of *Bacillus* spp., were further analysed by 16S rRNA gene sequencing. *Clostridium haemolyticum* strain LP 2361/89 in Paper IV and *C. chauvoei* strain AN 2548/02 in Papers IV and V were also analysed by this technique. Almost complete sequences were obtained by cycle sequencing of PCR products amplified from genomic DNA. The nucleotide sequences were determined with the ABI PRISM 3100 Genetic Analyzer and the DNA fragments were both in the forward and reverse direction. This approach resulted in DNA fragments that overlapped and cover the whole length of the original amplicons obtained by PCR. The sequences of the gene fragments were assembled into one contig by using the program ContigExpress, Vector NTI. The raw sequence data are shown as coloured peaks representing the respective type of nucleotide. To compare the sequences, similarity searches were performed using the program BLAST in GenBank (Benson *et al.*, 2007).

For construction of the 16S rRNA-based phylogenetic trees, sequences obtained from this work were aligned manually with prealigned sequences retrieved from Ribosomal Database Project II (RDP-II; Cole *et al.*, 2005). Sequences retrieved from GenBank were also manually aligned with the prealigned sequences from RDP-II. The purpose of the alignment was to organise the sequences into a matrix where the rows contained the sequences and each column contained the nucleotides from a homologous position. The phylogenetic relationship among the isolates was calculated by Neighbour-Joining. The branch order and the horizontal distances in a phylogenetic tree reflect the relation between the isolates and this was also used for identification.

4 Main Results

4.1 Non spore-forming pathogenic bacteria in the biogas process (Papers I and II)

Levels of indicator bacteria and non spore-forming pathogens, such as *Salmonella* spp., were reduced after pasteurisation (in BGPs and under laboratory conditions). Before pasteurisation, pathogens were found in 65% of the samples and the most common pathogens were *Salmonella* spp. (Paper I). Following pasteurisation (70°C for 30 or 60 min) at laboratory scale (Paper II), no indicator bacteria or inoculated pathogenic bacteria were detected. The inoculated *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *E. coli* O157 were found in samples pasteurised for 30 min at 55°C, but they could not be found in samples pasteurised for 60 min at 55°C.

In samples taken directly after anaerobic digestion in the BGPs, indicator bacteria were detected in 28% of the samples. In digested residues in storage wells on farms, the quantity of indicator bacteria increased compared with storages for digested residues at BGPs (Paper I) (*Figure 6*). In digested residues kept in storage wells on farms, salmonella was detected in 17% of the samples. At one BGP (Paper I), *Salmonella enterica* subsp. *enterica* serovar Agona was isolated on two sampling occasions before pasteurisation. It was also isolated on four sampling occasions in storage wells on farm sites (Table *4*). One strain from farm site 1 had the same PFGE pattern as one strain obtained before pasteurisation. The other strain obtained before pasteurisation was identical to the strain from farm site 2.

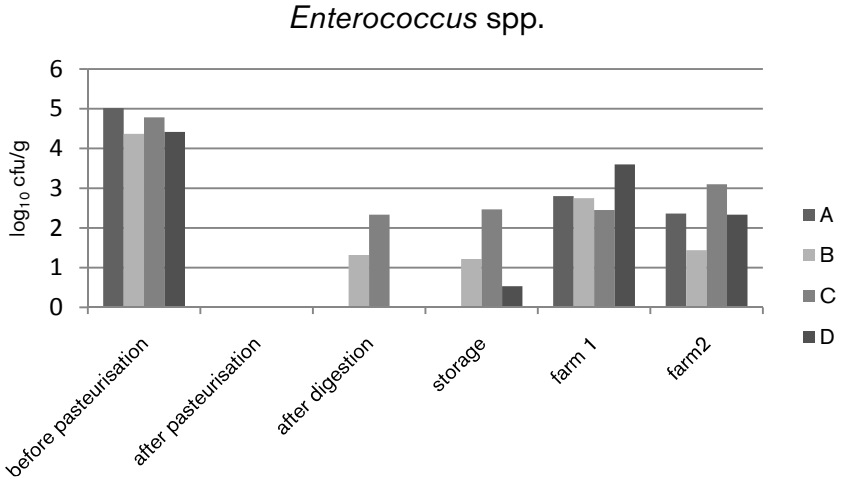

Figure 6. Mean values of *Enterococcus* spp. quantities on six sampling locations from biogas plant A-D

Table 4. *Pathogens isolated from BGP A-D at 3 of the 6 sampling locations. The number of sampling occasions on which the same species were isolated from the same sampling site is shown within parentheses.*

Sampling location / Biogas plant	Before pasteurisation	Storage wells at farm site 1	Storage wells at farm site 2
A	*C. jejuni* (2)		
	S. Schleissenheim[1]		
B	*C. coli*	*S.* Agona (2) [a]	*E. coli* O157
	E. coli O157		*S.* Agona (2) [b]
	S. Agona (2) [a, b]		
C	*C. jejuni*	*C. coli*	
	C. coli (2)		
	E. coli O157		
	S. Agona		
D	*L. monocytogenes*		

[1] S. *enterica* subsp. *enterica* serovar Schleissenheim

[a] One *S.* Agona before pasteurisation and one at farm site 1 had same PFGE pattern.

[b] One *S.* Agona before pasteurisation and two at farm site 2 had same PFGE pattern.

4.2 Hygiene in transportation vehicles (pilot study)

From the samples obtained by compresses from tanker lorry A, which was disinfected using lye, only small number of coliforms grew. From all lorries, growths of coliform bacteria were detected in samples from the lid area in the back, and at the bottom at the front of the tank. Only small numbers grew in lorry A and B. The numbers of bacteria on tyres and mudflaps were high on all three lorries. Compress samples from the two tanker lorries B and C had high quantities of bacteria inside the tank, around hatches and lids (Table *5*). In tank C, slaughterhouse waste was still found after cleansing and disinfection.

The bacterial growth of coliforms and total viable counts of bacteria on the contact plates were high (>10 cfu/cm^2) and could not be used for evaluation of the sampling method by contact plates (Table *5*). The results from sampling by poured physiological saline reflected the results of the compresses (Table *5*).

4.3 Screening of spore-forming bacteria in biowaste (Paper III)

All species of *Bacillus* spp. and *Clostridium* spp. found in cattle manure, slaughterhouse waste and BGPs samples are shown in Table *6*. From the strains identified by 16S rRNA sequencing phylogenetic trees were created (Paper III). Potentially new members of both genus *Clostridium* and genus *Bacillus* were found in the screening study, especially strains SH-C1, SH-C52, BG-C36, BG-C122 and BG-C151, which have high genetic similarity to each other and to an uncultured bacterium (Leser *et al.*, 2002) (*Figure* 7).

The most common *Bacillus* spp. found in biowaste, after pasteurisation and after digestion were *B. cereus, B. subtilis* and *B. pumilus*. The most common *Clostridium* sp. was *C. perfringens*. Pathogenic clostridia such as *C. sordellii* were detected in manure, slaughterhouse waste, before and after pasteurisation, but not after digestion. *Clostridium septicum* was found in slaughterhouse waste and *C. botulinum* was found before and after pasteurisation, but not after digestion. In one BGP, *C. sporogenes/C. botulinum* was found after pasteurisation. These two clostridia are difficult to distinguish by phylogenetic analysis based on 16S rRNA sequencing due to high sequence similarity and hence they are presented as alternatives in the results.

Table 5. *Results from hygiene study of transport vehicles.*

Compresses $\log_{10}$ cfu/g	tanker lorry A	tanker lorry B	tanker lorry C
Manhole hatch	<1	1.60	4.17
Bottom valve	<1	1.30	2.70
Lid back	2.53	2.30	4.84
Pipe on the lid back	nd	nd	4.93
Baffle[1]	<1	1.08	5.18
Top back in the tank [1]	<1	<1	4.96
Bottom front in the tank[1]	1.30	1.08	<1
Bottom, opening back	<1	3.08	<1
Trailer, bottom valve	nd	1.90	Nd
Trailer, bottom, opening back	nd	1.84	Nd
Trailer, lid back	nd	2.15	Nd
Tyre	4.64	2.63	<1
Mudflap	5.63	4.63	3.64

Rinsing sample, $\log_{10}$ cfu/g, mean value of two	tanker lorry A	tanker lorry B	tanker lorry C
Direct cultivation	<1	2.82	<1
After centrifugation and enrichment [2]	4	> 4	<1

plastic dishes cfu/cm^2	tanker lorry A	tanker lorry B	tanker lorry C
Manhole hatch	> 10	> 10	> 10
Bottom, opening back	> 10	> 10	> 10

nd = not determined.

[1] These samples were taken with a long metal stick equipped with a claw clutch. The compresses were attached by the claw clutch. The claw clutch was cleaned in 70% ethanol and dried before and between every sampling occasion.

[2] One litre NaCl (aq) were added to the tanks of lorries A and B. Lorry C was filled with remains of cleansing water mixed with disinfectant when sampling (cf. *Figure 12*). This liquid was collected into a bottle and then distributed in two tubes.

Table 6. *Bacillus spp., Clostridium spp., Lysinobacillus spp. and Paenibacillus spp. found in the different sampling material.*

	Farms	Slaughterhouses	Before pasteurisation	After pasteurisation	After digestion
***Bacillus* spp.** [1]	*B. cereus* *B. pumilus* *B. subtilis* *B. weihensteph./ mycoides* *Bacillus* spp. *Paenibacillus amylolyticus* *Paenibacillus polymyxa*	*B. cereus* *B. clausii* *B. licheniformis* *B. lentus* *B. oleronius* *B. pumilus* *B. subtilis* *B. thuringiensis* *Bacillus* spp. *Paenibacillus amylolyticus*	*B. cereus* *B. clausii* *B. licheniformis* *B. pumilus* *B. subtilis* *Bacillus* spp. *Lysinobacillus sphaericus* *Lysinobacillus* sp. *Paenibacillus polymyxa*	*B. cereus* *B. clausii* *B. licheniformis* *B. pumilus* *B. subtilis* *Bacillus* spp. *Lysinobacillus sphaericus* *Lysinobacillus* sp. *Paenibacillus polymyxa*	*B. cereus* *B. licheniformis* *B. megaterium* *B. pumilus* *B. subtilis* *Bacillus* spp. *Lysinobacillus sphaericus* *Lysinobacillus* sp. *Paenibacillus polymyxa*
***Clostridium* spp.**	*C. bifermentans* *C. butyricum* *C. neonatale* *C. perfringens* *C. ramosum* *C. sordelli* *Clostridium* spp.	*C. bifermentans* *C. butyricum* *C. cellobioparum* *C. glycolicum* *C. limosum* *C. perfringens* *C. septicum* *C. sordellii* *Clostridium* spp.	*C. aurantibutyricum* *C. barati* *C. bifermentans* *C. botulinum* *C. butyricum* *C. celatum* *C. durum* *C. formicoaceticum* *C. glycolicum* *C. limosum* *C. novy* *C. paraputrificum* *C. perenne* *C. perfringens* *C. sardiniensis* *C. sordellii* *C. subterminale* *C. tertium* *C. tyrobutyricum* *Clostridium* spp.	*C. acetobutylicum* *C. aurantibutyricum* *C. bifermentans* *C. botulinum* *C. butyricum* *C. durum* *C. glycolicum* *C. irregular* *C. limosum* *C. oceanicum* *C. perfringens* *C. sordellii* *C. sporogenes/C. botulinum* *C. subterminale* *Clostridium* spp.	*C. acetobutylicum* *C. aurantibutyricum* *C. barati* *C. bifermentans* *C. butyricum* *C. durum* *C. glycolicum* *C. limosum* *C. sardiniensis* *C. perfringens* *Clostridium* spp.

[1] Including *Lysinobacillus* spp. and *Paenibacillus* spp

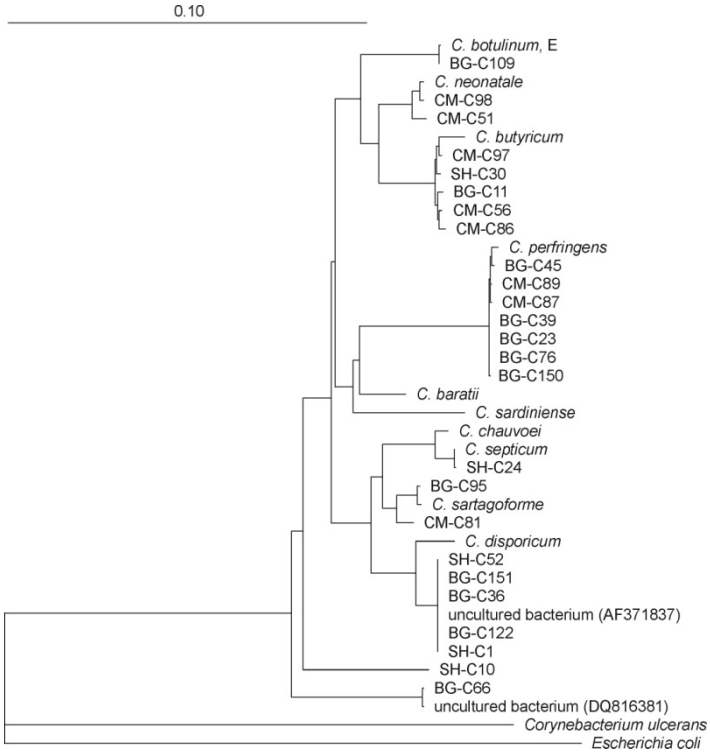

Figure 7. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between strains of *Clostridium* spp. related to *Clostridium perfringens*. isolated from cattle manure (CM), slaughterhouse waste (SH) and biogas plant (BG). The length of the scalebar is equivalent to 10 nucleotide substitutions per 100 positions.

The number of various species of *Bacillus* spp. in samples from manure, slaughterhouse waste and samples from different stages in the biogas process seemed to be nearly the same in all samples. The total numbers of *Bacillus* spp. also seemed to be similar in all samples.

The number of various species of clostridia was reduced in samples after digestion compared with the diversity of species in other samples. The total numbers of *Clostridium* spp. were also reduced through the biogas process. *Bacillus* spp. seemed to pass through the biogas process from biowaste to digested residues relatively unaffected. The numbers of *Bacillus* spp. and *Clostridium* spp. are shown in (Table *6*) and the quantities of spore-forming bacteria in *Figure 8*.

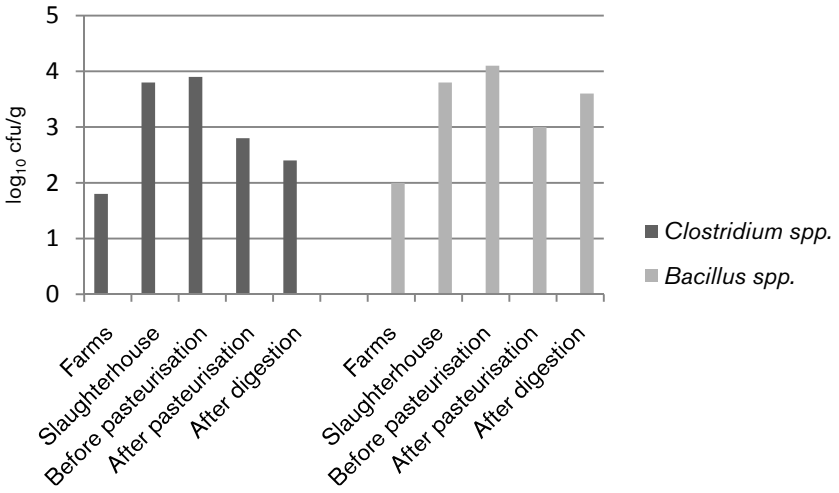

Figure 8. Mean values of the quantities of bacteria representing *Bacillus* spp., *Clostridium* spp., *Lysinobacillus* spp. and *Paenibacillus* spp. in the different sampling material. The two latter genera were included in genus *Bacillus*.

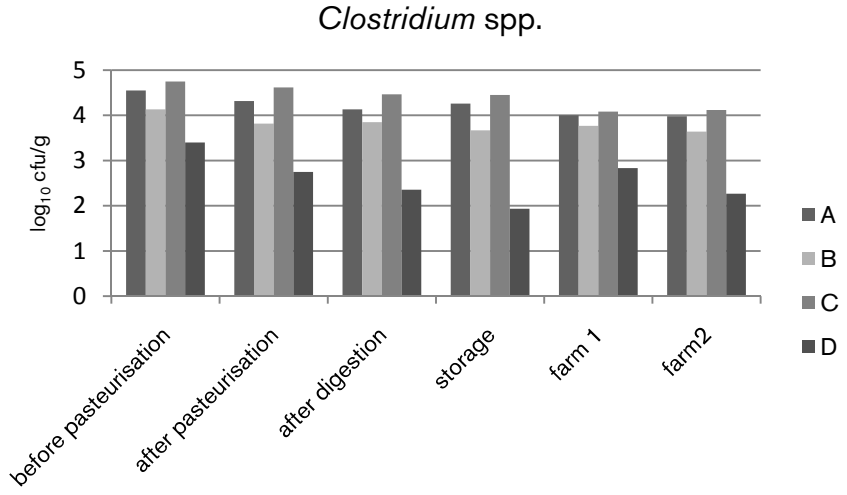

Figure 9. Mean values of *Clostridium* spp. quantities on six sampling locations from biogas plant A-D.

4.4 Pasteurisation of spore-forming bacteria (Papers I, II and IV)

Spore-forming bacteria were not reduced by pasteurisation at 70°C for 60 min. *Clostridium* spp. and *Bacillus* spp. were determined at viable bacterial counts in all samples, the mean value for all samples of *Clostridium* spp. being 4.4 $\log_{10}$ cfu/g and for *Bacillus* spp., 4.8 $\log_{10}$ cfu/g (Paper I) (*Figure 9*). During pasteurisation in laboratory scale, *C. perfringens* was not affected by heat treatment at 70°C for 60 min. The mean quantity of *C. perfringens* in the original substrate was 4.8 $\log_{10}$ cfu/g and the mean quantity after pasteurisation was 4.4 $\log_{10}$ cfu/g (Paper II).

In Paper IV, four of the inoculated species *C. chauvoei, C. perfringens* type C, *C. septicum*, and *C. sordellii* were detected both before and after pasteurisation. *Clostridium haemolyticum* was only detected in two cases, before and after pasteurisation. Before inoculation the numbers of bacteria were counted on FAA plates and in a Bürker chamber. *Clostridium* spp. were counted in a Bürker chamber to 6-8 $\log_{10}$ /mL in TGY broth. Spores were observed but were difficult to count. In uninoculated control samples, *C. septicum* was detected from two BGPs and *C. sordellii* was detected in samples from three BGPs (Table 7).

Table 7. *Detection of clostridia in the uninoculated samples analysed in the pasteurisation study.*

Biogas Plants	Before pasteurisation	After pasteurisation
A	*C. septicum, C. sordellii*	*C. septicum, C. sordellii*
B	-	*C. sordellii*
C	*C. sordellii*	*C. septicum*
D	-	-

4.5 Digestion of samples with pathogenic clostridia (Paper IV)

Strains of *C. septicum* and *C. sordellii,* inoculated in vials with digester material, could be detected in most samples and at the end of the digestion. *Clostridium chauvoei* inoculated in vials was detected throughout the whole digestion process in substrate from one BGP. In substrate from the other three BGPs, the presence of *C. chauvoei* varied and could not be observed throughout the whole sampling series. Inoculated *C. perfringens* type C and *C. haemolyticum* were detected only during the first few days of digestion in samples from all four BGPs. In uninoculated samples, both *Clostridium septicum* and *C. sordellii* could be detected in some samples. The quantities of indigenous flora and inoculated pathogens at the start of the trial and after the retention time are shown in *Figure 10*.

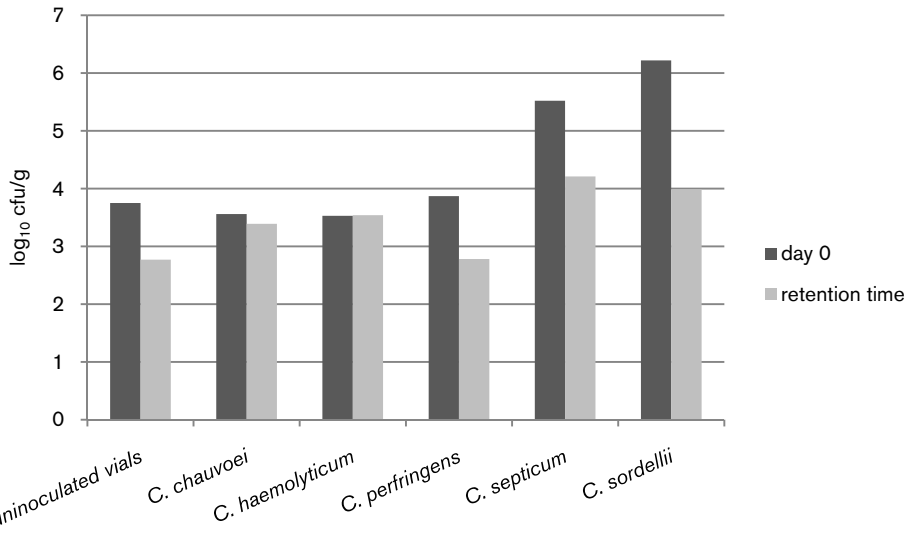

Figure 10. Mean values of the total quantities of indigenous flora and inoculated flora from all four biogas plants. The diagram shows uninoculated, which only contains indigenous flora, and inoculated samples at start (day 0) and after retention time (12 days for thermophilic digestion and 25 days for mesophilic digestion).

4.6 *Clostridium chauvoei* in muscle samples (Paper V)

The primer pair based on the region 16S-23S rDNA and designated 23UPCH and IGSC4 gave better bands when visualized under UV-light (*Figure 5*), and was more reliable than the primer pair based on the flagellar gene of *C. chauvoei*, designated CCF516 and CCR516.

In clinical cases of blackleg, *C. chauvoei* was detected in 32% of the tested material by culture followed by biochemical identification. When the same samples were analysed by culture followed by PCR, 56% were above the detection limit for *C. chauvoei*. DNA preparation without preculture, gave 9-26% above the detection limit. When the samples were taken by pressing swabs into the muscle tissue followed by culture on FAA plates and then analysed by PCR, 36% to 47% were above the detection limit. Storing the swabs for 1, 3 or 6 days, at room temperature before analysis, seemed to have little influence on the results.

Three samples from the BGPs taken before pasteurisation and one faecal sample were above the detection limit by PCR for *C. chauvoei*, but samples taken after pasteurisation and after digestion, and soil and silage samples, were all below the detection limit.

4.7 Detection level of clostridia by PCR in biowaste (Papers IV and V)

The detection level of the method used was 100-200 cfu/g for *C. chauvoei*, *C. haemolyticum* and *C. perfringens* type C. For *C. septicum* and *C. sordellii* the detection level was 1 cfu/g. Substrate before pasteurisation, after pasteurisation and digester substrate showed similar results.

4.8 PCR and sequencing (Papers III-V)

4.8.1 DNA preparation

From the DNA preparation methods evaluated, it was concluded that the most reliable method was boiled lysate with previous culture on FAA plates as an enrichment step. For phenol-chloroform and the two commercial kits, the detection level was approximately ten times higher than for boiled lysate (data not shown).

4.8.2 PCR

Positive and negative controls were always used when samples were analysed by PCR, and the results were only included in the study if they tested positive and negative, respectively. All reference strains used in this work tested positive by PCR with their specific primer pairs, and negative with the other primers.

5 General Discussion

5.1 Spore-forming bacteria

Biowaste contains spore-forming bacteria, such as *Clostridium* spp. and *Bacillus* spp. They probably occur as spores that are heat tolerant. The results of this work show that *Bacillus* spp., from manure and slaughterhouse waste, generally pass unaffected during pasteurisation and digestion at BGPs. The impact of digestion on *Clostridium* spp. differs according to species. Furthermore, *Bacillus* spp. and *Clostridium* spp. are affected by heat and pressure (Licciardello and Nickerson, 1963; Peleg *et al.*, 2005, Margosch *et al.*, 2006) and the effects of carbohydrates supplies (Volkova *et al.*, 1988).

Various species of clostridia sporulate under different conditions. Most cultures of *Clostridium* spp. contain vegetative cells, bacteria in varying degrees of sporulation and spores (Yang *et al.*, 2009). The exact mix is hard to discern. In Paper IV, the ambiguous results of *C. haemolyticum* and *C. perfringens* type C during digestion can be attributed to lack of spores. The method used afforded no strict control over sporulation. This study was performed a couple of years ago; today more knowledge about clostridial sporulation is available (Yang *et al.*, 2009). If that study were to be repeated, another spore preparation method would be used.

Clostridium perfringens was commonly detected in manure, slaughterhouse waste, both before and after pasteurisation, and after digestion (Papers I-III), which confirms previous studies (Larsen *et al.*, 1994; Chauret *et al.*, 1999; Aitken *et al.*, 2005). The results reported in Paper IV indicate that *C. perfringens* type C apparently does not remain in the digester regardless of whether it was the result of mesophilic or thermophilic digestion (Paper IV). This is in contrast to one earlier study (Olsen and Larsen, 1987), in which *C. perfringens* type C was not reduced in small-scale digesters at 35°C or 53°C. The experiment in Paper IV cannot be repeated using the same

digester substrate and it was unclear if the bacteria were present as spores or as vegetative cells.

Bearing in mind that *C. perfringens* type C can cause serious diseases in domestic animals such as hemorrhagic enterotoxinaemia in pigs, further studies are necessary before recommendations can be made regarding the spreading of digestive residues on fields.

The normal gut flora from most animal species contain spore-forming bacteria. Most of them are harmless for animals and humans (Timoney *et al.*, 1988; Gyles and Thoen, 1993). In faecal samples from healthy cows, *C. chauvoei* and *C. sordellii* were detected (Papers III and V). Spores probably are produced and pass unaffected through the digestive systems of animals and subsequently into manure, which is sent to BGPs. Slaughterhouse waste materials, including digestive tract content, also contain pathogenic spore-forming bacteria. *Clostridium sordellii* found in slaughterhouse waste, remains after pasteurisation, but not after digestion (Paper III). However, in Paper IV it was found that *C. septicum* and *C. sordellii* remained in the inoculated samples following pasteurisation and thrived in the digester. A considerable quantity of bacteria was inoculated into the digester vials; probably the quantity of these bacteria is far less in uninoculated samples (Paper IV). In uninoculated samples, *C. septicum* and *C. sordellii* were detected both before and after pasteurisation and following digestion (Paper IV). The most probable sources of these bacteria were manure and animal by-products. The consequences for animal health of spreading *C. septicum* and *C. sordellii* may be negligible, as these bacteria are commonly present in manure from healthy animals (Gyles and Thoen, 1993; Munang'andu *et al.*, 1996; Songer, 2006b) and biowaste.

In substrate from one BGP, *C. botulinum* was found both before and after pasteurisation, but not after anaerobic digestion (Paper III). No growth of *C. botulinum* was detected after digestion. The environment in the digester might inhibit *C. botulinum*, but more studies need to be undertaken. Due to the production of extremely potent toxins, *C. botulinum* is one of the most dreaded bacteria. To distinguish different toxin types of *C. botulinum,* toxin analysis has to be performed (Timoney *et al.*, 1988; Quinn *et al.*, 1994c), but in this work *C. botulinum* was only identified by sequencing.

Growth of *C. chauvoei* was detected before and after pasteurisation. Following anaerobic digestion, no *C. chauvoei* could be detected, except when hydrochloric acid (HCl) was added to the digester (Papers IV). Addition of HCl to digesters increases the gas yield. Consequently one could suspect that HCl affects the microbial flora, which could favour *C. chauvoei* in the digester (Paper IV). However, without HCl, the condition in

the digester seemed to inhibit *C. chauvoei* and then the risk of spreading the bacteria via BGP residues was minimized.

In one study, it was shown that a small dose of burnt lime (CaO) reduced the quantity of *Clostridium* spp. spores during storage, when the pH value exeeded 12 (Bujoczek *et al.*, 2002). A high concentration of free ammonia might have been responsible for the decrease of clostridia. However, high ammonia levels reduce the methane yield as the conditions for methanogens become counterproductive.

The number of different species of clostridia decreases after digestion, compared with the number before and after pasteurisation (Table *6*, *Figure 11*). In contrast, the number of different *Bacillus* spp. species seemed to be nearly constant in manure samples, slaughterhouse samples and all different kinds of BGP samples (Table *6*). The sporulation of *Bacillus* spp. seemed to be more independent of environmental factors, *e.g.* available carbohydrates (Volkova *et al.*, 1988) or acidic conditions (Cotter and Hill, 2003), than for *Clostridium* spp., which may explain these results. When comparing different BGPs, a significant difference between the quantities of clostridia was observed in two studies (Paper I and III, *Figure 9*). This was noted during all the processing stages at the BGPs. It is unclear how, but the composition of the substrate may have influenced the results. *Bacillus* spp. pass through the biogas process relatively unaffected (Paper III, *Figure 8*). Fortunately, most *Bacillus* spp. are fairly harmless, with some exception such as *B. anthracis, B. licheniformis* and *B. cereus*.

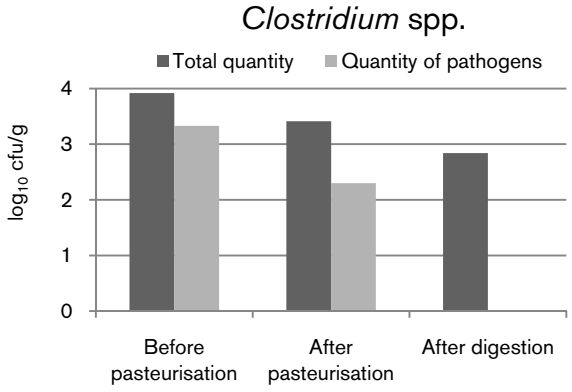

Figure 11. Mean values from two biogas plants of the quantities of bacteria representing *Clostridium* spp. in the different stages in the biogas process.

Paenibacillus spp., which belongs to the same order, *Bacillales*, as *Bacillus* spp., were frequently detected in biowaste and BGPs samples. *Paenibacillus*

polymyxa and *Paenibacillus amylolyticus* are quite harmless, whereas a close relative, *Paenibacillus larvae* caused American foulbrood in honeybees (Genersch, 2007). Since *P. polymyxa* and *P. amylolyticus* seemed to pass unaffected through the biogas process, *P. larvae* probably persists even after pasteurisation and digestion, thus constituting a risk of spreading *P. larvae* via digested residues.

5.2 Non spore-forming bacteria and other micro-organisms

A pasteurisation step at 70°C for 60 min before anaerobic digestion significantly reduced non spore-forming bacteria from incoming biowaste (Clements, 1983; Keller, 1983; Bendixen, 1999; Paper II). Hence, the subsequent handling of digested residues must be hygienically safe to avoid recontamination of pathogenic bacteria (Paper I).

The quantity of *Ascaris suum* eggs is sufficiently reduced already at 55°C (Bendixen, 1994; Plym-Forsehll, 1995; Aitken *et al.*, 2005; Paper II), whereas heat-resistant viruses (Haas *et al.*, 1995; Kim *et al.*, 2000) and prions (Huang *et al.*, 2007) are not reduced, and can persist after pasteurisation.

In previous studies, thermophilic digestion at 55°C reduced *Salmonella* spp. (Olsen and Larsen, 1987; Bendixen, 1993; Zábranská *et al.*, 2003; Iranpour *et al.*, 2006). In other studies it has been shown that *Enterococcus* spp. are particularly resistant to heat and can persist at 55°C. Thus, levels are not always sufficiently reduced (Larsen *et al.*, 1994; Kearns *et al.*, 1995; Paper II). However, the experiment described in Paper II showed that heat treatment at 55°C did not fulfil the criteria in EC regulation no.1774/2002, to reduce pathogens and ensure inactivation of salmonella, VTEC O157 and certain viruses. These results agree with previous studies on sewage sludge, regarding salmonella (Larsen *et al.*, 1994; Aitken *et al.*, 2005) and viruses (Bendixen, 1996; Bendixen, 1999).

In an investigation of anaerobic thermophilic digestion at 50°C, *Salmonella* spp., *Campylobacter* spp., *Listeria* spp. and *E. coli* were reduced below the detection limit within 24 h (Wagner *et al.*, 2008). However, the results in Paper II indicate that 55°C is not high enough for reduction of salmonella.

In terms of pathogen inactivation, mesophilic digestion at 37°C cannot be recommended as the only hygienic treatment for biowaste (Sahlström *et al.*, 2004). *Salmonella* spp. may be reduced, but not sufficiently, for digested residues to be regarded as hygienically safe (Kearney *et al.*, 1993b; Larsen *et al.*, 1994; Horan *et al.*, 2004; Smith *et al.*, 2005). *Salmonella* spp. can be detected continuously weeks and even months after mesophilic digestion

(Kearney *et al.*, 1993b; Bujoczek *et al.*, 2002; Gale, 2005). A mesophilic temperature is to close to the human body temperature at which most pathogens thrive.

Other than the temperature, acidic conditions (Plachá *et al.*, 2001; Salsali *et al.*, 2006) and alkaline conditions obtained *e.g.* by adding ammonia (Bujoczek *et al.*, 2002; Ottosson *et al.*, 2008), are inhibitory for *Salmonella* spp. in mesophilic digestion. Various serotypes of salmonella have differing tolerance of heat, acid, or hydrogen peroxide. An example is *Salmonella enterica* subsp. *enterica* serovar Enteridids phage type 4, which is more tolerant to these parameters than other serotypes of salmonella (Humphrey *et al.*, 1995). Safety margins should be considered, and pasteurisation at 70°C for 60 min is a minimum requirement for a hygienically acceptable product.

In earlier studies, regrowth of *Salmonella* spp. was found after storage of sludge (Gibbs *et al.*, 1995; Gibbs *et al.*, 1997; Ward *et al.*, 1999; Sahlström *et al.*, 2004). In winter, the reduction of salmonella in sludge is slower than in summer (Jepsen *et al.*, 1997; Plachá *et al.*, 2001). After land application of digested residues *Salmonella* spp., VTEC and *Campylobacter* spp. can be detected in soil for at least one month (Gerba and Smith, 2005; Nicholson *et al.*, 2005). In composts, *Salmonella* spp. can persist for months and *E. coli* and *Listeria* spp. for weeks (Lemunier *et al.*, 2005,). As these pathogens persisted in soil, recontamination of soil by digested residues spread on arable land should be avoided.

5.3 Pasteurisation

Most clostridia and *Bacillus* spp. probably occur as spores in biowaste, and pasteurisation at 70°C has a minimal or no effect on the persistence of spores. If they occur as vegetative cells, their persistence depends of their capacity to sporulate, which differs between *Bacillus* spp. and *Clostridium* spp. (Volkova *et al.*, 1988; Margosch *et al.*, 2006). Some variations between different species of clostridia have been reported, *e.g. C. perfringens* does not sporulate readily (del Mar Gamboa *et al.*, 2005).

For non spore-forming bacteria, pasteurisation of biowaste at 70°C for 60 min is an effective way to reduce most pathogens (Bendixen, 1999; Böhm *et al.*, 1999; Papers I and II). Important factors for inactivation of pathogens are temperature and treatment time, parameters which are easy to control (Bendixen, 1996), especially when batch-wise pasteurisation is used. Pasteurisation can be implemented in other technical designs, but the batch-wise method is preferred, as treatment time and temperature are both easily regulated and the results can be verified. When the first study was

performed (Paper I), one BGP used semi-continuous pasteurisation, which was built as a pipeline through which the biowaste was pumped for 15 min and then retained for 45 min. This semi-continuous system appeared to work according to specifications, but the exact duration of pasteurisation was difficult to regulate. One weakness with this system is if the pipelines became coated on the inside; the diameter will decrease. Consequently the biowaste will pass through the pipe faster than calculated, which will then reduce the pasteurisation effect. This BGP has now switched to batch-wise pasteurisation

Pasteurisation before anaerobic digestion (pre-pasteurisation) improves the hygienic quality and the methane yield, and reduces the retention time (Clements, 1983; Keller, 1983; Skiadas *et al.*, 2005). Pasteurisation after anaerobic digestion (post-pasteurisation) at 70°C for 30 min is more cost effective due to the reduced volume to process, thereby reducing energy consumption. The drawback is that the hygienic quality deteriorates quickly after the pasteurisation step (Keller, 1983). After digestion, the harmless digestion flora probably protects residues from growth of more pathogenic flora and post-pasteurisation most likely kills this flora.

5.4 Anaerobic digestion

In thermophilic anaerobic digestion, the degradation of substrate proceeds faster and the retention time is shorter than with mesophilic digestion (Zábranská *et al.*, 2000; van Lier *et al.*, 2001). Moreover, the reduction of non spore-forming bacteria is more efficient with thermophilic digestion than during mesophilic anaerobic digestion (Olsen and Larsen, 1987; Larsen *et al.*, 1994; Larsen, 1995; Sahlström *et al.*, 2004).

Generally, a greater diversity of micro-organisms is present in mesophilic than in thermophilic digestion. Consequently, mesophilic digestion is more efficient in destroying organic pollutants, with a few exceptions (McCarty, 2001). The bacteria involved in mesophilic digestion are also more tolerant to changes in environmental conditions, such as changes in pH, C/N ratio and composition of substrates. Mesophilic systems are therefore considered to be more stable than thermophilic systems. Changes in thermophilic digesters occur faster and are therefore less stable (Zinder, 1986), though some studies have shown the two systems to be equally stable and reliable (Zábranská *et al.*, 2000; van Lier *et al.*, 2001).

Release of volatile fatty acids reduces the pH, giving a bactericidal effect. Due to the volatile fatty acids and following acidification, a decrease in the salmonella count can be achieved under mesophilic digestion (Salsali *et al.*,

2006). Mesophilic digestion at high pH, by treatings of high ammonia levels, also inhibits the growth of bacterial pathogens and has a certain sanitizing effect (Bujoczek *et al.*, 2002; Zábranská *et al.*, 2003; Ottosson *et al.*, 2008). However, an increased ammonia concentration may inhibit the methanogenic bacteria in the biogas process and also increase ammonia emission.

Differences could also be shown between semi-continuous and batch-wise mesophilic digestion, though the decrease in *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. was slower in semi-continuous digestion (Kearney *et al.*, 1993a).

Following continuous thermophilic anaerobic digestion, *Salmonella* spp. have been detected, and hence digested residues cannot be regarded as hygienically safe (Aitken *et al.*, 2005; Paper II). Thermophilic anaerobic digestion with a controlled, well defined retention period between in- and outflow, can be regarded as more hygienically safe than continuous systems. However, since *Salmonella* spp. may persist after thermophilic digestion at 55°C, a pre-pasteurisation step at 70°C ensures the hygienic quality (Clements, 1983; Keller, 1983; Bendixen, 1999).

Digester substrate is a living microbial culture, like leaven. Therefore, when performing laboratory studies in Paper IV, it was important to start the experiment as quickly as possible, to ensure the survival of the methane-producing bacterial flora. The digested material was immediately delivered from the BGPs, flushed with N_2/CO_2 to obtain the right atmosphere, and then inoculated. Hence, the experiment could not be repeated with the same substrate. Both FAA plates and TSC plates were used in parallel to ensure optimal growth of the clostridia (Paper IV).

5.5 Methods

As far as was feasible, standard methods were used to analyse pathogens and indicator bacteria in these studies, such as NMKL or routine analyses at SVA. In Papers III, IV and V, a PCR method for detection of spore-forming bacteria was used on samples which were often contaminated by swarming flora.

Before PCR was introduced as a confirmatory method, four different DNA preparation methods were compared. Soil, faeces and biogas samples were contaminated by indigenous flora and loss of bacterial DNA under preparation is also expected. The DNA loss was probably significant for phenol-chloroform and the two commercial kits. The detection level was about ten times less sensitive than for DNA prepared as boiled lysate (data

not shown). In addition to the loss of DNA phenol-chloroform is unhealthy to handle. For boiled lysate, the detection level was 10^2 cfu/g despite the contamination flora. The detection level might seem to be poor. Sasaki *et al.* (2001) reported a detection level of 10^1 cfu/g for *C. chauvoei* in organs from ruminants that died from blackleg. However, organ samples are generally less contaminated than manure or biogas substrate samples. One disadvantage with boiled lysate was that DNA concentrations could not be measured in the DNA template, but the advantages outweigh the drawbacks.

The DNA in both viable and non-viable bacteria is amplified by PCR. If only viable cells are to be detected, an enrichment step can be used (Burtscher and Wuertz, 2003; Uzal *et al.*, 2003a). Culture before DNA preparation and PCR will dilute DNA of non-viable bacteria. The PCR reaction can be hampered by numerous substances, including irrelevant DNA, humic acids, VFA, fats and proteins (Rossen *et al.*, 1992). However, the cultivation step before PCR also reduces the influence of inhibitory substances.

Phylogenetic trees were constructed from 16S rRNA sequences of members of the genus *Clostridium* obtained from cattle manure, slaughterhouse waste and substrates from BGPs (Paper III). In earlier studies, the genus *Clostridium* was shown to be a phylogenetically heterogeneous group (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999), which is confirmed by our results. The phylogenetic tree (Paper III) shows that *C. botulinum* is also a heterogeneous species (Stackebrandt *et al.*, 1997) and different toxin types of *C. botulinum* are present in different clusters. *Clostridium botulinum* can actually be regarded phylogenetically as at least four different species. *Clostridium botulinum* (BG-C109) was found both before and after pasteurisation in substrate from one BGP. *Clostridium sporogenes/C. botulinum* (BG-C8) was found after pasteurisation (Paper III). The toxin types cannot be determined by 16S rRNA sequencing due to the fact that phylogenetically identical or very similar *C. botulinum* strains may also carry different toxin genes. To distinguish between these, toxin analyses can be performed, but only if toxins are expressed during culture (Timoney *et al.*, 1988; Quinn *et al.*, 1994c). New members of genus *Clostridium* were found in the study, as shown in Paper III.

Phylogenetic trees were also calculated for the *Bacillus* spp. found in cattle manure, slaughterhouse waste and substrates from BGPs. It is well known that it can be difficult to distinguish between *B. anthracis*, *B. cereus* and *B. thuringiensis* when using 16S rRNA sequencing, due to the close similarity in this gene (Sacchi *et al.*, 2002) and between *B. pumilus* and

Bacillus safensis, but *B. safensis* has only been isolated from special environments (Satomi *et al.*, 2006). Some of the sequenced *Bacillus* spp. strains in Paper III could not be identified exactly to species level and some probably represent new members of the genus *Bacillus*. Sequencing of *Bacillus* spp. caused problems, probably due to sequence length polymorphisms.

5.6 Comparison of culture and PCR of *Clostridium chauvoei*

To diagnose clinical cases of suspected blackleg, muscle samples were taken at autopsies of cattle. In most cases the quantity of *C. chauvoei* is high in muscle tissue, and can easily be detected by culture and confirmed by biochemical methods. But verifying by biochemical tests requires growth in pure culture, which might need more time for subculture. Culture of *C. chauvoei* is expensive, time consuming and the samples are frequently contaminated with other bacteria (Sasaki *et al.*, 2000b; Uzal *et al.*, 2003a), especially in samples other than muscle tissue from cattle that died from blackleg. Culture and identification by PCR in muscle tissue samples are independent of overgrowth of contaminating flora (Paper V). These results are consistent with a study of an outbreak of blackleg, where culture and identification by PCR gave better results than biochemical analysis (Kuhnert *et al.*, 1997). Likewise, Uzal *et al.* (2003a) demonstrated that DNA preparation from culture before PCR gave better results than DNA preparation applied directly to biomass. Moreover, the PCR method is much faster than identification by biochemical tests. The biochemical detection method takes at least 4 days, and the PCR 2 days. Some clinical cases of blackleg may be caused by mixed infection with *C. chauvoei* and *C. septicum* (Sternberg *et al.*, 1999). In the study by Kuhnert *et al.* (1997) the culture became overgrown by *C. septicum* but gave better results for *C. chauvoei* in the PCR.

Without pure culture and collection of strains, no strains can be retained for subsequent studies in the future, which is a disadvantage of using PCR detection as the sole method. Therefore, PCR should be used as a complementare identification method for contaminated muscle samples.

DNA preparation was performed directly on muscle tissue samples from cattle that died from blackleg, before analysis by PCR (Paper V). Of these, only 12% were above the detection limit, compared with samples cultured before DNA preparation, where 56% were above the detection limit. In a study by Sasaki *et al.* (2001) organs, such as muscle, liver and spleen, from cattle experimentally infected with *C. chauvoei* were subjected to DNA

preparation before PCR and by culture followed by biochemical identification. Detection by PCR gave better results than culture (Sasaki *et al.*, 2001). These results differ from those in Paper V, in which DNA preparation and PCR proved positive in only 12% of the samples. For detection by culture followed by biochemical identification, 32% were positive (Paper V). Only a few of the meat juice samples and samples from muscle tissue minced in physiological saline were above the detection limit for PCR, and probably some inhibitory substances were present in the samples. It is known that inhibitors of the enzymatic reaction of PCR amplification exist in organs (Takeuchi *et al.*, 1997).

Before the trials in Papers IV and V, different PCR primer pairs for *C. chauvoei* were tested. The primer pair based on the spacer region of the16S-23S rRNA genes (23UPCH and IGSC4) (Sasaki *et al.*, 2000a) was chosen, since this primer pair gave better bands when visualizing under UV-light, than the primer pair based on the flagellar gene (CCF516 and CCR516) (Kojima *et al.*, 2001). Kuhnert *et al.* (1997) used a specific primer pair (designated CC16S-L and CC16S-R) based on the16S rRNA gene for detecting of *C. chauvoei* in muscle tissue from cattle that died from blackleg. The sequence differences in the16S rRNA genes are few in strains closely related to *C. chauvoei*, *e.g. Clostridium carnis* and *C. septicum* (Kuhnert *et al.*, 1997).

5.7 Recontamination of digested residues in vehicles

The study in Paper I highlights the problem of cleansing the transportation tanks between transportation to and from BGPs. Even so, the pasteurisation step was efficient regarding reducing non spore-forming bacteria, but transportation by vehicles to the farms caused that *S.* Agona was detected again in storage wells at the farms (Paper I). *Salmonella* Agona, isolated before pasteurisation at BGP and in the storage wells at two different farms, had the same PFGE pattern. Most likely, this recontamination had occurred during transportation, as the same vehicles were used to transport both manure and slaughterhouse waste to BGPs and to transport digested residues to the farms. One BGP solved the problem by constructing a vehicle with separate tanks for incoming untreated manure and outgoing digested residues. However, the results in Paper I suggested a pilot study on the efficiency of cleansing transportation vehicles. The cleaning process must be effective in order to prevent recontamination, both of the exterior of the vehicles and of the interior of the tank. All studied BGPs have their own types of vehicles with cleansing and disinfection routines.

In the pilot study, tanker lorry A was disinfected with lye and found to be fairly clean after disinfection. The two tanker lorries B and C were not sufficiently clean, especially around manhole hatches and lids in lorry C (Table *5*). Slaughterhouse waste still remained after washing and disinfection of lorry C, which could be an explanation of the results (*Figure 12*). In a test comparing disinfectants tested on coliforms and *Enterococcus* spp., Virkon S® was more effective than lye (Ekvall *et al.*, 2005). Tanker lorry A, cleaned with lye was probably more carefully cleansed before disinfection. The tyres and mudflaps were not sufficiently cleansed. No washing system has been developed for this particular purpose, with it is high demands on efficiency. It is difficult to ensure that all parts of the interior are sufficiently clean, particularly around baffles, gaskets, lids and bottom valve.

Figure 12. Inside of a tank after cleansing and disinfection. Manure can be seen in the tank. (Photo: Nils-Gunnar Ericsson, November 2004)

Concerning sampling methods, the contact plates were easy to deal with, but the results were difficult to evaluate. The compresses gave good discriminating results, but were difficult to use. Physiological saline poured into the tank gave discriminating results, but the application needs one person to climb up on top of the vehicle. As a method to assess cleansing efficiency, the compresses are the most useful, due to the fact that the compresses were simpler to analyse in the laboratory. Generally, samples taken with compresses give more distinguishable answers than physiological

saline or contact plates. However, taking samples by compress is more difficult from a hygienic point of view than the use of contact plates or pouring physiological saline. Some cleansing controls are needed. Ekvall *et al.* (2005) suggested testing of coliforms or *Enterococcus* spp.

The cleaning of the vehicles has not proved hygienically safe, as shown in Paper I with the detection of *S.* Agona in the farm well and the results in the pilot study. In conclusion, the cleaning barriers are not good enough to prevent the spreading of pathogens and one solution could be vehicles using separate tanks for transport to and from BGPs.

5.8 Hygiene quality of digested residues

To reduce the risk of spreading pathogens, pasteurisation at 70°C for 60 min is recommended before anaerobic digestion. If manure and animal by-products are present in the substrate, a pasteurisation step or equivalent treatment, has to be included in the biogas process, regulated by EC regulation no. 1774/2002 and no. 208/2006. A pasteurisation step before anaerobic digestion reduces *Salmonella* spp., VTEC O157, parasites and other non spore-forming micro-organisms to hygienically acceptable levels (Bendixen, 1996; Papers I and II) (Table 8). However, after digestion it is of great importance to avoid recontamination of the digested residues.

Even though most pathogenic bacteria are reduced during the pasteurisation step in the biogas process, spore-forming bacteria are not (Larsen *et al.*, 1994; Papers I and II). With reference to their spore-forming capacity, *Clostridium* spp. and *Bacillus* spp. are extremely heat-tolerant and consequently require sterilisation (130°C at 3 bar for 20 min) to be inactivated, which is not economically feasible. Even if spore-forming bacteria pass through the biogas process, the pasteurisation step before digestion has advantages, besides the reduction of non spore-forming bacteria, since pasteurisation before thermophilic digestion increases the digestibility of sludge (Skiadas *et al.*, 2005).

Spore-forming bacteria can pose a hygienic risk when digested residues are spread on arable land, especially to previously unaffected areas. If areas free of pathogenic clostridia become contaminated, it will be difficult to get rid of the contagion, as many pathogenic clostridia can persist for long periods of time in soil (Mitscherlich and Marth, 1984; Gyles and Thoen, 1993; Bujoczek *et al.*, 2002; del Mar Gamboa *et al.*, 2005). Caution should be taken before spreading digested residues in such areas. The accessibility of spores in soil may have seasonal variations. For *C. chauvoei*, heavy rainfall can contribute to spreading of the spores (Munang'andu *et al.*, 1996; Useh *et*

al., 2006). Shrubbery clearing of pasture may also make the resting spores in the soil more accessible for cattle.

According to EC regulation no. 1774/2002, spreading of digested residues on pasture is prohibited during a period of 3 weeks prior to grazing. Bacterial spores can persist far longer than 3 weeks. In Sweden, the recommendation is to use digested residues on arable land, but not on pasture. However, fields used for crops one year, may be used as pasture next year, and spore-forming bacteria has the ability to persist for more than one year in the environment.

The risk of spreading pathogenic spore-forming bacteria should be weighed against the disadvantages of using artificial fertiliser, *i.e.* long-term sustainability due to the limited resources of phosphorus (Enocksson *et al.*, 2002; Muga and Mihelcic, 2008). However, the advantages of using digested residues as fertiliser are noteworthy. More studies on the impact of spore-forming bacteria from the biogas process, and their persistence in soil are needed so that the risk can be better understood and dealt with.

Table 8. Reduction of different groups of bacteria depending on treatment.

Treatment / Bacteria	130°C at 3 bar for 20 min	70°C for 60 min	Termophilic digestion at 55°C	Mesoplilic digestion at 37°C	Other ways of treatment for reduction
Salmonella spp.	Complete reduction	Complete reduction	No guaranties for complete reduction	Low reduction	Low pH High pH
Enterococcus spp.	Complete reduction	Complete reduction	No guaranties for complete reduction	Low reduction	-
Bacillus spp.	Complete reduction	Not reduced	Not reduced	Not reduced	-
Clostridium spp.	Complete reduction	Probably not reduced	Probably low reduction	Probably low reduction	High pH availabillity of carbohydrates

6 Concluding remarks and future research

Pathogenic bacteria in substrate transported to biogas plants can later pose a hygiene problem when the digested residues are spread on arable land. This risk must be tackled in an acceptable manner as the advantages should be weighed against the disadvantages of using artificial fertiliser. Due to limited natural resources of artificial fertilisers, such as phosphorus, the digested residues may become more valuable in the future. Therefore, it is of great importance to be able to use digested residues without biosecurity risks. A pasteurisation stage at 70°C for 60 min in the biogas plants is advantageous as the contents of pathogens such as *Salmonella* spp., VTEC and parasites are reduced. However, spore-forming bacteria such as *Clostridium* spp. and *Bacillus* spp. are not totally reduced and can persist unaffected. The impact of anaerobic digestion on *Bacillus* spp. and *Clostridium* spp differs. The number of species of clostridia decreases following digestion, compared with *Bacillus* spp., where the number of species appeared to remain constant.

The microbial flora in digesters differs between biogas plants and changes in a plant over time. Due to many unknown parameters, micro-biological experiments with full-scale biogas plants are difficult to perform. The environment in the digester seems to have big influence on reduction of pathogenic spore-forming bacteria.

In this work, *C. botulinum* was detected before and after pasteurisation, but not after anaerobic digestion. Toxins produced by *C. botulinum* are some of the most potent toxins known, causing a dreaded, life-threatening disease. If the bacteria can be reduced by anaerobic digestion, this would be very valuable, but further studies are needed to confirm these preliminary results.

In the laboratory scale trial (Paper IV), *C. perfringens* type C was detected, but only during the first few days of digestion. Regarding *C. perfringens* type C, causing hemorrhagic enterotoxinemia in pigs, more studies should be

undertaken before digestive residues can recommended to be spread on arable land.

Clostridium chauvoei appeared to be reduced during digestion, except when hydrochloric acid was added to the digester in order to increase the gas yield. More studies on the effect of hydrochloric acid on digestion need to be made to gain knowledge about its impact on *C. chauvoei* survival. If digested residues containing *C. chauvoei* are spread, there is a risk of causing a blackleg outbreak in ruminants. In muscle samples from cattle that died from blackleg, the quantity of *C. chauvoei* was high, but conventional analysis by culture followed by biochemical identification may be hampered by contaminant flora. Detection of *C. chauvoei* by culture followed by PCR is faster, simpler and safer than the conventional method. A recommendation to bacteriological laboratories is to introduce PCR as the identification method for analysis of samples from suspected blackleg cases.

The most important findings in this thesis were:

- Non spore-forming bacteria such as *Salmonella* spp. and VTEC were not detected after pasteurisation stage at 70°C for 60 min.
- Recontamination was observed during transportation and has to be avoided.
- Anaerobic digestion seemed to decrease both the number of species of clostridia and the quantities of clostridia.
- Detection and identification of *C. chauvoei* by culture in combination with PCR proved to be better than culture with biochemical identification in samples from suspected blackleg cases.
- Future studies should focus on the effects of anaerobic digestion on important pathogenic spore-forming bacteria, especially pathogenic *Clostridium* spp.

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The effect of hygienic treatment on the microbial flora of biowaste at biogas plants

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Abstract

In Sweden, full-scale, commercial biogas plants (BGP), which process low-risk animal waste, operate a separate pre-pasteurisation at 70 °C for 60 min as required by EEC regulation 1774/2002. The purpose of this study was to establish if, during pasteurisation and further processing and handling in full-scale BGPs, pathogens in biowaste could be sufficiently reduced to allow its use on arable land.

Four BGPs were sampled on six occasions during 1 year. Sampling was performed from six locations during biogas production. The samples being analysed quantitatively to detect indicator bacteria (*Escherichia coli*, *Enterococcus* spp. and coliforms) and spore-forming bacteria (*Clostridium* spp. and *Bacillus* spp.) and qualitatively for bacterial pathogens (salmonella, listeria, campylobacter and VTEC O157).

Salmonella was the most frequently isolated pathogen before pasteurisation In general, the treatment adequatly reduced both indicator and pathogenic bacteria. Spore-forming bacteria were not reduced. However, recontamination and regrowth of bacteria in biowaste was frequently noted after pasteurisation and digestion.

Keywords: Anaerobic digestion; Pasteurisation; Biogas; Biowaste; Fertiliser

1. Introduction

Anaerobic digestion (AD) in full-scale, commercial biogas plants (BGP) is an environmentally friendly option for the treatment of biowaste. Biowaste consists of what is manure from pig and cattle farms, household waste, waste from food and drug industry, etc. In Europe, there is growing interest in biogas production and also in recycling of biowaste nutrients from urban areas to food production on arable land (IEA Bioenergy workshop, 1999; www.adnett.org). This is a prerequisite for tenable farming in the future and digested residues may be applied as fertiliser on agricultural land. The AD process generates biogas from which methane can be extracted for use as a renewable fuel for engines, both stationary and vehicular. The number of BGPs is increasing in Sweden; there are currently ten large-scale operational BGPs and many are in the planning stage. Biowaste is known to contain pathogens, i.e. salmonella, listeria and campylobacter (Gibbs et al., 1995; Ilsøe, 1993; Larsen, 1995). The digested residues must, therefore, first be adequately treated and proven hygienically safe before be by spreading on arable land according to European regulation, EEC 1774/2002, can recycle it. In Sweden, however, use on pastureland is not recommended.

When this study was performed, in 1999–2001, the regulation of the Swedish Board of Agriculture was still

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in force (SJVFS 1998; 34, K14) (Swedish Board of Agriculture, 1998), which required the same treatment of low-risk animal waste, in general defined in the same way as Category 3 material, see below, in the current EEC legislation. The EEC 1774/2002, divides animal by-products into three categories depending on the expected degree of pathogen content. Category 1 material possibly including prions, must be incinerated and is not permitted for biogas production. Category 2 material must be sterilised in 133 °C at 3 bar pressure for 20 min before further treatment in BGPs. Category 3 material includes animal by-products from healthy animals approved for human consumption and required 70 °C for 60 min before digestion.

Pasteurisation of biowaste at 70 °C for 1 h is an effective way of heat treatment to reduce most pathogens (Bendixen, 1999; Böhm et al., 1999). Pasteurisation in a BGP can be performed batch wise or as a continuous process. The batch-wise method is preferred, as treatment time is more easily regulated and the pathogen reducing effect can be verified. In all except one Swedish BGP using Category 3 material, pasteurisation is performed batch wise before AD. The incoming biowaste is minced before heating up for pasteurisation. The heating up occurs with steam or heat exchanger. After pasteurisation the biowaste will be digested in around 20–25 days in mesophilic AD or in 10–20 days in thermophilic AD (Fig. 1).

As many pathogens are difficult to detect and quantify, indicator bacteria are generally used to monitor the bacterial level and show the effect of disinfections (Larsen et al., 1994). An increased number of indicator bacteria indicate a possible increase in pathogens. *Escherichia coli* and *Enterococcus* spp. can be used as indicator bacteria (Gibbs et al., 1995; Ilsøe, 1993). *Enterococcus* spp. are more resistant to different kinds of waste treatments, but coliform bacteria are closer related to salmonella (Larsen et al., 1994). Spore-forming bacteria grow as vegetative bacteria under favourable conditions, but when growth conditions are poor they can survive as persistent spores for very long periods of time. Both *Clostridium* spp. and *Bacillus* spp. are spore-forming bacteria, but *Clostriduim* spp only grow anaerobically. Spore-forming bacteria are often present in manure and most of them are harmless, but some of them (for example *Cl. chauvoie*, *Cl. botulinum*, *B. anthracis*) are very pathogenic to humans and animals.

Previous studies have investigated the ability of thermophilic AD treatment of pig slurry and biowaste to reduce the content of pathogens such as *Salmonella* spp. (Larsen et al., 1994; Plym-Forsell, 1995) within the retention period, but not batch wise. *Salmonella* spp. is common in manure and can under favourable conditions survive for more than 1 year in the environment (Mitscherlich and Marth, 1984). All serovars of *Salmonella* spp. are potentially pathogenic zoonotic infections in man and domestic animals, which makes *Salmonella* spp. an important consideration in biowaste disposal. Another zoonotic pathogen of interest is *Listeria monocytogenes*, commonly found in soil, silage, faeces and in sewage sludge (De Luca et al., 1998; Donald et al., 1995). *Listeria* spp. can survive and even proliferate at 1–45 °C in digested residues from BGPs (Junttila et al., 1988). *E. coli* O157, also a zoonotic pathogen, is frequently found (Albihn et al. 2003) in bovine manure, a common biowaste used in BGPs. It is thus a possible source of environmental contamination (Kudva and Hovde, 1998). *Campylobacter jejuni* and *C. coli* are often present in slurry and raw sewage sludge (Kearney et al., 1993a, b; Sahlström et al., 2004). *Campylobacter* spp. are also present in cattle and pig manure.

The aim of this study was to establish if pathogens are sufficiently reduced and the digested residues can be regarded safe for use as fertiliser when biowaste is processed in BGPs. Additionally the study would survey

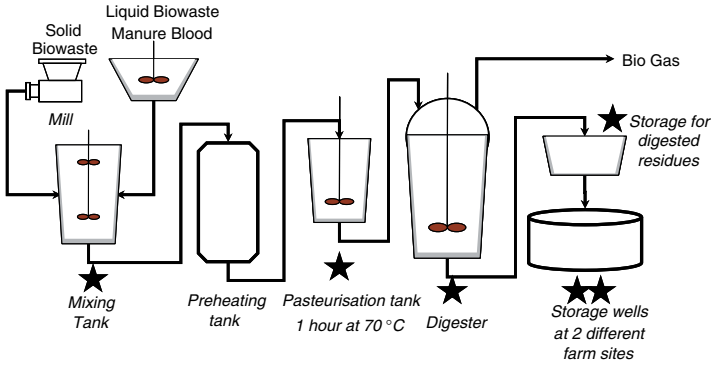

Fig. 1. Schematic picture over the flow of biowaste through a BGP. The locations of sampling in this study are marked with a black star (★).

the risk of recontamination of the digested residues during storage and transportation.

2. Materials and methods

2.1. Biogas plants and sampling

Four Swedish commercial full-scale BGPs situated in southern and central Sweden were studied. At the time of this study, these were the four BGPs which processed low-risk animal waste, as defined in SJVFS 1998; 34, K14 (Swedish Board of Agriculture, 1998), which requires a separate pasteurisation at 70 °C for 60 min.

The BGPs received slurry from pig and dairy farms, between 5 and 20 farms per plant, also biowaste from food processing plants, households and restaurants. Sewage sludge was not treated in these BGPs. All categories of waste were mixed, pasteurised and digested together. All four BGPs had a separate pasteurisation step at 70 °C for 60 min; at plant C this was semi-continuous while the other three used batch-wise pasteurisation (Table 1). The semi-continuous system was built as a pipeline through which the biowaste was pumped for 15 min and then held for 45 min. BGP A used thermophilic (50–55 °C) AD, whereas the other three used mesophilic (30–38 °C) AD (Table 1). Biogas plant D had two digesters. Contents in digesters and pasteurisation tanks were continuously and mechanically stirred. Dry matter content in incoming substrate is around 7–10%, 3% in the digester and 4–6% in digested residues. All BGPs in this study had continuous surveillance systems for the process including several alarmed check points for treatment duration, temperature and the flow of biowaste through the plant in order to minimise the risk of contamination.

Biowaste at the BGPs was sampled six times evenly distributed during 1 year starting with the first plant in October 1999 and ending with the fourth plant in January 2001. Samplings were performed before and after pasteurisation, after AD, in storage for digested residues at the plant and from intermediate storage wells for the digested residues at two different farm sites (Fig. 1). Total numbers of samples were 139; 24 before and 24 after pasteurisation, 29 after digestion (plant D had two digesters), 23 in storage for digested residues, 39 from farm wells (The samples from the 2 farms connected to each plant are added together). A few samples are missed due to practical problems such as empty storage wells at the time of sampling. The four plants were sampled in rotation during the whole period, in that way the samples were evenly distributed among the four seasons.

Local BGP staff performed the sampling according to detailed instructions, such as to let the biowaste run through the tap before sampling and to chill the samples directly after sampling. All samples weighed approximately 100 g, and were collected in clean vessels and stored in clean pots. They were kept cold (<12 °C) during transportation and arrived for cultivation at the laboratory within 24 h of sampling.

2.2. Analytical methods

Cultivation of indicator and spore-forming bacteria was quantitative, whereas pathogens were analysed qualitatively. The method used for detection of bacteria was the standard Nordic Committee on Food Analysis (NMKL, 1990–1999), in all cases, except for the method used to detect *Clostridium perfringens*, *Campylobacter* spp. and *E. coli* O157. The National Veterinary Institute, Uppsala, Sweden, supplied the media unless otherwise stated. The statistical calculations are the geometric mean values.

2.2.1. Quantitative methods

For quantitative analysis, 10-fold dilutions were made in saline solution (0.9% NaCl).

Enterococcus spp. (NMKL no. 68, 2nd ed., 1992) were counted on Slanetz-Bartley agar after incubation at 45 °C for 48 h.

Coliform bacteria (NMKL no. 44, 4th ed., 1995) were analysed by first mixing the inoculum with violet–red bile agar and incubating at 37 °C for 24 h. Suspected colonies were then counted and further analysed by cultivation in brilliant green bile lactose broth incubated at 37 °C for 24 h.

Table 1
Technical information about the four BGPs

	A	B	C	D
Number of connected farms	5–10	20	20	5
Manure (m^3/year)	20,000	38,000	24,000	9000
Total volume of biowaste (m^3/year)	24,000	79,000	35,000	37,000
Gas-production (GWh/year)	4	22	10–15	23
Pasteurisation	Batch wise	Batch wise	semi-continuous	Batch wise
Anaerobic digestion	thermophilic	mesophilic	mesophilic	mesophilic

The thermotolerant coliforms and *E. coli* (NMKL no. 125, 3rd ed., 1996) were analysed by first mixing the inoculum with violet red bile agar and incubating at 45 °C for 24 h. Suspected colonies were then counted and further analysed by cultivation in lactose tryptone lauryl sulphate broth incubated at 45 °C for 24 h. Kovacs' indole reagent was used to detect *E. coli*.

C. perfringens was detected and counted on tryptose–sulphite–cycloserine agar incubated at 37 °C for 24 h in anaerobic jars. Five suspected colonies were further analysed on egg yolk agar and horse blood agar and incubated at 37 °C for 24 h in anaerobic jars.

Bacillus spp. (NMKL no. 67, 4th en., 1997) were cultivated on horse blood agar incubated at 30 °C for 24 h, and suspected colonies were counted.

The coliform bacteria and *C. perfringens* were obtained with a detection limit of 10 cfu/ml and *Enterococcus* spp. and *Bacillus* spp. were obtained with a detection limit of 100 cfu/ml.

2.2.2. Qualitative methods

Salmonella spp. (NMKL no. 71, 5th ed., 1999). Twenty-five gram was pre-enriched in buffered peptone water incubated at 37 °C for 24 h and then inoculated in Rappaport-Vassiliadis broth incubated at 42 °C for 24 h. An aliquot from the selective enrichment broth was streaked on xylose–lysine–desoxycholate agar and brilliant green-phenol red agar and incubated at 37 °C for 24 h. Suspected colonies were confirmed by biochemical tests (mannitol, sorbose phosphate, sucrose, indole/β-galactosidase, urea, mallonate, triple sugar iron agar, and lysine decarboxylase) and serotyping of *Salmonella* spp. was performed by agglutination according to the Kauffmann-White scheme (WHO, 1997).

L. monocytogenes (NMKL no. 136, 1990). Ten gram was pre-enriched in Listeria enrichment broth, and incubated at 30 °C for 48 h. An aliquot was then streaked on Listeria-selective agar (Oxford agar) and incubated at 37 °C for 24 h and 48 h. Suspected colonies were confirmed by biochemical tests (Gram-staining, catalase test, rhamnose, glucose, lactose, maltose, sucrose, xylose, aesculin and motility tests).

C. jejuni and *C. coli*. Two swabs were pre-enriched in a Preston broth incubated at 42 °C for 24 h, then streaked on Preston agar and incubated at 42 °C for 48 h. Suspected colonies were further analysed and confirmed by motility and biochemical tests (motility tests by phase-contrast microscopy, oxidase test, catalase test and hippurate hydrolysis).

For analysis of *E. coli* O157, 10 g was pre-enriched in peptone-water incubated at 37 °C for 6–8 h. The samples were then analysed by immuno-magnetic-absorbent assay with immunomagnetic beads (Dynabeads anti-*E. coli* O157; Dynal, Oslo, Norway) to isolate VTEC O157, thereafter incubated on sorbitol-MacConkey agar with cefixime and potassium tellurite at 37 °C for 24 h. Suspected colonies were further analysed with a latex agglutination test (Oxoid DR 622, Oxoid, Basingstoke, England). The suspected colonies were streaked on horse-blood-agar and confirmed by PCR (Hoorfar et al., 2000) and API 20e (Bio Mérieux AB, Marcyl l'Etoile, France).

2.2.3. PFGE

Salmonella serotypes found on more than one sampling occasion or sampling place were typed by macrorestriction enzyme analysis followed by pulsed-field gel electrophoresis (PFGE), as described by Palmgren et al. (2002). Three different macrorestriction enzymes were used: *Xba*I, *Bln*I and *Spe*I (Amersham Biosciences, Uppsala, Sweden).

3. Results

After pasteurisation, growth of indicator bacteria was observed in one out of 24 (4%) samples. This observation was made on the third sampling occasion from BGP B, were *Enterococcus* spp. was found (5.5 $\log_{10}$ CFU/g) (Fig. 3). In samples taken directly after AD, indicator bacteria were detected in 7/29 (24%): *Enterococcus* spp. were detected in 6/29 (21%), and coliform bacteria in one out of 29 (3%) (Figs. 2 and 3). In the storage tanks for digested residues at BGPs, indicator bacteria were detected in 11/23 (48%) of the samples. The results for thermotolerant coliforms and *E. coli* were nearly the same as the results for coliform bacteria (data not shown). In the digester and in the storage for digested residues at the plant some bacterial growth was noted, but after the transportation to the storage wells at farm sites, the number of indicator bacteria increases.

Spore-forming bacteria were not reduced by the process and *Cl. perfringens* and *Bacillus* spp. were determined as viable counts in all samples. The mean value for *Cl. perfringens* were 4.4 $\log_{10}$ CFU/g (Fig. 4) and the mean value for *Bacillus* spp. were 4.8 $\log_{10}$ CFU/g.

Growth of pathogens are shown in Table 2. Pathogens were found in 13/24 (54%) of samples before pasteurisation, of which *Salmonella* spp. were isolated in 4/24 (17%) of the samples. In digested residue stored in wells at farm sites, salmonella was detected in 4/24 (17%) of the samples. *Salmonella enterica* subsp. *enterica* serovar Agona was isolated on two sampling occasions at BGP B before pasteurisation and isolated on four sampling occasions in storage wells at farm sites (Table 2). One strain from farm site 1 had the same PFGE pattern as one strain obtained before pasteurisation. The other strain obtained before pasteurisation was identical to the strain from farm site 2. The two other strains from farm sites 1 and 2 differed from each other and from the remaining strains obtained before pasteurisation.

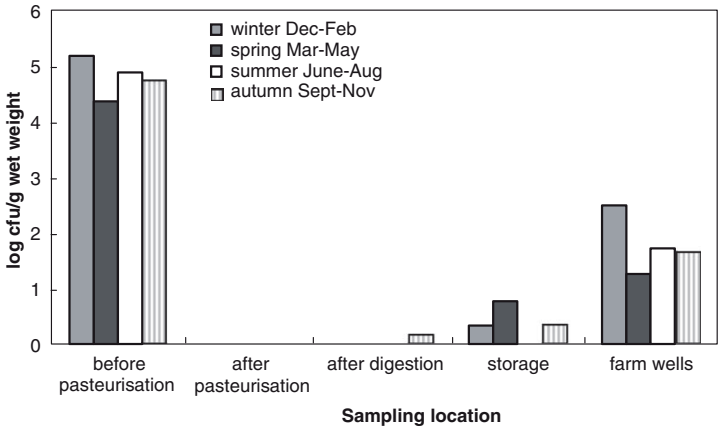

Fig. 2. Mean value of coliform bacteria on six sampling locations from each of the four BGPs presented according to season.

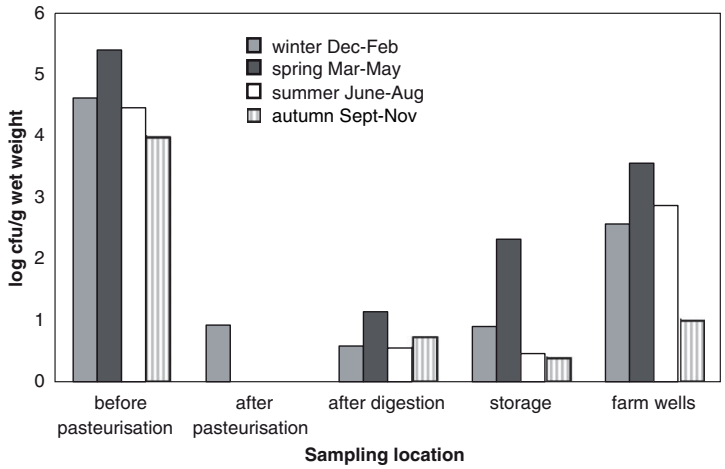

Fig. 3. Mean value of *Enterococcus* spp. on six sampling locations from each of the four BGPs presented according to season.

In one sample from BGP B, on the third sampling occasion, *S. enterica* subsp. *enterica* serovar Brandenburg was found after pasteurisation (Table 2).

4. Discussion

In this study, BGPs using a pasteurisation step at 70 °C for 60 min before AD significantly reduced all studied pathogens as well as all indicator bacteria in incoming biowaste. These results agree with previous studies on sewage sludge, which showed that *Salmonella* spp. could not be detected for more than 30 min at 70 °C (Bendixen, 1996; Mitscherlich and Marth, 1984). However, neither pasteurisation nor AD reduced spore-forming bacteria.

However, the numbers of samples are too low for reliable statistical analyses of the results. But our results indicate that the numbers of indicator and spore-forming bacteria do not change over season. The indication of a reduction of clostridia after digestion and in storage may depend on different dry matter content (Fig. 4).

Even though the pasteurisation was efficient, both pathogens and indicator bacteria were again detected in storage wells at farm sites. The increase of bacteria after transportation and storage can depend on persistence of bacteria below detection level after pasteurisation.

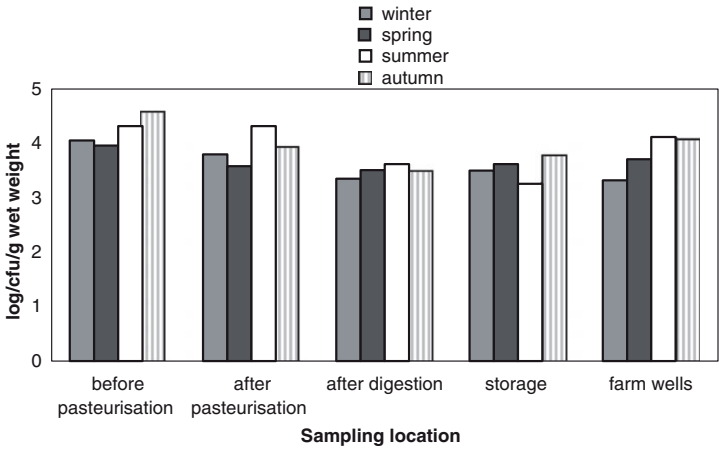

Fig. 4. Mean value of *C. perfringens* on six sampling locations from each of the four BGPs presented according to season.

Table 2
Pathogens isolated from BGP A–D at four of the six sampling locations

Sampling locations				
BGP	Before pasteurisation	After pasteurisation	Storage wells at farm site 1	Storage wells at farm site 2
A	*C. jejuni* (2) *S.* Schleissenheim[a] (1)			
B	*C. coli* (1) *E. coli* O157 (1) *S.* Agona (2)	*S.* Brandenburg (1)	*S.* Agona (2)	*E. coli* O157 (1) *S.* Agona (2)
C	*C. jejuni* (1) *C. coli* (2) *E. coli* O157 (1) *S.* Agona (1)		*C. coli* (1)	
D	*L. monocytogenes* (1)			

At the locations after digestion and in the storage well at the BGPs no pathogens were found. The number of sampling occasions on which the same species were isolated from the same sampling site is shown within parentheses.
[a]S. *enterica* subsp. *enterica* serovar Schleissenheim.

Another explanation to the high number of indicator bacteria at the farm wells could be a high amount of bacteria in the farm wells before the digested residues are added to the wells. Most probably, however, this recontamination occurred during transportation, as the same vehicles were used for both in-transportation of manure and out-transportation of digested residues. However, more samples have to be analysed before any conclusions can be drawn. The transportation tanks were flushed either with hot water, about 70 °C at plant B and C, or cold water about 10 °C at plant A and D. Due to a stirring system inside the tank, it was difficult to accomplish complete cleaning and some material remained in the tank.

The findings of *Salmonella* Agona with the same PFGE pattern isolated before pasteurisation and in the storage wells at two different farm sites at two different sampling occasions in BGP B, support the theory about contamination during transportation. This study highlights the problem of cleaning of transportation tanks between in and out transportation. To solve the problem, after this study, one BGP constructed a vehicle with separate tanks for untreated manure and digested residues.

As the pasteurisation process seemed to follow specifications, also at BGP B, without disturbances in treatment time, temperature, stirring, etc, the detection of *Salmonella* Brandenburg and *Enterococcus* spp. on

one occasion after pasteurisation was surprising. No explanation other than a contamination in connection with sampling could be found. No other pathogens or growth of indicator bacteria were noticed in pasteurised biowaste on the three subsequent sampling occasions, after the local staff had repeatedly been informed about the importance of a strictly hygienic sampling technique.

Spore-forming bacteria persisted at all sampling locations, as expected from earlier studies (Mitscherlich and Marth, 1984; Olsen and Larsen, 1987). If there are any pathogenic spore-forming bacteria in the incoming manure they persist in the digested residues. Therefore, spore-forming bacteria can pose a hygienic problem when biowaste is spread on arable and pasture land as they can cause several serious diseases such as black leg and other clostridial diseases (Hang'ombe et al., 2000; Sternberg et al., 1999; Wierup and Sandstedt, 1983).

Ward et al. (1999) expounded that *Salmonella* spp. and coliforms do not regrow in digested residues if effectively pasteurised (70 °C for 30 min). In our study, however, we found a potential risk for regrowth of bacteria when treated biowaste became recontaminated during handling following pasteurisation. Hence, biowaste cannot be considered entirely free from *Salmonella* spp. or other pathogens. *Salmonella* spp. can survive for a long time outside the host when environmental conditions are suitable (Gibbs et al., 1995).

Temperature and treatment time, which are important factors for inactivation of pathogens during pasteurisation, can be easily regulated (Bendixen, 1996), particularly when batch-wise pasteurisation is used. Biogas plant C in this study used a semi-continuous pasteurisation step, biowaste passing through a pipeline. Depending on the hygienic quality of the pasteurised biowaste, it appeared to work according to specifications, but the exact duration of treatment is difficult to regulate. For example, if the pipelines become coated on the inside, the pipe diameter decreases and consequently the biowaste will pass through the pasteurisation step faster than calculated, which will decrease the pasteurisation effect.

Degradation proceeds faster in thermophilic AD and giving a shorter retention time than in mesophilic AD. Moreover, the reduction of bacteria is more efficient during thermophilic AD than during mesophilic AD (Olsen and Larsen, 1987). Even in our study, BGP A with thermophilic AD had fewer indicator bacteria, both after AD and in the storage tank at the BGP, than the other BGPs. However, thermophilic AD is not batch wise and therefore not as hygienically safe as a separate pasteurisation. In sewage sludge, *Salmonella* spp. are often found after mesophilic AD (Sahlström et al., 2004) and in other studies *Salmonella* spp. were detected after weeks and even months of mesophilic AD (Kearney et al., 1993a, b). Therefore, the pasteurisation is a necessity to support AD if the biowaste is to be safely used on arable land.

The growing interest in Europe for biogas production (IEA Bioenergy workshop, 1999; www.adnett.org) and the use of digested residues on arable land makes it important to consider and regulate biosecurity aspects. If the hygienic quality of digested residues is acceptable, the biowaste can be recycled back to the food production, it may be used as a fertiliser in agriculture. If not, other solutions for digested residues must be elaborated i.e. incineration. Moreover, it should be considered that spore-forming bacteria are not reduced. Therefore, in Sweden the official recommendation is that digested residues from BGPs despite only pasteurisation, should be applied on arable land and not on grasslands for animal pasture.

5. Conclusions

The study shows that a pre-pasteurisation step before AD gives an adequate reduction of indicator bacteria and the pathogenic bacteria detected in this study. However, to ensure the hygienic standard of the digested residues the post-AD handling must be in a strictly hygienic manner to avoid recontamination and regrowth.

Spore-forming bacteria will still persist after the pasteurisation and digestion. This may be a risk when using digested residues on arable land. Thus, further studies have to be done in order to find out if the digested residue have an acceptable hygienic standard regarding the risk of recontamination and spore-forming bacteria.

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A laboratory study of survival of selected microorganisms after heat treatment of biowaste used in biogas plants

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Abstract

The aim of the study was to assess the effect of pasteurisation, as set by the European regulation EC 1774/2002, on selected pathogens and indicator organisms.

Unpasteurised substrate (biowaste), including animal by-products from a full-scale biogas plant was heat treated under laboratory conditions at 70 °C and 55 °C for 30 min and 60 min.

Heat treatment at 55 °C for 60 min was not sufficient to achieve a hygienically acceptable product. Heat treatment at 70 °C for 30 min and 60 min was effective in reducing pathogenic bacteria, *Ascaris suum* eggs, Swine vesicular disease virus and indicator organisms. However, this level of pasteurisation will still not reduce the quantity of Clostridia spores, or completely inactivate heat-resistant viruses such as Porcine parvovirus or *Salmonella* phage 28B.

The results still give cause for some concern regarding the use of digested residue from biogasplants in agriculture.

Keywords: Anaerobic digestion; Fertiliser; *Campylobacter*; Enterococci; *E. coli* O157

1. Introduction

In full-scale biogas plants (BGP) in Sweden, different kinds of biowaste, though not sewage sludge, are used to produce biogas. Animal by-products (ABP) such as manure, slaughter by-products, blood, lipids and other biowaste, e.g. biological household waste separated at source and waste from food industries, can contain pathogens infectious to humans and animals. The digested residue is used as a fertiliser and soil improver, and must therefore be hygienically safe, to avoid spreading diseases through the environment to all forms of animal life and humans. Hygienically safe or acceptable is here defined as indicator bacteria and salmonella being below detectable limits with the methods used in this study or with other similar standard methods.

At the time of the present study, Swedish law requires BGPs that use animal waste to pasteurise the incoming substrate at 70 °C for 60 min before digestion, to ensure a hygienically acceptable product. In May 2003, a new European regulation (EC 1774/2002) concerning ABPs was implemented, replacing laws set by the member states of the EU that previously regulated the use of animal waste. The EC-regulation divides ABPs into three categories, depending on the expected degree of pathogenic contamination. Category 1 material, which could contain

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prions, must be incinerated. Category 2 material, including carcasses (of animals other than those in category 1 material), must be sterilised (133 °C at 3 bar for 20 min) before further treatment in a BGP. Manure is included in Category 2 material, but it is allowed to be used in BGPs without sterilisation, if not deemed contagious by the authorities. Category 3 material includes ABPs from healthy animals approved for human consumption, and requires pasteurisation at 70 °C for 60 min before use in a BGP, if it is kept separate from material in Categories 1 and 2. As Category 3 material and manure may contain agents that are potentially infectious for people and animals, it is important that the pasteurisation process is sufficiently thorough to kill pathogens. The substrate used in BGPs is rarely investigated in terms of pathogenic microorganisms and their reduction after heat treatment. The difference between this substrate and other media is the heterogeneity and variation of the mixture and the potentially rather large (maximum 12 mm) particles. The effect of pasteurisation can be studied using pathogenic microorganisms or indicator bacteria or both. *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter coli*, *C. jejuni* and verotoxin-producing *Escherichia coli* O157 (VTEC) have all been isolated from substrate in full-scale BGPs. All are zoonotic agents and important bacteria causing infectious disease amongst humans and animals and many are resistant to environmental conditions.

The aim of the present laboratory study was to assess the effect of heat treatment at 70 °C on the survival of selected pathogens and indicator organisms in substrate from a large-scale BGP in Sweden. Heat treatment at 55 °C under similar conditions was also investigated due to the well-founded commercial interest in lower treatment temperatures.

We tested the microorganisms described below, as well as indigenous indicator bacteria such as coliforms, thermotolerant coliforms, presumptive *E. coli* and enterococci in survival trials. The substrate was heat-treated and the survival of the inoculated pathogens was determined after 15 (only for *A. suum*), 30 min and 60 min.

Clostridium perfringens is commonly found in substrate from BGPs and was chosen here as an indicator for spore-forming bacteria (Carrington, 2001). Porcine parvovirus (PPV) causes reproductive failure in swine and was selected as a viral indicator (Lund et al., 1996) due to its high thermo-resistance (Haas et al., 1995; Kim et al., 2000). Swine vesicular disease virus (SVDV) is not as resistant to heat as PPV (Herniman et al., 1973; McKercher et al., 1980), but has been used in inactivation studies regarding slurry (Turner et al., 1998). SVDV is a picornavirus and belongs to the genus *Enterovirus*, together with Bovine and Porcine enterovirus (King et al., 2000). In sanitation stipulations for biogas reactors, picornavirus is proposed as an indicator (Lund et al., 1996). Various types of bacteriophages have been suggested as viral indicators and indicators of faecal contamination (Havelaar et al., 1991). They are similar to viruses in structure, but easier and cheaper to analyse and harmless to all animals. *Salmonella* typhimurium phage 28B (Lilleengen, 1948) has been successfully added to sewage and used to trace leakage from sewage to groundwater (Johansson et al., 1998; Carlander et al., 2000) and has also been used as a process evaluator for liquid composting (Eller, 1995). However, it has not previously been compared with viruses with respect to heat resistance. *Salmonella* phage 28B does not occur naturally in either environmental samples or faeces. *A. suum* eggs are one of the most heat-resistant parasitic ovas, and are therefore suitable as an indicator of parasite survival (Feachem et al., 1983).

2. Methods

2.1. Substrate

The substrate used in this study was untreated, mixed biowaste from a large-scale BGP in Sweden, consisting of waste from food industries, biological household waste separated at source, and Category 3 ABPs such as manure, blood, fat, etc. The substrate was collected in clean vessels from a tap on the homogenisation tank before pasteurisation at the BGP. It was then chilled in a water bath (+8 °C) before transportation in a cold-box to the National Veterinary Institute (SVA), Uppsala, where it was kept in a refrigerator (+4 °C) at SVA before the pasteurisation trial started within 24 h. Because several trials were performed, there were several batches of substrate; hence, the content and subsequently the structure of the substrate may have varied, as raw material used in large-scale BGP normally varies over time.

An analysis was performed before every pasteurisation trial in order to check for pathogens in the original substrate and to check that no phage was present in the substrate to interfere with the *Salmonella* serovar typhimurium type 5 host bacteria.

The supplier of media in this study was SVA, unless otherwise stated.

2.2. Bacterial strains

Pathogenic bacteria inoculated into the substrate were from the strain collection at SVA: *Salmonella enterica* subsp. *enterica* serovar typhimurium, Culture Collection, University of Gothenburg (CCUG) 31969, *L. monocytogenes* CCUG 15527, *E. coli* O157 CCUG 8018 and *C. jejuni* CCUG 11284. The bacterial strains were stored at −70 °C. They were streaked onto blood agar plates and incubated at 37 °C for 24 h, before enrichment in serum broth at 37 °C for 24 h. The final concentration of bacteria in the suspension was approx 10^8 CFU ml^{-1}, except for *C. jejuni*, which had a concentration of approx 10^7 CFU ml^{-1}. The concentration was determined based on 10-fold dilutions in buffered peptone water and colony counts on agar media, as described under Bacterial analysis, except

for *L. monocytogenes* for which horse blood (5%) agar plates were used. Indicator bacteria enumerated were indigenous faecal bacteria such as coliforms (37 °C), thermotolerant coliforms (44 °C), presumptive *E. coli*, enterococci and *C. perfringens*.

2.3. Virus and cells

The PPV strain 893/76 originally isolated at the Danish Institute for Food and Veterinary Research, Lindholm, Denmark, was grown in PK-15 (pig kidney) cells, American Type Culture Collection (ATCC) CCL33, using as cell culture medium (CCM) Eagle's minimal essential medium (MEM) (Eagle, 1959), supplemented with non-essential amino acids (acids (L-Alanine 8.9 mg l^{-1}, L-Asparagine monohydrate 15 mg l^{-1}, L-Aspartic acid 13.3 mg l^{-1}, L-glutamic acid 14.7 mg l^{-1}, L-proline 11.5 mg l^{-1}, L-serine 10.5 mg l^{-1}) and containing 4% foetal bovine serum (FBS). The suspension was frozen and thawed twice, clarified by compressed air filtration through 3.0 μm and 0.45 μm filters (Millipore, Billerica, MA, USA), aliquoted and stored at −70 °C. The final titre of PPV suspension was 6.9 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ per 50 μl. The titre was determined as described under Virus analysis.

The SVDV strain 27/72 was grown in IB-RS-2 (pig kidney) cells (both obtained from Pirbright Laboratory, Institute for Animal Health, UK) using Eagle's MEM containing 2% FBS. After full cytopathogenic effect, the virus-infected cells and supernatant were frozen and thawed twice, and clarified by centrifugation at 2500×g for 20 min. The virus suspension was aliquoted and stored at −70 °C. The final titre of SVDV was 8.1 $\log_{10}$ $TCID_{50}$ per 50 μl, determined as described under Virus analysis.

2.4. Propagation of Salmonella typhimurium phage 28B

Salmonella typhimurium phage 28B and its host *Salmonella enterica* serovar typhimurium type 5 (Lilleengen, 1948) were obtained from the Swedish Institute for Infectious Disease Control, Solna. The bacteriophage was propagated in Nutrient Broth (Oxoid) against its host strain to a concentration of approx 10^{10} PFU ml^{-1}. Determination of the concentration was performed in the same way as described under Analysis of *Salmonella* phage 28B. The bacterial host was killed by adding chloroform (10 ml l^{-1}). The phage solution was then centrifuged for 30 min at 4300×*g* and filtered through 0.45 μm membrane filters to remove cell debris.

2.5. Parasites

A. suum eggs were obtained from roundworms in fattening pigs at the slaughterhouse in Uppsala, Sweden, via the Department of Parasitology, SVA. The *A. suum* eggs were stored in 0.1 M H_2SO_4 at 4 °C before the pasteurisation trial.

2.6. Inoculation of pathogens and pasteurisation trial

The substrate was distributed into 250 ml glass bottles. The bacterial pathogens were inoculated into the substrate to a final concentration of approx 10^5 CFU g^{-1} of each bacterium. One bottle with a mix of bacterial pathogens was used for each temperature–time combination and one bottle was not heated and analysed as positive control. Indicator bacteria were not inoculated, whereas indigenous indicator bacteria were enumerated in the analysis. The viral agents were treated in separate bottles at the desired temperature. The inoculation ratio for the viruses was 1:10 and the theoretical initial virus mean concentration was 7.2 $\log_{10}$ $TCID_{50}$ g^{-1} for PPV and 8.4 $\log_{10}$ $TCID_{50}$ g^{-1} for SVDV. The phage was inoculated into the substrate to a final concentration of approx 10^8 PFU g^{-1}. *A. suum* eggs were separately heat-treated in substrate kept in small tea-bag-sized polyethylene bags with a pore size of 0.20 μm. Each bag contained about 10^4 eggs. The concentrations of inoculated pathogens in this study were based on numbers of indicator bacteria normally found in BGP in previous studies (Herniman et al., 1973; Haas et al., 1995) and a worst-case scenario.

Heat treatment was performed in a water bath at 70 °C or 55 °C, with continuous mixing in the bottles. The temperature was continuously controlled with a thermosensor in the substrate, and timekeeping began when it reached the desired level (70 °C or 55 °C) in the whole substrate. The temperature was reached after 14–20 min of heating in the water bath, and thus it simulated the actual process in the biogas plant. Samples for analysis were taken directly after inoculation and after 30 min and 60 min. In addition, analysis of *A. suum* eggs was also performed after 15 min.

Substrate samples containing pathogenic bacteria, viruses, *A. suum* eggs (in bags) or *Salmonella* phage 28B were kept in bottles similar to the ones used in the experiment, at approx 20 °C during the trial, as control samples. The purpose of the controls was to study the effect of the substrate on the reduction of the organisms. Additionally, a suspension of the two viruses was kept in CCM, while bags with *A. suum* eggs were kept in 0.1 M H_2SO_4 at approx 20 °C as controls. The pasteurisation trial and analysis of the pathogens and indicator organisms were repeated three times.

2.7. Bacterial analysis

S. typhimurium, *C. jejuni*, *E. coli* O157, *L. monocytogenes* and *C. perfringens* were analysed according to the methods described in Sahlström et al. (2004). Suspected colonies of *L. monocytogenes* in the positive control samples were confirmed by Gram staining rendering visible Gram-positive colonies including *L. monocytogenes*. Counting of indicator bacteria was performed after 10-fold dilutions in peptone–water according to NMKL 91:2:1988. The NMKL methods used were: for enterococci, NMKL 68:2:1992, where samples were plated onto enterococcus

agar according to Slanetz and Bartley (Oxoid) and incubated at 44 °C for 48 h; for coliform bacteria, NMKL 44:4:1995 utilising violet red bile agar (Difco) and incubated at 37 °C for 24 h; and for thermotolerant coliform bacteria and presumptive *E. coli*, NMKL 125:3.1996. The same agar as for coliform bacteria was used, but it was incubated at 44 °C for 24 h. Confirmation of coliform bacteria was performed by gas production in Brilliant Green broth. Confirmation of thermotolerant coliforms was performed in lactose tryptone lauryl sulphate broth (LTLSB) (Oxoid). For examination of presumptive *E. coli*, Kovaćs reagent for detecting indole was added to the inoculated LTLSB tubes.

2.8. Virus analysis

Samples were centrifuged in a microcentrifuge at 7000×*g* for 2 min, as this has been shown to ensure sufficient virus recovery to obtain an adequate initial virus titre of the spiked substrate, with regard to SVDV (Turner et al., 1999). To reduce cytotoxicity where appropriate for analysis of SVDV, the centrifuged samples were filtered through 0.45 μm sterile filters (Millipore, Billerica, MA, USA), followed by treatment with a PD-10 desalting column containing Sephadex™ G-25 Medium (GE Healthcare, Chalfont St. Giles, UK). This treatment was shown not to reduce the virus titre. Cytotoxicity and viral interference studies were performed using substrate treated as above. Cytotoxicity tests were performed by diluting the treated substrate 3-fold in cell culture medium, inoculating onto IB-RS-2 cells, and observing cytotoxicity for up to 4 days. As control cell culture medium was used. The viral interference studies were performed by titrating SVDV in the 3-fold dilutions of the treated substrate, inoculating onto IB-RS-2 cells and observing the virus titres after 4 days. SVDV titrated in cell culture medium was used as control.

Titration of samples and titre calculations were performed using a $TCID_{50}$ quantal assay (Kärber, 1931).

Cells and CCM used were as described under Virus and cells, with an extra addition to the CCM of 0.75 μg ml^{-1} of fungizone (Bristol–Myers Squibb, NY, USA). CCM without serum was used as sample dilution medium and as a negative control. Samples from each treatment were diluted 10-fold (or 3-fold where a low virus titre was expected) and added to microtitre plates containing cells, with eight 50-μl replicates per dilution. After 4 days (SVDV) or 7 days (PPV) of incubation at 37 ± 1 °C in $5 \pm 1\%$ CO_2 in a humidified atmosphere, the cells in the microtitre plates were analysed microscopically to determine the cytopathogenic effect. For PPV, a specific immunoperoxidase test (Emmoth et al., 2004) was used to visualise the infected cells prior to microscoping.

2.9. Analysis of Salmonella phage 28B

Samples of 10 g were combined with 90 g buffered peptone–water and mixed in a Stomacher. Counts were made after 10-fold dilutions in sodium chloride. *Salmonella* phage 28B was analysed by a double-agar layer method (Adams, 1959). The host strain *Salmonella* serovar *typhimurium* type 5 was cultured in Nutrient Broth at 37 °C for 4 h. A mixture of 1 ml sample, 1 ml cultivated host and 3 ml soft agar (70% Blood agar base (CM55, Oxoid) and 30% Nutrient Broth) was spread on a dry CM55 agar plate and incubated at 37 °C for 18 h. Clear zones (plaques) were counted as PFU.

2.10. Analysis of A. suum ova

After pasteurisation, the bags with *A. suum* eggs were placed in 0.1 M H_2SO_4 at approx 20 °C to mature. After 4 weeks, 1000 eggs from each bag were counted, and scrutinised for developed larvae. The number of developed larvae among the heat-treated eggs was compared with the number of developed larvae in the unpasteurised control, which was kept in 0.1 M H_2SO_4 at approx 20 °C.

3. Results

3.1. Bacteria

The pathogenic bacteria that were inoculated and analysed in the trial could not be detected in the original substrate. No inoculated pathogenic bacteria or indicator bacteria, except for *C. perfringens*, could be found after heat treatment at 70 °C for 30 min or 60 min. *S. typhimurium* and *E. coli* O157 were once detected after 30 min at 55 °C, but after 60 min at 55 °C, no inoculated pathogenic bacteria could be found. The reduction of the indicator bacteria at 55 °C and 70 °C is presented in Table 1. The number of *C. perfringens* (mean of 4.8 log_{10} in the original substrate) was not significantly (Students' *T*-test; $P < 0.05$) affected by heat treatment (mean 4.4 log_{10} after 60 min at 70 °C).

3.2. Viruses

PPV or SVDV could not be detected in the original substrate from the BGP. Virus titres before and titre reductions after treatment at 55 °C and 70 °C are presented in Table 2. PPV was reduced by a mean of 3.2 (min 3.0 and max 3.3) log_{10} units after 60 min at 70 °C. SVDV was inactivated to below the detection level after 30 min at 70 °C, which gave a mean reduction of $\geqslant 6.1$ (min $\geqslant 5.6$ and max $\geqslant 6.4$) log_{10} units. For the three SVDV experiments at 70 °C, the viral interference studies showed that the lowest dilution of the treated substrate that could be used for the titre reduction calculations was 1.7, 1.8 and 2.4 log_{10} $TCID_{50}$ g^{-1}, respectively, due to somewhat different cytotoxicity and viral interference of the treated substrates. Thus, the mean detection limit concerning SVDV for the substrate was found to be 2.0 log_{10} $TCID_{50}$ g^{-1}, based on the results from the viral interference study.

Table 1
Content of indigenous indicator bacteria in the substrate before and after 30 min and 60 min (pre-heating time 14–20 min) in 55 °C and 70 °C, respectively

Bacteria	Before heat treatment ($n = 6$)[a]		55 °C 30 min ($n = 3$)		55 °C 60 min ($n = 3$)		70 °C 30 min ($n = 3$)		70 °C 60 min ($n = 3$)	
	min	max	min	max	min	max	min	max	min	max
Enterococci	4.1	5.4	0	3.1	0	3.7	0	0	0	0
Coliforms 37 °C	4.3	5.6	0	2.7	0	0	0	0	0	0
Coliforms 44 °C	4.1	5.4	0	2.9	0	0	0	0	0	0
Presumptive *E. coli*	4.1	5.4	0	2.7	0	0	0	0	0	0
C. perfringens	3.9	5.4	4.0	4.9	3.9	5.0	4.0	4.4	4.0	4.6

The figures ($\log_{10}$ cfu g^{-1}) represent the minimum and maximum amount of bacteria from the results of the three trials (n = number of samples).
[a] Includes samples from both 55 °C and 70 °C trials.

Table 2
Reduction of PPV, SVDV and *Salmonella* phage 28b (Ph 28b) after heat treatment

Virus	Before heat treatment ($n = 6$)		55 °C 30 min ($n = 3$)		55 °C 60 min ($n = 3$)		70 °C 30 min ($n = 3$)		70 °C 60 min ($n = 3$)	
	min	max	min	max	min	max	min	max	min	max
PPV	6.4	7.6	0.7	1.5	1.0	2.1	2.2	3.1	3.0	3.3
SVDV	7.3	8.8	1.1	1.6	1.5	2.1	$\geqslant 5.6$[a]	$\geqslant 6.4$[a]	$\geqslant 5.6$[a]	$\geqslant 6.4$[a]
Ph 28b	7.8	7.9	nd	nd	nd	nd	0.0	0.1	0.2	0.2

Initial content of PPV, SVDV and Ph 28b in the spiked substrate and reductions after 30 min and 60 min (pre-heating time 14–20 min) at 55 °C or 70 °C are presented. The figures for spiked substrate (virus $\log_{10}$ $TCID_{50}$ g^{-1} and phage $\log_{10}$ pfu g^{-1}) represent the minimum and maximum initial amount of viruses and phage and include spiked samples from both 55 °C and 70 °C trials ($n = 6$). The figures after each heating step represent the minimum and maximum $\log_{10}$ reductions from the three trials (n = number of samples). nd = not determined.
[a] Virus titre fell below detection limit.

As regards the control samples in substrate, a mean reduction of $0.5\log_{10}$ units for PPV and of $0.2\log_{10}$ units for SVDV was observed. This reflects the inactivating capacity of the substrates from the BGP, as the controls in cell-culture medium were stable (results not shown).

3.3. Salmonella phage 28B

According to a check performed three times, in which substrate without added phages was analysed, no phage was present in the substrate to interfere with the *Salmonella* serovar *typhimurium* type 5 host bacteria. No reduction of *Salmonella* phage 28B was observed after treatment at 70 °C for 30 min. After 60 min at 70 °C, the *Salmonella* phage 28B had not significantly decreased (Students' *T*-test, $P < 0.05$) from a mean of $7.9\log_{10}$ PFU g^{-1} before heat treatment to a mean of $7.7\log_{10}$ PFU g^{-1} after heat treatment.

3.4. A. suum

No developed larvae of *A. suum* could be detected in the samples treated for 15 min, 30 min or 60 min at 55 °C and at 70 °C. The development of larvae in the control samples kept at approx 20 °C in 0.1 M H_2SO_4 was 88.5%. The development of larvae was not affected in the unpasteurised control in the substrate.

4. Discussion

Pasteurisation at 70 °C for 30 min and 60 min is enough to ensure a hygienically safe product, as regards the *A. suum* eggs and the bacteria included in this study, except for *C. perfringens*. However, *Salmonella* phage 28B was scarcely affected, and PPV was not reduced sufficiently at 70 °C for 60 min. Carrington (2001) reports an average level of 10^4 g^{-1} enteroviruses in raw sewage sludge. Based on those figures, Carrington (2001) suggests a $4\log_{10}$ reduction of virus, which would ensure virus reduction to undetectable levels, hence a sufficient hygienic level of sewage sludge. In a similar study, Lund et al. (1996) demonstrated a reduction in PPV of $1.4\log_{10}$ units after a 60 min pasteurisation at 70 °C and these authors opposed the requirement of a $4\log_{10}$ reduction as too high for such a heat-resistant virus as PPV. However, in the present study, PPV reduction reached a minimum of $3.0\log_{10}$ units after 60 min pasteurisation at 70 °C. Depending on the initial amount of virus in the substrate, a reduction of $3\log_{10}$ may be too low to achieve an adequate hygienic level in the digested residue, according to Carrington (2001). However, the EU Commission has now proposed a reduction of $3\log_{10}$ units for validation of other processes that could replace the 60 min pasteurisation at 70 °C (SANCO/2632 and 2634/2005) for ABPs and manure.

However, in contrast to bacteria, viruses are unable to grow and increase in number outside their host. One can only speculate about the possible amount of virus in substrate in a BGP. Taking into account the new regulation (EC 1774/2002), which divides ABPs into three different categories with regard to the risk of contamination, the possibility of high virus quantities in Category 3 material, which is used as substrate at BGPs, should be low. However, there is still a risk of subclinically infected animals.

For example, PPV infection is widespread in the swine population and the virus is excreted in the faeces (Klingeborn, 2004). In addition, concern about cross-contamination or other residual infectivity in Category 3 material was recently addressed by Böhm (2004). His results show that bovine parvovirus in slurry only reaches a mean reduction of 2.6 $\log_{10}$ after 60 min heat treatment at 70 °C, which indicates that also other heat stable viruses such as circo- and caliciviruses would survive.

Owing to their properties of being easy and safe to analyse and handle, bacteriophages ought to be excellent indicators of viral contamination. *Salmonella* phage 28B was almost unaffected by treatment at 70 °C for 60 min. Compared with the reduction in the two other viruses (SVDV and PPV), *Salmonella* phage 28B proved considerably more resistant to heat. It might therefore be too conservative an indicator organism for evaluating a one-hour pasteurisation process at 70 °C. For prolonged processes such as composting, however, *Salmonella* phage 28B can be used as an indicator for human and animal viruses (Eller, 1995). For shorter processes, other less heat-resistant bacteriophages could be more suitable (Moce-Llivina et al., 2003).

In agreement with findings by Feachem et al. (1983), *A. suum* eggs were inactivated after 15 min at 55 °C. However, heat treatment for 30 min and 60 min at 55 °C did not ensure inactivation of all indicator bacteria, viruses and the phage. Even some pathogenic bacteria, such as *E. coli* O157 and *S. typhimurium*, survived the heat treatment at 55 °C for 30 min. Amongst indicator bacteria, enterococci are particularly resistant to heat, as demonstrated earlier by for example Kearns et al. (1995).

Owing to their spore-forming capacity, *Clostridium* spp. are extremely heat-resistant and consequently require sterilisation (130 °C at 3 bar for 20 min) to be inactivated. Such a treatment is an economically unreasonable requirement for commercial BGP in practice. In this study, *C. perfringens* was analysed as an indicator for other *Clostridium* spp. that cause severe disease amongst cattle. Moreover, *Clostridium tyrobutyricum* can cause problems in the dairy industry, e.g. late blowing of cheese (Dasgupta and Hull, 1989). The present study indicates that problems with spore-forming clostridia may increase if *Clostridium* spp. are spread through digested residue on grassland grazed by dairy cows. Such application is prohibited 3 weeks before grazing according to Article 22:1c in EU regulation 1774/2002, if ABPs are included in the substrate used at the BGP. However, bacterial spores are able to survive far longer than 3 weeks. The risk with such use of digested residue could be a spread of spore-forming bacteria causing diseases as blackleg, botulism, tetanus and anthrax to areas still free from these diseases.

In this study, the effect of pasteurisation in full-scale BGPs under controlled laboratory conditions was evaluated. The substrate used was obtained from a full-scale BGP and the aim was to mimic the full-scale conditions as closely as possible. As described previously, the content of the substrate may have varied somewhat, as the substrate in full-scale BGPs tends to vary according to availability and season. Despite that, the pasteurisation must in all circumstances fulfil the need for adequate hygienisation of the substrate. The results from the three different trials performed gave similar results, which demonstrate that pasteurisation works as expected at 70 °C, but it does not always fulfil the criteria to reduce all pathogens at 55 °C.

As mentioned, EU regulation 1774/2002 is proposed to be amended by SANCO/2632 and 2634/2005, which allow other processes demonstrated to achieve the same minimised risk as 70 °C for 60 min to be used in treatment of ABPs and manure. The method used here to evaluate pasteurisation could also be used as one way to evaluate other combinations of time and temperature before such methods can eventually be accepted for treatment of Category 3 material according to the imminent EC-regulation (2006).

Ward et al. (1999) showed that *Salmonella* spp. and faecal coliforms in thoroughly pasteurised substrate were not able to regrow within the solids if the substrate was not re-contaminated from the surroundings. The heat-up time in this laboratory study mimics the time in the heat-exchanger in a full-scale biogas plant. However, there are other differences between a full-scale BGP and the almost ideal conditions in a laboratory study. Achieving a uniform temperature level in the whole substrate at full-scale might not be as easy. This was also evident in the present laboratory-scale study, because uneven temperatures were observed within the bottles during heating of the substrate. Safety margins should therefore be considered, and 60 min pasteurisation at 70 °C would be the minimum acceptable. Our results still raises some concerns regarding the use of digested residue from BGPs in agriculture.

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III

Diversity of spore forming bacteria in cattle manure, slaughterhouse waste and substrates from biogas plants

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Abstract

Biogas, derived from digestion of biowaste, is a renewable source of energy. In addition to the production of biogas, digested residues are rich in plant nutrients and can be used as fertiliser. Biowaste contain various types of pathogenic micro-organisms and, therefore, the recommended treatment is pasteurisation at 70°C for 60 min. Pasteurisation reduces levels of pathogens such as *Salmonella* spp., but spore-forming bacteria levels (*Bacillus* spp. and *Clostridium* spp.) are not reduced. Most spore-forming bacteria are harmless inhabitants of the gut or in soil. However, some of them cause dreaded diseases, *e.g.* blackleg, botulism and anthrax. Spore-forming bacteria occurring in biowaste are contaminating the substrate used in biogas plants. In this study, the influence of the biogas process on *Bacillus* spp. and *Clostridium* spp. was investigated.

Ninety-seven faecal samples collected from healthy cattle, 20 samples from waste intended for biogas production from slaughterhouses and 60 samples from different stages in the biogas process were analysed. *Bacillus* spp. and *Clostridium* spp. were quantified and the various species were counted and subcultured. The isolates were identified by biochemical methods and by 16S rRNA sequencing. Phylogenetic trees were constructed from the sequences obtained from *Bacillus* spp. and *Clostridium* spp. found in manure, slaughterhouse waste and biogas plant substrates.

The most common *Clostridium* spp. found in biowaste and in biogas plant substrates was *C. perfringens*. *Clostridium botulinum* and *Clostridium sordellii* were found before and after pasteurisation, but not after anaerobic digestion. The most common *Bacillus* spp. were *Bacillus pumilus* and *Bacillus subtilis*. Some of the isolated strains were probably representing new members of the genera *Clostridium* and *Bacillus*. The number of species of clostridia decreased after digestion while *Bacillus* spp. remained constant. Some pathogenic spore-forming bacteria from biowaste can pass unaffected through biogas plants, but the results in this study indicate that levels of pathogenic clostridia are reduced in the digester.

Keywords: biogas, manure, phylogenetic trees, slaughter house waste, 16S rRNA.

1 Introduction

Biogas is a renewable source of energy, derived from digestion of biowaste, such as manure, food- and slaughterhouse waste. In addition to the production of biogas, digested residues can be used as fertiliser. Re-cycling of biowaste nutrients from urban areas, back to food production on arable land, is a prerequisite for tenable farming in the future. Digested residues from biogas plants are rich in plant nutrients and improve physical and chemical properties of the soil. Digested residues applied as fertiliser on arable land, reduces the need for artificial fertilisers.

However, biowaste intended for biogas plants contain various types of biological contaminants and pathogenic micro-organisms. Before digested residues are recycled by spreading on arable land, biowaste must be adequately treated and proven hygienically safe. To reduce the risk of spreading pathogens, pasteurisation at 70°C for 60 min of biowaste is recommended before anaerobic digestion. If manure or animal by-products are present in the substrate, a pasteurisation stage or other treatment, with equivalent effect regarding the reduction of pathogens have to precede anaerobic digestion. This is regulated in European Commission regulation EC nos. 1774/2002 and 208/2006. Pasteurisation is an effective way to reduce most pathogens, such as *Salmonella* spp., and indicator bacteria

(Bendixen, 1996; Bendixen, 1999; Bagge *et al.*, 2006; Sahlström *et al.*, 2008). However, spore-forming bacteria (*e.g. Bacillus* spp. and *Clostridium* spp.) are not reduced and can persist unaffected after pasteurisation and digestion (Olsen and Larsen, 87; Larsen *et al.*, 1994; Larsen, 1995; Bagge *et al.*, 2006; Sahlström *et al.*, 2008). Due to their survival during the biogas process, it is important to investigate the risk of spreading spore-forming bacteria by digested residues from biogas plants and to take the necessary measures.

Several kinds of spore-forming bacteria are harmless or even essential inhabitants in the gut of animal species (Timoney *et al.*, 1988; Gyles and Thoen, 1993). Some spores pass unaffected through the digestive systems. *Clostridium* spp. and *Bacillus* spp. are commonly found in manure from cattle (Larsen *et al.*, 1994; Chauret *et al.*, 1999; Aitken *et al.*, 2005). Pathogenic spore-forming bacteria may also be found in manure and hence in the substrate for biogas plants. Little is known about the activities of various pathogenic spore-forming bacterial species during pasteurisation and digestion.

Spore-forming bacteria grow as vegetative cells under favourable conditions. When growth conditions are poor or nutrient deprivation occurs, the bacteria can sporulate (Labbé and Remi-Shih, 1997). Spores can survive as very resistant dormant spores for many years in soil (Mitscherlich and Marth, 1984; Gyles and Thoen, 1993). When the growth conditions become more favourable, the spores germinate to vegetative cells. The spores are tolerant to heat, disinfectants and desiccation. *Clostridium* spp. only grow under anaerobic conditions (Gyles and Thoen, 1993; Songer and Post, 2005b), whereas Bacillus spp. are aerobic (Quinn *et al.*, 1994a).

Pathogenic spore-forming bacteria of special concern for animal health are *Bacillus anthracis, Clostridium botulinum, Clostridium chauvoei*, *Clostridium haemolyticum, Clostridium perfringens, Clostridium septicum, Clostridium sordellii* and *Clostridium tetani*. All these bacteria are lethal or cause serious clinical diseases in farm animals, which results in suffering for the animals and extra costs for farmers.

Bacillus anthracis causes anthrax, a per-acute, life-threatening, dreaded disease, in animals and humans, especially the pulmonary form after inhalation of *B. anthracis* spores (Bravata *et al.*, 2005; Songer and Post, 2005a). *Bacillus cereus* causes food poisoning in humans (Quinn *et al.*, 1994a) and can rarely cause mastitis in cattle (Schiefer *et al.*, 1976; Turnbull *et al.*, 1979). *Bacillus licheniformis* and *Bacillus pumilus* can cause mastitis and *B. licheniformis* can cause abortions in cattle (Songer and Post, 2005a; Nieminen *et al.*, 2007). In addition to *B. anthracis* and the above mentioned species,

most *Bacillus* spp. are harmless saprophytes, which are commonly found in the environment.

The disease caused by *C. botulinum* is an intoxication, botulism, which arise from ingestion of preformed toxin in food or feed (Timoney *et al.*, 1988; Deprez, 2006). *Clostridium botulinum s*pores and vegetative cells can be found in soil, decaying vegetation and manure (Dahlenborg *et al.*, 2001; Dahlenborg *et al.*, 2003; del Mar Gamboa *et al.*, 2005; Songer and Post, 2005b). In cattle and sheep, *C. chauvoei* causes a disease with high mortality called blackleg (Sternberg *et al.*, 1999; Songer and Post, 2005b). *Clostridium chauvoei* can be present in soil and faeces (Gyles and Thoen, 1993; Hang'ombe *et al.*, 2000; del Mar Gamboa *et al.*, 2005). When a pasture becomes contaminated, the disease can usually occurs annually in susceptible animals (Timoney *et al.*, 1988) grazing the same pasture. *Clostridium perfringens* causes many different diseases in animals and humans depending on toxin types produced (Gyles and Thoen, 1993; Quinn *et al.*, 1994b; Songer and Post, 2005b). In humans, food poisoning is common. Usually *C. perfringens* is found in the intestines of animals and humans (Songer and Post, 2005b). *Clostridium septicum* causes malignant edema in connection with infected wounds in many animal species (Songer, 1997). *Clostridium sordellii* can cause gas gangrene in infected wounds on cattle, sheep and horses (Quinn *et al.*, 1994b). Both *C. septicum* and *C. sordellii* are common inhabitants in soil and in the intestinal tract of many animals (Timoney *et al.*, 1988; Gyles and Thoen, 1993; Munang'andu *et al.*, 1996; Songer, 2006).

In earlier studies, it has been shown that *Clostridium* spp. and *Bacillus* spp. survived the pasteurisations stage in biogas plants (Olsen and Larsen, 1987; Bagge *et al.*, 2005; Sahlström *et al.*, 2008). When these bacteria are included in biowaste intended for biogas production, there is a risk of spreading these bacteria to new areas via digested residues. To estimate this risk, more knowledge about the bacterial flora in the digested residue is needed.

Standard methods for identification of *Bacillus* spp. and *Clostridium* spp. in samples are based on culture followed by biochemical identification. Biochemical identification methods have limitations for these bacteria. Therefore, phylogenetic analyses based on 16S rRNA gene sequences can be used as a complement to biochemical identification. However, high sequence similarity in the 16S rRNA gene of closely related species makes it difficult to distinguish such species from each other by phylogenetic analysis. *Bacillus anthracis, B. cereus* and *B. thuringiensis*, for instance, have high 16S rRNA sequence similarity (Sacchi *et al.*, 2002).

Both *Bacillus* spp. and *Clostridium* spp. have a Gram-positive structure of the cell wall and are spore-forming bacteria, but phylogenetic analysis based

on 16S rRNA gene has revealed divergence into two evolutionary lineages (Collins *et al.*, 1994; Stackebrandt and Rainey, 1997; Stackebrandt *et al.*, 1999). In phylogenetic analysis it was also demonstrated that the genus *Clostridium* is a very heterogeneous group with 19 distinct clusters. Most of the clostridial species were shown to belong to cluster I. Pathogenic clostridia of concern for animal health (*C. botulinum*, *C. chauvoei*, *C. novy*, *C. perfringens*, *C. septicum* and *C. tetani*), were found to be affiliated to cluster I. Some other pathogenic clostridia of concern for animal health were included in cluster XI, for instance *C. difficile* and *C. sordellii*. *Clostridium botulinum* have been shown to represent a phylogenetically heterogeneous group, present in many subclusters of cluster I and more than one toxin type can be affiliated to one single subcluster. Furthermore, species have been described, that are phylogenetically very close to *C. botulinum*, but they lack the toxin gene, *e.g. Clostridium sporogenes*.

Many bacterial species have multiple 16S rRNA operons, and the homologous constituting genes are not necessarily identical within one strain (Stackebrandt and Rainey, 1997). These sequence differences are known as polymorphism. Members of the genera *Bacillus* and *Clostridium* have unusually large numbers of rRNA operons, which sometimes makes interpreting of the sequence data difficult, particularly if there are also sequences length polymorphisms.

Non pathogenic spore-forming bacteria can be used as indicators and reflect the effect on the more pathogenic ones. Spore-forming bacteria from biowaste survive after pasteurisation and digestion. Therefore, these spore-forming bacteria may pose a hygienic problem, when spreading digested residues as fertiliser.

The aim of this screening study was to identify and quantify species of *Bacillus* spp. and *Clostridium* spp. in manure, slaughterhouse waste and in substrates from different stages in biogas plants process.

2 Materials and methods

2.1 Collection of samples

2.1.1 Farms:

Ninety-seven individual faecal samples were taken from 10 dairy farms, farm A to J, in the county of Uppland in Sweden. Faeces were collected as individual samples from the floor behind each cow. From each farm 8-10

faecal samples were collected at one occasion between January and April 2003. All cattle were healthy at the sampling occasion. The samples were taken by local veterinarians during regular visits. Approximately 100 g of each sample were collected and stored in clean pots.

At the sampling occasion a questionnaire was filled in concerning feeding, health, manure spreading practice, size of the farm and grazing routines. The biggest farm housed between 100-120 cows and around 200 heifers. The smallest farm housed 12 cows and 5 heifers (Table 1).

2.1.2 Slaughterhouses:

Two slaughterhouses, U and K, took samples from waste intended for biogas production once a day during ten days. The slaughterhouse U was located close to the farms included in this study. The samples from this slaughterhouse were fetched daily by employees at the National Veterinary Institute (SVA). Samples from slaughterhouse K were daily sent by mail to SVA. The samples were taken by the employees at the slaughterhouse. Approximately 100 g of each sample were collected and stored in clean pots.

2.1.3 Biogas plants:

Samples were collected from two biogas plants, K and L. From both biogas plants, samples were taken from the homogenisation tank (before pasteurisation, BP), after pasteurisation (AP) and after digestion (AD), once a week during ten weeks. The employees at the biogas plants carried out the sampling. The samples were without delay sent by mail to SVA. Approximately 100 g of each sample were collected and stored in clean pots.

The slaughterhouse K was located close to the biogas plant K. This slaughterhouse sent the animal by-products to biogas plants K. Hydrochloric acid (HCl) was added to the digester of biogas plant L to increase the gas production, but the amount was not available.

2.2 Culture

2.2.1 Quantitative methods

The samples were analysed immediate after arrival at the laboratory. Ten gram of substrate (faeces, slaughterhouse waste or biogas plant material) were placed in a bottle and heated at 65°C for 10 min. Quantitative analyses were made after performing 10-fold dilution series in peptone saline solution (0.9%).

The detection level was based on methods from the Nordic Committee on Food Analysis (NMKL, 91:3:2001), and for *Clostridium* spp. on Tryptose

Sulphite Cycloserine agar plates (TSC, perfringens agar base, Oxoid, Basingstoke, England) the level was reported to be 10 cfu/mL. The detection levels for *Clostridium* spp. on Fastidious Anaerobic Agar plates (FAA, LabM, Bury, Lancashire, England), with 5% defibrinated horse blood, and for *Bacillus* spp. on horse blood agar plates, were reported to be 100 cfu/mL.

2.2.2 Clostridium spp.

For analysis of *Clostridium* spp. the samples were cultured on TSC and FAA. One millilitre from different steps in the 10-fold dilution series was mixed with melted TSC and 0.1 mL was streaked onto FAA plates. All agar plates were incubated at 37°C for 24 h in anaerobic jars (ANAEROgen®, Oxoid, Basingstoke, Hampshire, England). Following incubation different species of *Clostridium* spp. were quantified and subcultured.

Suspected colonies of *C. perfringens* were counted and from each sample, 10 colonies were subcultured on egg yolk agar and horse blood agar and incubated in anaerobic jars at 37°C for 24 h. From each sample, two *C. perfringens* isolates (lecithinase positive and with haemolysis), were stored at -20°C.

Suspected colonies of *Clostridium* spp. other than *C. perfringens* were counted and subcultured onto horse blood agar. Following anaerobic incubation at 37°C for 24 h, the isolated bacteria were Gram-stained. *Clostridium* spp. were identified as Gram-positive rods. The identification of clostridia was based on fermentation of glucose, maltose, lactose, sucrose, starch, mannitol and fructose, production of lecithinase, tryptophanase, urease and hydrolysis of aesculin. Other strains suspected to represent *Clostridium* spp. were identified by 16S rRNA sequencing. All isolates in pure culture were stored at -20°C.

2.2.3 Bacillus spp.

For analysis of *Bacillus* spp., 0.1 mL from different steps in the 10-fold dilution series was cultured on horse blood agar incubated at 30°C for 24 h. Following incubation, different species of *Bacillus* spp. were counted and subcultured.

Suspected colonies of *Bacillus* spp. were counted and subcultured on horse blood agar. *Bacillus cereus* was confirmed by culture on Mossel Cereus Selective agar (MCS, Merck, Darmstadt, Germany) at 37°C for 24 h. Other strains of *Bacillus* spp. were analysed by Gram staining, catalase test, Voges-Proskauer (VP) test, and production of lecithinase and tryptophanase, fermentation of glucose, arabinose, mannitol and citrate and reduction of

nitrate. *Bacillus* spp. were identified as catalase positive, Gram-positive rods. Other strains suspected to represent *Bacillus* spp. were identifying by 16S rRNA sequencing. All isolates in pure culture were stored at -20°C.

2.3 16S rRNA sequencing

2.3.1 DNA preparation

One loopful (approximately 5 μL of bacteria collected with a 10 μL loop) of colony material was suspended in 500 μL of phosphate-buffered saline (PBS, Merck, Darmstadt, Germany) and centrifuged for 10 min at 7,200 x g. The supernatant was discarded and the pellet was washed again by the same procedure. The pellet was re-suspended in 250 μL of double distilled H_2O. The bacterial cells were lysed by boiling the suspension for 10 min before quickly cooling on ice and then stored at -20°C until further analysis.

2.3.2 Amplification

A segment corresponding to about 90-95% of the 16S rRNA genes of *Clostridium* spp. and *Bacillus* spp. was amplified with universal primers (RIT593 and kag-001) designed for members of the phylum *Firmicutes* (Johansson *et al.*, 1998; Johansson *et al.*, 2006). The PCR mixture (50 μL total volume) comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs (Applied Biosystems, Foster City, CA, USA), 10 pmol of each oligonucleotide primer, 1 U AmpliTaq DNA polymerase (Applied Biosystems) and 1 μL of template DNA. The template DNA was used at two concentrations, undiluted and diluted 10^{-1}. The amplification program for *Bacillus* spp. consisted of an initial denaturation at 96°C for 20 s followed by 30 cycles of denaturation at 96°C for 20 s, primer annealing at 55°C for 35 s, extension at 72°C for 45 s and a last extension at 72°C for 2 min. The amplification program for *Clostridium* spp. consisted of 30 cycles of denaturation at 96°C for 32 s, primer annealing and extension at 60°C for 2.15 min, and a last extension at 72°C for 4 min.

The PCR experiments were performed in 200 μL tubes with individual lids in a MJ research Peltier Thermal Cycler (PTC 200) (MJ Research Inc, Watertown, Massachusetts, USA). The PCR products were separated by electrophoresis in 1.5% (w/v) agarose gel (Agarose NA, GE Healthcare, Uppsala, Sweden), stained with ethidium bromide and visualized by ultraviolet transillumination. *Mycoplasma capricolum* subsp. *capricolum* (Calif. Kid^T) was used as positive PCR control. A PCR mixture with water instead of template was used as negative PCR control.

2.3.3 Sequencing of the 16S rRNA gene

Amplicons of 50 strains of *Clostridium* spp. and 51 strains of *Bacillus* spp. that could not be typed by standard methods were analysed by 16S rRNA gene sequencing. The amplicons were purified prior to sequencing by using the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience Europe, GmbH, Freiburg, Germany).

The purified amplicons were sequenced with previously described forward (RIT583, RIT584, RIT538, kag-006) and reverse (RIT631, kag-011, kag-002) primers (Johansson *et al.*, 1998; Johansson *et al.*, 2006). The amplicons were diluted and cycle sequencing reactions were carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Foster City, CA, USA) in combination with ethanol/EDTA/sodium acetate precipitation, according to the protocol of the manufacturer. Thermocycling was performed in a GeneAmp 2700 Thermocycler (Applied Biosystems), which resulted in seven overlapping DNA fragments. These fragments were subjected to electrophoretic separation and on-line detection on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The resulting sequences were assembled into a contig corresponding to about 90-95% of the 16S rRNA gene by using the program ContigExpress, Vector NTI suite ver. 9.0. Sequence similarity searches were done in GenBank (Benson *et al.*, 2007). The 16S rRNA gene sequence of *Clostridium* spp. and *Bacillus* spp. found in manure, slaughterhouse waste and biogas plant substrates were deposited in GenBank under the accession numbers listed in Table 2.

2.3.4 Phylogenetic analyses

For construction of the 16S rRNA based phylogenetic tree, sequences obtained from this work were aligned manually with sequences retrieved from GenBank and prealigned sequences retrieved from the Ribosomal Database Project II (RDP-II, Cole *et al.*, 2005). The sequences retrieved from GenBank and RDP-II were selected from the high score list obtained from the sequence similarity searches. The phylogenetic relationships among the isolates and the sequences retrieved from GenBank and RDP-II were calculated by Neighbour-Joining.

The GenBank accession numbers of the 16S rRNA gene sequences of the type and reference strains from *Clostridium* spp. and *Bacillus* spp. used for construction of phylogenetic trees are presented in Table 3 and Figure 1. *Escherichia coli* with accession number J01695 and *Corynebacterium ulcerans* NCTC 7910^{T} with accession number X84256 were used as outgroups for constructing phylogenetic trees for all *Clostridium* spp. and *Bacillus* spp isolates used in this study.

3 Results

All species of *Bacillus* spp. and *Clostridium* spp. found in cattle manure, slaughterhouse waste and samples from biogas plants are presented in Table 4. The quantities of *Bacillus* spp. and *Clostridium* spp. are presented in Figures 2-3.

3.1 Clostridium spp.

The most common *Clostridium* sp. found in manure, slaughterhouse waste and samples from different stages in the biogas process was *C. perfringens*.

Clostridium botulinum was found before and after pasteurisation but not after anaerobic digestion in biogas plant K (Table 4). After pasteurisation in plant K, *C. sporogenes/C. botulinum* was found. These two clostridia are difficult to distinguish by phylogenetic analysis based on 16S rRNA sequencing due to high sequence similarity.

Clostridium sordellii was found in cattle manure, slaughterhouse waste and before pasteurisation and remains after pasteurisation, but not after digestion (Table 4). Phylogenetically, all *C. sordellii* strains found in this studies formed a homogeneous group, in spite of different habitats (Fig. 6). *Clostridium septicum* was found at one occasion in slaughterhouse waste (Table 4). *Clostridium tyrobutyricum* was found before pasteurisation in biogas plant K and *Clostridium butyricum* was found in 5 cattle from 3 farms.

Strains SH-C1, SH-C52, BG-C36, BG-C122 and BG-C151 were isolated at different occasions and in different substrates, but they showed high sequence similarity to each other and to the uncultured bacterial DNA clone P-2117-S959-2 (accession number AF371837, Leser *et al.*, 2002) (Fig. 5). The sequence of P-2117-S959-2 was found among the clones from the gastrointestinal tract of pigs where DNA was extracted from intestinal luminal contents without previous culture. Closest relatives to SH-C10 were *Clostridium baratii*, *Clostridium sardiniense* or an uncultured bacterial DNA clone P-2171-s959-3 (not show in the phylogenetic tree) (accession number AF371841, Leser *et al.*, 2002). However, strain SH-C10 is probably a new member of the genus *Clostridium* (Fig. 5). A close relative to BG-C66 is an uncultured bacterial DNA clone aab17c11 (accession number DQ816381), found among the clones from a caecum sample of a mouse (Rawls *et al.*, 2006) (Fig. 5). The closest relative to BG-C95 is *Clostridium sartagoforme*, the closest relatives to BG-C51 is *Clostridium thiosulfatireducens* and *Clostridium subterminale*. CM-C81 is probably representing a new member of the genus *Clostridium* (Fig. 5).

Strain BG-C4 showed sequence similarity to uncultured bacterium F36 (accession number DQ232855) found among the clones from anaerobic sludge (Kim *et al.,* unpublished data) (Fig. 6). Some of the other clostridia strains are probably also representing new members of the genus *Clostridium*. The closest relative to CM-C52 is *Clostridium glycolicum* and the closest relatives to CM-C99 are *Clostridium xylanolyticum* and *Clostridium aerotolerans* and the closest relative to BG-C130 and BG-C131 *Clostridium limosum* (Fig. 6).

3.2 Bacillus

The 16S rRNA gene sequences of *Bacillus* spp. were sometimes shorter (90-95% of the complete gene) than for *Clostridium* spp., which seemed to be caused by a sequence length polymorphism close to the 5´-end.

The most common *Bacillus* spp. found in manure, slaughterhouse waste, before and after pasteurisation and after digestion were *B. cereus, B. subtilis* and *B. pumilus*.

Bacillus pumilus strains CM-B53, CM-B54, CM-B84, CMB93, SH-B30 and BG-B50 were phylogenitcially similar to each other in spite of the fact that they were found in different substrates and at different sampling occassions (Table 2, Fig. 7). The same situation was observed for *B. subtilis* strains CM-B55, CM-B82, CM-B92, SH-B1, SH-B13, BG-B2, BG-B7, BG-B28 and BG-B38 (Table 2, Fig. 7).

Bacillus subtilis is closely related to *B. velezensis* (also referred to as *B. amyloliquefaciens*), and strains may be difficult to identify by 16S rRNA sequencing, such as SH-B29 (Fig. 7). CM-B72 is probably representing *Bacillus weihenstephanensis* or *Bacillus mycoides* (Fig. 8).

Some of the sequenced *Bacillus* spp. strains could not be exactly identified and some of them probably represent new members of the genus *Bacillus*. In one cluster of *Bacillus* spp. SH-B7, SH-B27 and BG-B27 are located. The closest relative to SH-B7 is *Bacillus flexus* and for BG-B27 *Bacillus firmus* is the first alternative and *B. flexus* the second. The closest relative to SH-B27 is *"Bacillus pichinotyi"*, but this species has so far no standing in nomenclature. CM-B91 probably represents a new member of the genus *Bacillus* or *Lysinobacillus*. The closest relative to this strain is *L. sphaericus*. Both BG-B18 and BG-B109 are in the same cluster as *Bacillus fordii*.

Paenibacillus amylolyticus, *Paenibacillus polymyxa* and *Lysinobacillus sphaericus* were detected frequently in biowaste and biogas plants samples (Tables 2 and 4).

3.3 Number of spore-forming bacteria

The number of species of *Bacillus* spp. in samples from manure, slaughterhouse waste and samples taken from different stages in the biogas process seemed to be nearly the same (Table 4). Also the quantities of *Bacillus* spp. seemed to be similar in samples from slaughterhouse and different stages in the biogas process, but in manure samples the quantity of *Bacillus* spp. differ (Fig. 2).

The number of species of clostridia decreased in samples taken after digestion compared to the number of species in other samples (Table 4, Fig. 3). Also the quantities of clostridia decreased during the biogas process, especially in biogas plant L (Fig. 4).

3.4 The farms

The quantity of bacteria and number of species of spore forming bacteria in manure samples from individual cattle, were approximately the same at each farm, but differed between the farms. Of the 97 cattle in this study, 34% were without *Clostridium* spp. in their faeces and 49% were without *Bacillus* spp. It may depend of the fodder, and no correlation can be found between these farms as judged from the questionnaire.

4 Discussion

The phylogeny of the genus *Clostridium* based on 16S rRNA gene sequences has been previously described in detail (Collins *et al.*, 1994; Stackebrandt and Rainey, 1997). The genus is a heterogeneous group, which comprises about 170 species. The strains found in this study represent about 42 species (Table 3 and 5) and their phylogeny is shown in Figures 5-6.

The genus bacillus comprises about 150 species. The strains of *Bacillus* spp. found in this study represent about 17 species, *Lysinobacillus* spp. 2 species and *Paenibacillus* spp. 2 species (Table 3 and 5 and Figures 7-8).

4.1 Clostridium spp.

Clostridium botulinum and *C. sporogenes/C. botulinum* were found before and after pasteurisation but not after anaerobic digestion. The environment in the digester may inhibit growth of *C. botulinum*, but more studies are needed.

Clostridium sordellii and *C. septicum* are common inhabitants in the intestinal tract of many animal species (Timoney *et al.*, 1988; Gyles and Thoen, 1993; Munang'andu *et al.*, 1996; del Mar Gamboa *et al.*, 2005) and reach biogas plants via biowaste. In this study probably *C. sordellii* in the manure samples only pass through the intestinal tract in the cattle since they were healthy. *Clostridium sordellii* found in maure, slaughterhouse waste, before and after pasteurisation represented a phylogentically homogeneous group.

If *C. tyrobutyricum* is present in cheese, it can produce butyric acid, which is a problem for cheese production in dairies. The so called "late blowing" of hard cheese cause economic losses in dairy industries (Dasgupta and Hull, 1989). In this study *C. tyrobutyricum* was found only before pasteurisation. Jo *et al.* (2008) showed that spores levels of *C. tyrobutyricum* are not reduced by anaerobic digestion. Occurrence of C. *tyrobutyricum* in digested residues used as fertiliser may contaminate fodder or silage. Spores in consumed fodder are excreted in the dung and subsequently in the milk. In similar ways, *C. butyricum* can disturb cheese production and this bacterium was found at more than one occasion in the manure samples.

4.2 Bacillus

Bacillus pumilus was a common isolate in all investigated substrates and most of the islolates were identified by sequencing. However, it is difficult to distinguish between *B. pumilus* and *B. safensis* by 16S rRNA sequencing. As *B. safensis* has only been found in very special environments like spacecraft surfaces and assembly-facility surfaces at the laboratory in Kennedy Space Center in Florida (Satomi *et al.*, 2006), therefore, our isolates probable represents *B. pumilus*.

The strains of *B. pumilus* named CM-B53, CM-B54, CM-B84, CM-B93 in manure were derived from different farms and individuals. *Bacillus pumilus* named SH-B30 was found in waste from slaughterhouse K, that sent animal by-products to biogas plant K, where BG-B50 was found before pasteurisation (Table 2 and 4). All these *B. pumilus* strains were phylogentically similar to each other (Fig. 7). In spite of the fact that they were found in different substrates and at different samplings this observation demonstrates a high similarity in the 16S rRNA gene of this bacterium.

The strains of *B. subtilis* named CM-B55, CM-B82 and CM-B92 in manure were derived from different farms. *Bacillus subtilis* named SH-B1 and SH-B13 were both from the same slaughterhouse, but sampled at different times, likewise BG-B28 and BG-B38, which were both collected before

pasteurisation at biogas plant L, but at different sampling times. BG-B2 was sampled before pasteurisation at biogas plant K and BG-B7 after digestion at the same plant.

Bacillus cereus found in cattle manure, slaughterhouse waste and biogas plant substrate are normally easily identified by biochemical methods. Strains of *B. cereus* with deviating biochemistry are difficult to identify by 16S rRNA sequencing because of the similarity to *B. antrhacis* and *B. thuringiensis* (Sacchi *et al*., 2002).

Paenibacillus polymyxa and *Paenibacillus amylolyticus* were detected frequently in biowaste and biogas plants samples. These species are quite harmless, but a close relative, *Paenibacillus larvae*, causes American foulbrood in honeybees (Genersch, 2007). The previous name for *P. polymyxa* was *Bacillus polymyxa*. The Family *Lysinobacillaceae* is also a new taxon of the order *Bacillales*. *Lysinobacillus sphaericus*, which was previously called *Bacillus sphaericus*, was frequently found in substrates from the biogas plants.

4.3 Number of spore-forming bacteria

Both the number of species of *Bacillus* spp. and the quantity indicates that *Bacillus* spp. seemed to pass through the biogas process from biowaste to digested residues relatively unaffected (Table 4, Fig. 2). The quantities of *Bacillus* spp. in manure differ between the farms (Fig. 2).

In contrast, *Clostridium* spp. seemed to be more sensitive for pasteurisation and digestion. The quantities of clostridia decrease through the biogas process, especially in biogas plant L. In plant L a decrease could be shown after pasteurisation and after digestion. The addition of HCl to the digester in plant L may influence the quantity of clostridia, but it could not explain the reduction after pasteurisation. The differences in quantity of clostridia between biogas plant K and L can be difficult to explain, but may depend on the composition of used substrates and/or differences in the biogas process between the biogas plants (Fig. 4).

4.4 The farms

At some farms the quantities of spore-forming bacteria in manure samples were high in most of the cattle and in other farms low (Figs. 2 and 3). An explanation could not be deduced from the results of the questionnaire. The composition of the gut flora may depend on the fodder quality. The individual flora seemed to be similar within each herd.

Different fodder given to cattle appears to cause the same amount of spore-forming bacteria in the manure samples. Farm G, the only without any silage-use, had about the same amount of spore-forming bacteria in the manure samples as the other farms. Farm I, which did not use hay, had very high levels of *Bacillus* spp. in the manure samples (data not shown). Low quality of fodder has been shown to result in high quantities of *Bacillus* spp. (Scheldeman *et al.*, 2006).

The lengths of the stubble can have an influence on the quantity of spore-forming bacteria in manure, but no differences could be observed on these farms. Half of the farms had short length stubble, 5 cm, and the other half around 8-12 cm (Table 1).

None of the farms in this study spread manure on pasturage, but four farms spread manure on fields for hay and silage. At two of the farms that spread manure on the silage fields, their cattle had only few clostridia in the manure. One farm (A) took manure from other farms for spreading on fields for grain, but they never spread manure on pasturage or fields for silage. The cattle on this farm have high levels of clostridia in the manure samples, but the levels of *Bacillus* spp. were low (Fig. 3). Six farms only have storage as treatment for the manure, farm D was sending the manure to the local biogas plant, farm G used composting as treatment of the manure and farm C treated the manure with lime (Penac®). No significant differences could be shown between these farms.

The results of the questionnaire did not show any differences between the farms correlated to the handling of manure, silage, cow health or the quantities of spore forming bacteria. The hygienic quality of the feed has not been determined, but it may influence the flora of the spore forming bacteria in the gut.

4.5 Risk of spreading pathogenic spore-forming bacteria

The normal gut flora and faeces from most animal species contain various kinds of spore-forming bacteria (Timoney *et al.*, 1988; Gyles and Thoen, 1993), most of them are harmless and some of them are essential. Ingested pathogenic spore-forming bacteria can cause diseases, but can also pass unaffected through the digestive systems of animals. Manure sent to biogas plants may include spores of pathogenic bacteria. As expected from earlier studies, spore-forming bacteria persisted in digested residues from biogas plants (Olsen and Larsen, 1987; Aitken *et al.*, 2005; Bagge *et al.*, 2006). Therefore, spore-forming bacteria can pose a hygiene problem when

digested residues are spread on arable land as fertiliser if spores of pathogenic spore-forming bacteria occur.

The risk of spreading *Bacillus* spp. is higher than that of *Clostridium* spp., as *Bacillus* spp. in manure and slaughterhouse waste seemed to pass unaffected through the biogas process. Fortunately, most *Bacillus* spp. are fairly harmless, except *B. anthracis*. Pathogenic clostridia found in this study, such as *C. botulinum*, *C. septicum* and *C. sordellii*, were not detected after digestion. There are probably differences in the survival of *Clostridium* spp. and *Bacillus* spp. spores. Many factors, such as supply of carbohydrates, pressure, temperature and acid conditions, have influences of the survival (Volkova *et al.*, 1988; Cotter and Hill, 2003; Peleg *et al.*, 2005; Margosch *et al.*, 2006). The amount of *Clostridium* spp. spores may be reduced if the pH is above 12, which has been shown in one study, where low doses of burnt lime (CaO) were added during storage of biowaste (Bujoczek *et al.* 2002). The impact of anaerobic digestion on various kinds of spore-forming bacteria is not fully known, and further studies are needed.

5 Conclusion

Biowaste, such as manure and slaughterhouse waste, contains spore-forming bacteria, including those that can cause serious diseases such as *C. botulinum, C. sordellii* and *C. septicum*. In biogas plants, biowaste is pasteurised and digested. None of the pathogenic clostridia were found after digestion and the number of clostridial species decrease after digestion. This observation indicates that clostridia are more sensitive than *Bacillus* spp. to the process in the digester.

Potentially new members of both genus *Clostridium* and genus *Bacillus* were detected in the screening study of cattle manure, slaughterhouse waste and substrates by biogas plants.

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Tables and Figures

Table 1. *Conditions at farms A-J from which manure samples were obtained for the study.*

Farm:	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
No. cows	54	25	32	100-120	58	92	12	55	20	18
No. young animals	45	55	70-80	200	100	-	5	50-60	10	14
Coarse fodder,	H, S	S	H, S	H, S	H, S	H, S	H	H, S	S	H, S
Length of stubble, cm	8-10	10	12	4-5	10-12	5	5	>10	5	< 5
Bedding	straw	straw	straw	straw, shavings	shavings	straw	straw	straw	straw	straw
Manure	solid	solid	solid	liquid	liquid	liquid	solid	Solid-cows, liquid-heifers	solid	solid
Manure treatment	storage	storage	lime	digestion	storage	storage	compost	storage	storage	-
No. samples, cows	5	5	5	5	5	5	5	5	5	5
No. samples, heifers	5	4	5	5	5	5	5	5	5	3

H = hay
S = silage

Table 2. *Accession number (Acc. No.) for 16S rRNA gene sequences of Bacillus spp., Clostridium spp., Lysinobacillus spp. and Paenibacillus sp. found in manure, slaughterhouse waste and during different stages in the biogas process.*

Strain	Origin	***Bacillus* spp., *Lysinobacillus* spp. and *Paenibacillus* sp.**	Acc. No. in GenBank
CM-B53	Farm A/ 492	*Bacillus pumilus*	EU869221
CM-B54	Farm A/ 506	*Bacillus pumilus*	EU869222
CM-B55	Farm A	*Bacillus subtilis*	EU869223
CM-B68	Farm F	*Bacillus* sp. (probably represents *B. cereus*)	EU869224
CM-B72	Farm H	*Bacillus* sp. (probably represents *B. weihenstephanensis* or *B. mycoides*)	EU869225
CM-B77	Farm D	*Bacillus subtilis*	EU869226
CM-B82	Farm I	*Bacillus subtilis*	EU869227
CM-B84	Farm I	*Bacillus pumilus*	EU869228
CM-B91	Farm E	*Bacillus* sp. (probably represents a new member of the genus *Bacillus*)	EU869229
CM-B92	Farm E	*Bacillus subtilis*	EU869230
CM-B93	Farm E	*Bacillus pumilus*	EU869231
CM-B94	Farm J	*Bacillus subtilis*	EU869232
BG-B1	K BP 1	*Bacillus* sp. (probably represents *B. cereus*)	EU869246
BG-B2	K BP 1	*Bacillus subtilis*	EU869247
BG-B7	K AD 1	*Bacillus subtilis*	EU869248
BG-B11	L BP 1	*Bacillus licheniformis*	EU869249
BG-B15	L AP 1	*Bacillus clausii*	EU869250
BG-B18	L AP 1	*Bacillus* sp.	EU869251
BG-B24	K BP 2	*Bacillus pumilus*	EU869252
BG-B26	K AD 2	*Bacillus pumilus*	EU869253
BG-B27	L BP 2	*Bacillus* sp.	EU869254
BG-B28	L BP 2	*Bacillus subtilis*	EU869255
BG-B29	L BP 2	*Bacillus* sp. (probably represents *B. cereus*)	EU869256
BG-B38	L BP 3	*B. subtilis*	EU869257
BG-B44	K BP 4	*Lysinobacillus sphaericus*	EU869258
BG-B50	K BP 5	*Bacillus pumilus*	EU869259
BG-B57	K AD 5	*Bacillus subtilis*	EU869260
BG-B62	L AD 5	*Bacillus megaterium*	EU869261
BG-B63	L AD 5	*Bacillus licheniformis*	EU869262
BG-B79	K BP 7	*Bacillus pumilus*	EU869263
BG-B81	K AP 7	*Bacillus subtilis*	EU869264
BG-B109	L AP 10	*Bacillus* sp.	EU869265
BG-B111	L AD 10	*Lysinobacillus sphaericus*	EU869266
BG-B112	K BP 4	*Lysinobacillus* sp. (probably represents *L. fusiformis* or *L. sphaericus*)	EU869267
SH-B1	U 1	*Bacillus subtilis*	FJ549006
SH-B2	U 1	*Bacillus licheniformis*	FJ549007
SH-B3	U 1	*Bacillus pumilus*	FJ549008
SH-B4	U 1	*Bacillus oleronius*	FJ549009
SH-B7	U 4	*Bacillus* sp.	FJ549010
SH-B13	U 6	*Bacillus subtilis*	FJ549011
SH-B18	U 10	*Bacillus* sp. (similar to *B. thuringiensis* or *B. cereus*)	FJ549012
SH-B20	K 1	*Bacillus* sp. (similar to *B. cereus* or *B. thuringiensis*)	FJ549014
SH-B21	K 1	*Bacillus subtilis*	FJ549015
SH-B23	K 1	*Bacillus pumilus*	FJ549016
SH-B27	K 3	*Bacillus* sp.	FJ549017
SH-B29	K 4	*Bacillus* sp. (similar to *B. subtilis* or *B. velezensis*)	FJ549018
SH-B30	K 5	*Bacillus pumilus*	FJ549019
SH-B34	K 7	*Paenibacillus amylolyticus*	FJ549020
SH-B35	K 7	*Bacillus licheniformis*	FJ549021
SH-B40	K 9	*Bacillus clausii*	FJ549022

Strain	Origin	*Clostridium sp.*	Acc. No. in GenBank
CM-C50	Farm A	*Clostridium ramosum*	EU869233
CM-C51	Farm A	*Clostridium neonatale*	EU869234
CM-C52	Farm A	*Clostridium sp. (related to C. glycolinum)*	EU869235
CM-C56	Farm B	*Clostridium butyricum*	EU869236
CM-C76	Farm D	*Clostridium bifermentans*	EU869237
CM-C81	Farm D	*Clostridium sp. (probably represents a new member of the genus Clostridium)*	EU869238
CM-C86	Farm I	*Clostridium butyricum*	EU869239
CM-C87	Farm I	*Clostridium perfringens*	EU869240
CM-C89	Farm E	*Clostridium perfringens*	EU869241
CM-C96	Farm J	*Clostridium sordellii*	EU869242
CM-C97	Farm J	*Clostridium butyricum*	EU869243
CM-C98	Farm J	*Clostridium neonatale*	EU869244
CM-C99	Farm J	*Clostridium sp. (related to C. xylanolyticum and C. aerotolerans)*	EU869245
BG-C4	K AP 1	*Clostridium sp.*	FJ384366
BG-C8	K AP 1	*Clostridium sporogenes/ botulinum*	FJ384367
BG-C9	K AP 1	*Clostridium subterminale*	FJ384368
BG-C11	K BP 1	*Clostridium butyricum*	FJ384369
BG-C22	K BP 2	*Clostridium sordellii*	FJ384370
BG-C23	K BP 2	*Clostridium perfringens*	FJ384371
BG-C29	K AP 2	*Clostridium irregulare*	FJ384372
BG-C36	K AD 3	*Clostridium sp.*	FJ384373
BG-C39	L BP 3	*Clostridium perfringens*	FJ384374
BG-C42	K BP 3	*Clostridium glycolicum*	FJ384375
BG-C45	K AP 4	*Clostridium perfringens*	FJ384376
BG-C51	L AD 4	*Clostridium sp.*	FJ384377
BG-C66	L BP 5	*Clostridium sp.*	FJ384378
BG-C76	K AP 6	*Clostridium perfringens*	FJ384379
BG-C95	K AP 7	*Clostridium sp. (related to C. sartagoforme)*	FJ384380
BG-C96	K AP 7	*Clostridium sordellii*	FJ384381
BG-C109	K AP 7	*Clostridium botulinum*	FJ384382
BG-C122	K AD 8	*Clostridium sp.*	FJ384383
BG-C128	K BP 8	*Clostridium glycolicum*	FJ384385
BG-C130	K AP 8	*Clostridium sp.*	FJ384386
BG-C131	K AD 8	*Clostridium sp.*	FJ384387
BG-C135	L BP 8	*Clostridium sordellii*	FJ384388
BG-C150	K BP 10	*Clostridium perfringens*	FJ384389
BG-C151	K AD 10	*Clostridium sp.*	FJ384390
SH-C1	U 2	*Clostridium sp.*	FJ424472
SH-C5	U 1	*Clostridium bifermentans*	FJ424473
SH-C10	U 2	*Clostridium sp.*	FJ424474
SH-C14	U 4	*Clostridium bifermentans*	FJ424475
SH-C19	U 1	*Clostridium sordellii*	FJ424476
SH-C20	U 7	*Clostridium bifermentans*	FJ424477
SH-C24	U 6	*Clostridium septicum*	FJ424478
SH-C30	U 9	*Clostridium butyricum*	FJ424480
SH-C52	K 9	*Clostridium sp.*	FJ424481
SH-C58	K 7	*Clostridium bifermentans*	FJ424482
SH-C65	K 2	*Clostridium bifermentans*	FJ424483

CM = cattle manure
SH = slaughterhouse waste from the slaughter houses U and K
BG = biogas plant K and L. BP = before pasteurisation, AP = after pasteurisation AD = after digestion
The number correspond to sampling time

Table 3. *Accession number (Acc. No.) for 16S rRNA gene sequences of type and reference strains of Bacillus spp., Clostridium spp., Lysinobacillus spp., Paenibacillus sp. and outgroups used for construction of phylogenetic trees.*

Strain	***Bacillus* spp., *Lysinobacillus* spp. and *Paenibacillus* sp.**	**Acc. No. in GenBank**
ATCC 14578	*Bacillus anhtracis*	AB190217
ATCC 14574^T	*Bacillus badius*	D78310
ATCC 14579	*Bacillus cereus*	DQ207729
DSM8716^T	*Bacillus clausii*	X76440
RA-24	*"Bacillus pichinotyi"*[a]	EU652096
IAM 12464^T	*Bacillus firmus*	D16268
681	*Bacillus flexus*	AB361590
R7190^T	*Bacillus fordii*	AY443039
DSM 13^T	*Bacillus licheniformis*	X68416
DSM 32^T	*Bacillus megaterium*	X60629
ATCC 6462	*Bacillus mycoides*	EF210295
DSM 9356^T	*Bacillus oleronius*	X82492
DSMZ 27^T	*Bacillus pumilus*	AY456263
ATCC 10792^T	*Bacillus thuringiensis*	AF290545
DSM 10^T	*Bacillus subtilis*	AJ276351
CR-502^T	*Bacillus velezensis/ B. amyloliquefaciens*	AY603658
WSBC 10204	*Bacillus weihenstephanensis*	AM747230
ATCC 14577	*Lysinobacillus sphaericus*	DQ286298
ATCC 7055^T	*Lysinobacillus fusiformis*	L14013
NRS 290^T	*Paenibacillus amylolyticus*	D85396

[a] This species name is not yet officially approved

strain	**Outgroup**	**Acc. No. in GenBank**
NCTC 7910 (T)	*Corynebacterium ulcerans*	X84256
ATCC 29521 (T)	*Bifidobacterium bifidum*	M38018
	Escherichia coli	J01695

Strain	***Clostridium* spp.**	**Acc. No. in GenBank**
DSM 5434 (T)	*Clostridium aerotolerans*	X 76163
ATCC 27638 (T)	*Clostridium baratii*	X68174
DSM 10716 (T)	*Clostridium bifermentans*	X73437
ATCC 25763 (T)	*Clostridium botulinum* type A	L37585
ATCC 7949	*Clostridium botulinum* type B	L37587
ATCC 17782	*Clostridium botulinum* type C	L37590
ATCC 9564	*Clostridium botulinum* type E	L37592
ATCC 27322	*Clostridium botulinum* type G	M59087
ATCC 19398	*Clostridium butyricum*	AB075768
ATCC 10092 (T)	*Clostridium chauvoei*	U51843
DSM 5521 (T)	*Clostridium disporicum*	Y18176
CIN 5 (T)	*Clostridium glycolicum*	AY007244
ATCC 9650 (T)	*Clostridium haemolyticum*	AB037910
DSM 2635 (T)	*Clostridium irregulare*	X73447
VA3187/2007	*Clostridium limosum*	EU118811
LCDC99-A-005	*Clostridium neonatale*	AF275949
ATCC 13124 (T)	*Clostridium perfringens*	M59103
DSM 1402 (T)	*Clostridium ramosum*	X73440
DSM 2632 (T)	*Clostridium sardiniense*	X73446
DSM 1292 (T)	*Clostridium sartagoforme*	Y18175
ATCC 12464 (T)	*Clostridium septicum*	U59278
ATCC 9714 (T)	*Clostridium sordellii*	M59105
ATCC 3584 (T)	*Clostridium sporogenes*	X68189
ATCC 25774 (T)	*Clostridium subterminale*	L37595
E 88	*Clostridium tetani*	AE015927
LUP 21	*Clostridium thiosulfatireducens*	AY024332
ATCC 4963	*Clostridium xylanolyticum*	X71855
p-2117-s959-2	Uncultured bacterium	AF371837
F36	Uncultured bacterium	DQ232855
aab17c11	Uncultured bacterium	DQ816381

Table 4. *Bacillus spp., Clostridium spp., Lysinobacillus spp. and Paenibacillus spp. found in the different sampling material*

	***Bacillus* spp.** [1]	***Clostridium* spp.**	
Farms	*B. cereus*	*C. bifermentans*	
	B. pumilus	*C. butyricum*	
	B. subtilis	*C. neonatale*	
	B. weihensteph./ mycoides	*C. perfringens*	
	Bacillus spp.	*C. ramosum*	
	Paenibacillus amylolyticus	*C. sordelli*	
	Paenibacillus polymyxa	*Clostridium* spp.	
Slaughterhouses	*B. cereus*	*C. bifermentans*	
	B. clausii	*C. butyricum*	
	B. licheniformis	*C. cellobioparum*	
	B. lentus	*C. glycolicum*	
	B. oleronius	*C. limosum*	
	B. pumilus	*C. perfringens*	
	B. subtilis	*C. septicum*	
	B. thuringiensis	*C. sordellii*	
	Bacillus spp.	*Clostridium* spp.	
	Paenibacillus amylolyticus		
Before pasteurisation	*B. cereus*	*C. aurantibutyricum*	*C. novy*
	B. clausii	*C. barati*	*C. paraputrificum*
	B. licheniformis	*C. bifermentans*	*C. perenne*
	B. pumilus	*C. botulinum*	*C. perfringens*
	B. subtilis	*C. butyricum*	*C. sardiniensis*
	Bacillus spp.	*C. celatum*	*C. sordellii*
	Lysinobacillus sphaericus	*C. durum*	*C. subterminale*
	Lysinobacillus sp.	*C. formicoaceticum*	*C. tertium*
	Paenibacillus polymyxa	*C. glycolicum*	*C. tyrobutyricum*
		C. limosum	*Clostridium* spp.
After pasteurisation	*B. cereus*	*C. acetobutylicum*	*C. limosum*
	B. clausii	*C. aurantibutyricum*	*C. oceanicum*
	B. licheniformis	*C. bifermentans*	*C. perfringens*
	B. pumilus	*C. botulinum*	*C. sordellii*
	B. subtilis	*C. butyricum*	*C. sporogenes/ C. botulinum*
	Bacillus spp.	*C. durum*	
	Lysinobacillus sphaericus	*C. glycolicum*	*C. subterminale*
	Lysinobacillus sp.	*C. irregulare*	*Clostridium* spp.
	Paenibacillus polymyxa		
After digestion	*B. cereus*	*C. acetobutylicum*	*C. glycolicum*
	B. licheniformis	*C. aurantibutyricum*	*C. limosum*
	B. megaterium	*C. barati*	*C. sardiniensis*
	B. pumilus	*C. bifermentans*	*C. perfringens*
	B. subtilis	*C. butyricum*	*Clostridium* spp.
	Bacillus spp.	*C. durum*	
	Lysinobacillus sphaericus		
	Lysinobacillus sp.		
	Paenibacillus polymyxa		

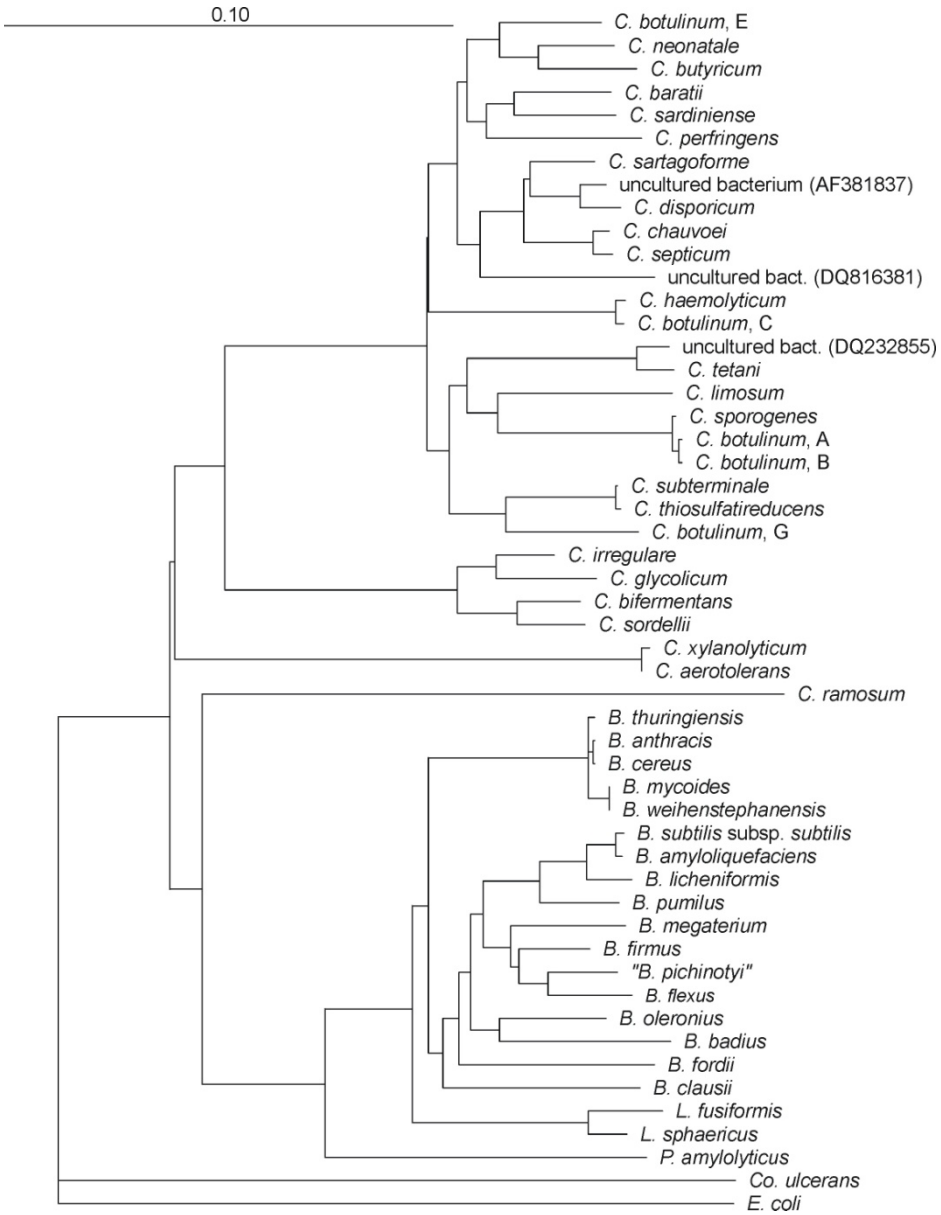

Figure 1. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between type and reference strains of *Bacillus* spp., *Clostridium* spp., *Lysinobacillus* spp. and *Paenibacillus* sp. The length of the scalebar is equivalent to 10 nucleotide substitutions per 100 positions.

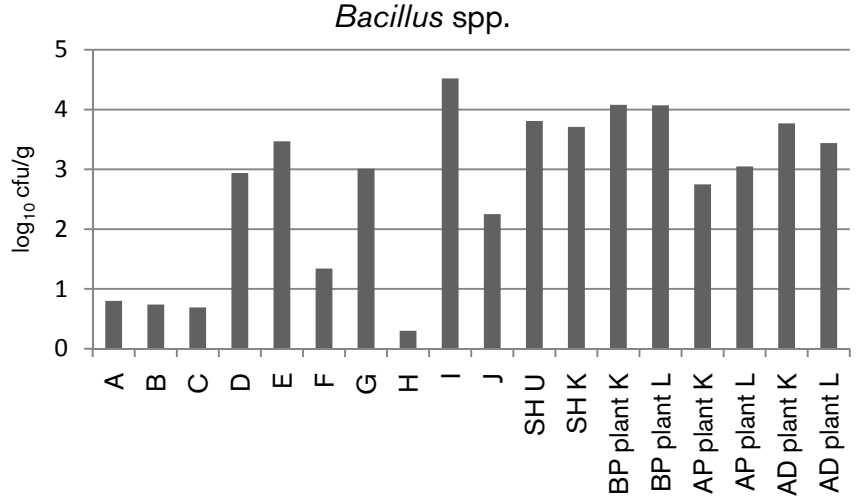

Figure 2. Mean values of the quantities of bacteria representing *Bacillus* spp. *Lysinobacillus* spp. and *Paenibacillus* spp. in the different sampling material.
SH = slaughterhouse waste from the slaughter houses U and K
BG = biogas plant K and L. BP = before pasteurisation, AP = after pasteurisation AD = after digestion

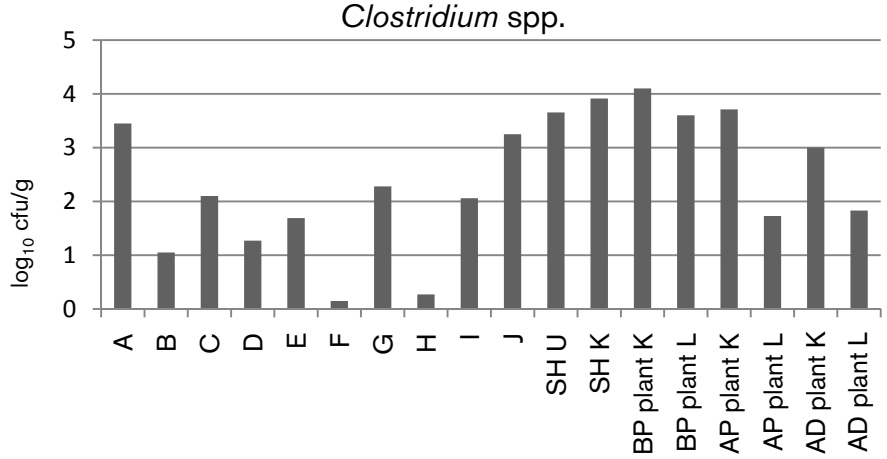

Figure 3. Mean values of the quantities of bacteria representing Clostridium spp. in the different sampling material
SH = slaughterhouse waste from the slaughter houses U and K
BG = biogas plant K and L. BP = before pasteurisation, AP = after pasteurisation AD = after digestion

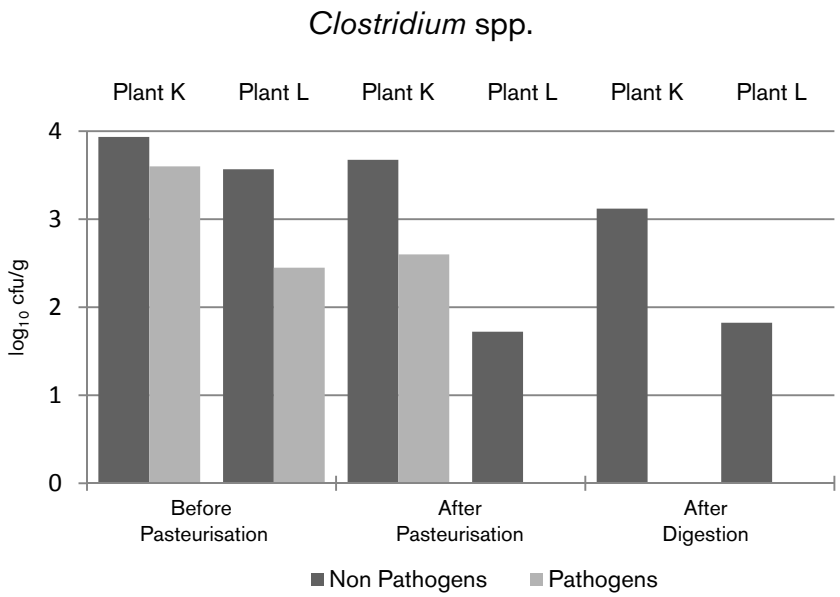

Figure 4. Mean values of the quantities of bacteria representing *Clostridium* spp. in the different stages in the biogas process.

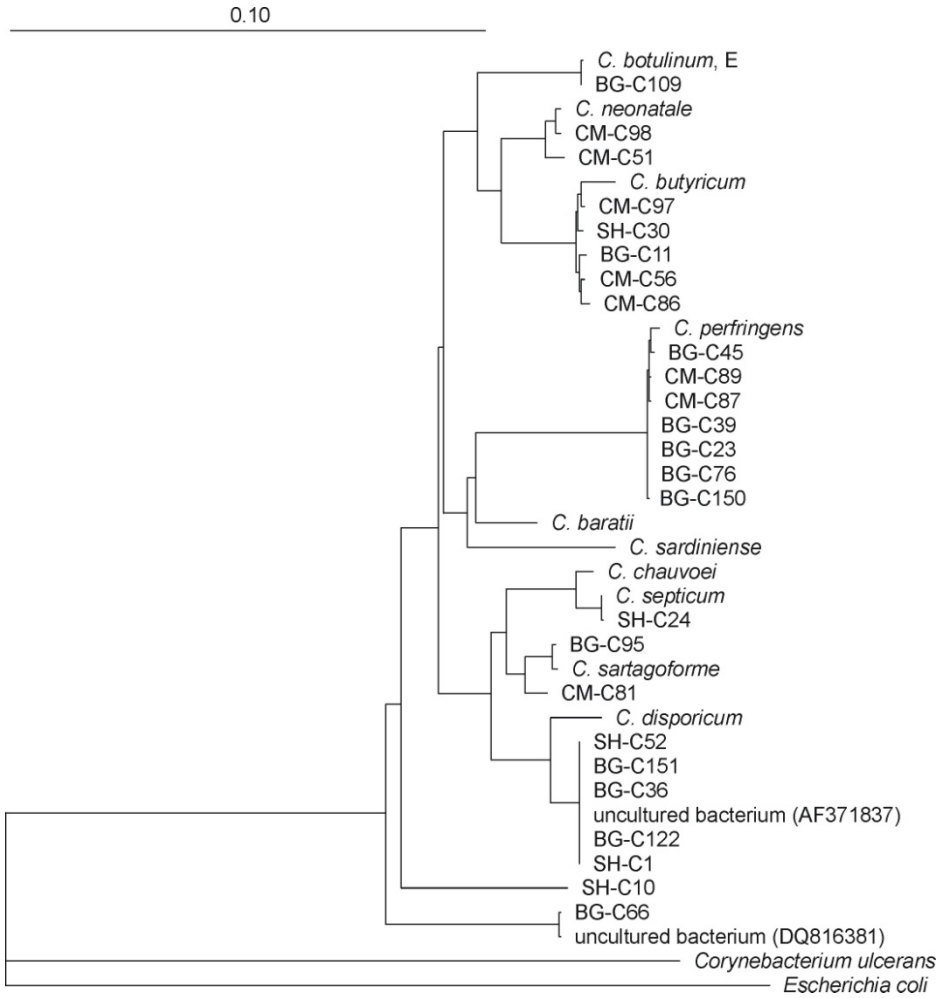

Figure 5. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between strains of *Clostridium* spp. related to *Clostridium perfringens*, isolated from cattle manure (CM), slaughterhouse waste (SH) and biogas plant (BG). The length of the scalebar is equivalent to 10 nucleotide substitutions per 100 positions.

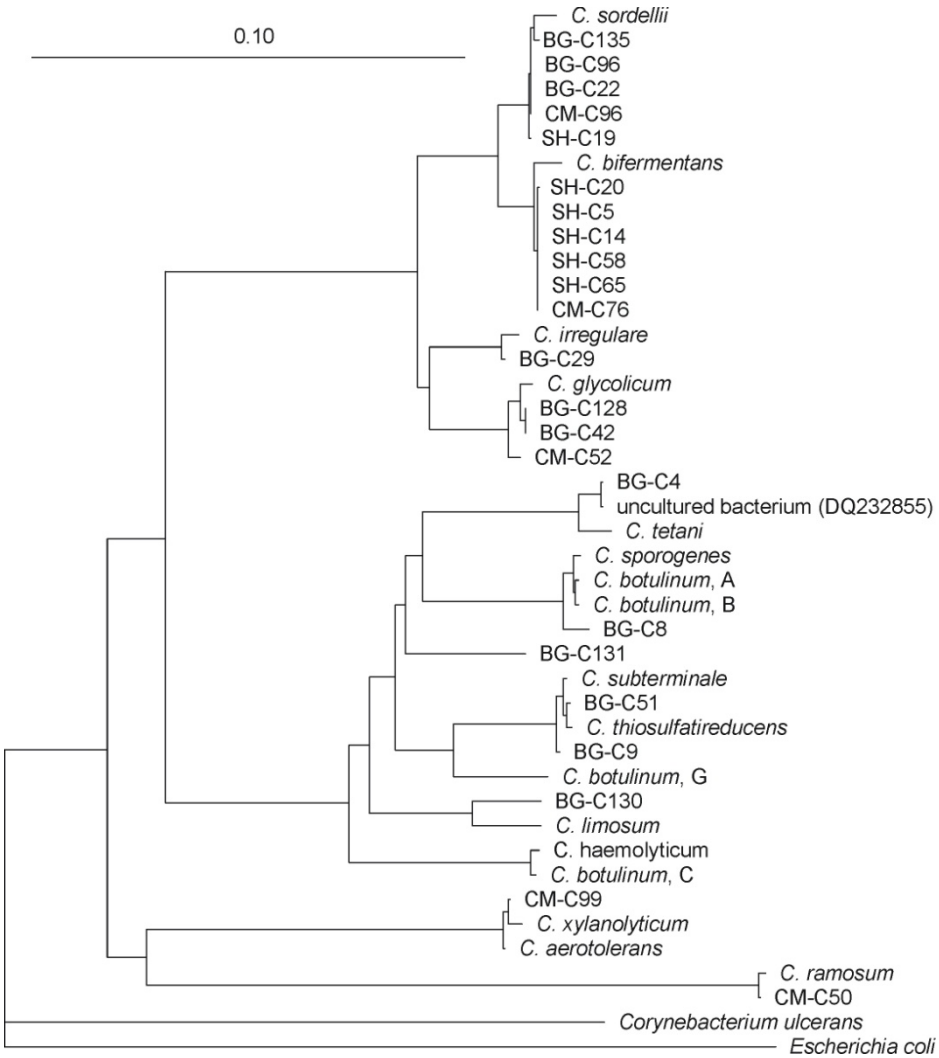

Figure 6. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between strains of *Clostridium* spp. related to *Clostridium sordelii,* isolated from cattle manure (CM), slaughterhouse waste (SH) and biogas plant (BG). The length of the scalebar is equivalent to 10 nucleotide substitutions per 100 positions.

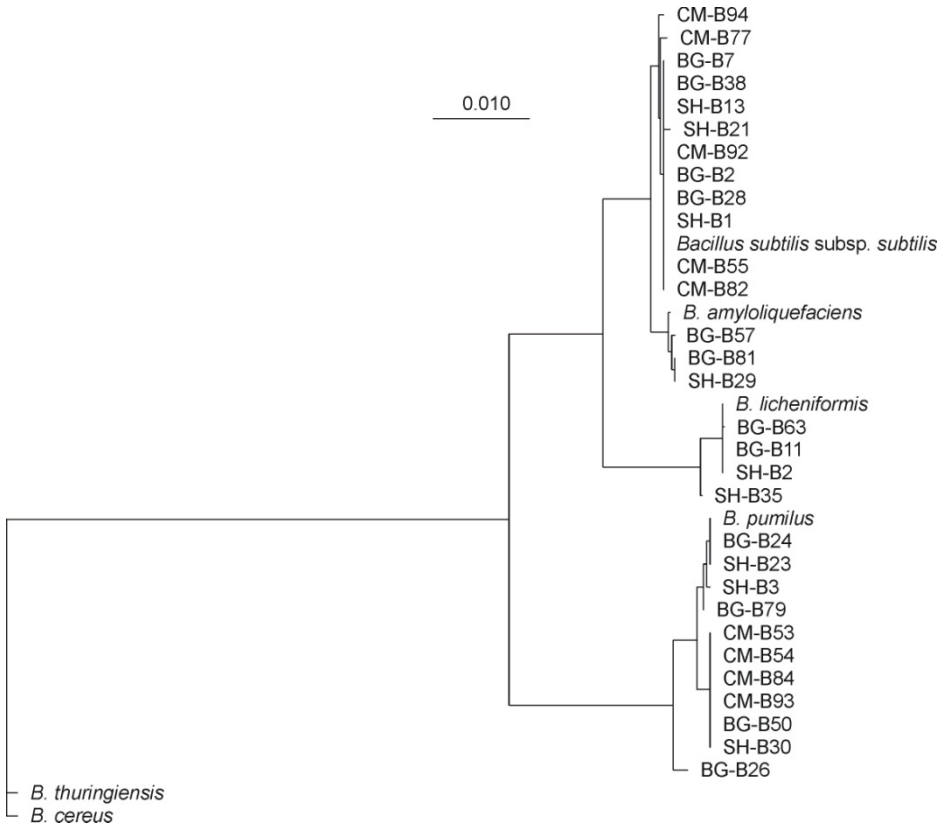

Figure 7. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between strains of *Bacillus* spp. related to *Bacillus subtillis*, isolated from cattle manure (CM), slaughterhouse waste (SH) and biogas plant (BG). The length of the scalebar is equivalent to 1 nucleotide substitutions per 100 positions.

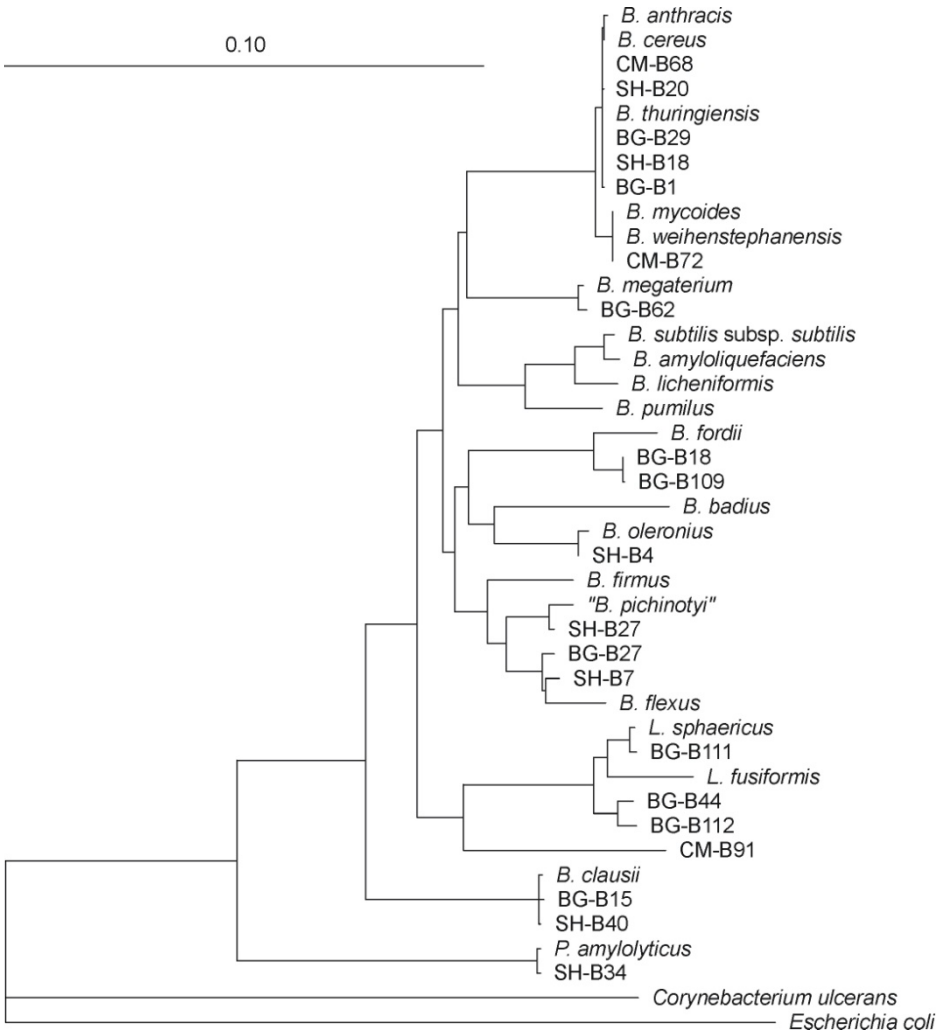

Figure 8. Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic relations between strains of *Bacillus* spp. related to *Bacillus cereus*, and *Lysinobacillus* spp. and *Paenibacillus* spp., isolated from cattle manure (CM), slaughterhouse waste (SH) and biogas plant (BG). The length of the scalebar is equivalent to 10 nucleotide substitutions per 100 positions.

IV

Survival of pathogenic clostridia after pasteurisation and during anaerobic digestion in biogas plants - a laboratory study

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Abstract

Biogas can be obtained by processing biowaste and the digested residues can be used as fertiliser. Pathogenic micro-organisms generally occur in the raw biowaste used as substrates at biogas plants. If the micro-organisms survive the biogas process, there is a risk of their spreading disease when the digested residue is spread on arable land. To prevent this, pasteurisation at 70°C for 60 min before anaerobic digestion can be used to significantly reduce micro-organisms such as salmonella. However, spore-forming bacteria, including pathogenic clostridia, generally persist after pasteurisation and anaerobic digestion. Little is known about the persistence of pathogenic spore-forming bacteria, especially differences between species.

In this laboratory scale study, five pathogenic clostridia (*Clostridium chauvoei, Clostridium haemolyticum, Clostridium perfringens* type C, *Clostridium septicum* and *Clostridium sordellii*) were inoculated into biogas plant substrates from homogenisation and digester tanks. Pasteurisation and digestion were simulated. To detect and identify clostridia, specific PCR primer pairs for each *Clostridium* spp. were used after culture on Fastidious Anaerobic Agar plates and DNA preparation.

Clostridium chauvoei, *Cl. perfringens* type C, *Cl. septicum* and *Cl. sordellii* were detected both before and after pasteurisation. *Clostridium septicum* and *Cl.*

sordellii were detected following anaerobic digestion whereas neither *Cl. perfringens* type C nor *Cl. chauvoei* could be detected throughout the digestion. The impact of anaerobic digestion differs between species of pathogenic spore-forming bacteria, which should be taken into consideration when planning the use of residues as fertiliser.

Keywords: biogas, pathogens, *Clostridium* spp., pasteurisation, digestion.

1 Introduction

Biogas is an expanding renewable, CO_2-neutral, energy alternative produced by anaerobic digestion of biowaste. The essential part of the digestion is an anaerobic microbial process. The digested residues are rich in plant nutrients and can be spread on arable land, which reduces the need for artificial fertilisers. However, when digested residues are used as fertiliser, the content of heavy metals, organic pollutants and pathogenic micro-organisms must be minimized. At most full-scale biogas plants in Sweden, only sorted biowaste is accepted, in order to minimize undesired contamination of the substrate. Common substrates in biogas plants originate from kitchen waste, food industry waste and animal by-products (ABP) from slaughterhouses, cattle manure and pig slurry. The types of microbiological contaminants that can be found in biowaste include bacteria, fungi, parasites and viruses (Larsen *et al.*, 1994; Bendixen, 1996). Consequently, humans and animals can become infected with pathogenic micro-organisms from biowaste if it is used as a fertiliser and spread in the environment (Gerba and Smith, 2005).

According to EU regulations (EC nos. 1774/2002 and 208/2006) for ABP Category 3 and manure, it is compulsory that biowaste be pasteurised at 70°C for 60 min or subjected to an equivalent treatment before anaerobic digestion in a biogas plant. Heating at 70°C for 60 min reduces indicator bacteria and salmonella (Bendixen, 1996; Bagge *et al.*, 2006; Sahlström *et al.*, 2008), but some spore-forming bacteria, heat-resistant viruses and prions can persist unaffected (Huang *et al.*, 2007; Sahlström *et al.*, 2008). In addition to raised temperature, acidic conditions are inhibitory to *Salmonella* spp. in mesophilic digestion (Salsali *et al.*, 2006). The quantity of *Clostridium* spp. spores decreases during storage if pH exceeds 12 (Bujoczek *et al.*, 2002).

Spore-forming bacteria (*e.g.* *Bacillus* spp. and *Clostridium* spp.) belong to the normal flora of the gut and faeces from most animal species (Timoney *et al.*

1988; Gyles and Thoen 1993). Spore-forming bacteria grow as vegetative cells under favourable conditions. But when growth conditions are poor they can survive as inactive spores for very long periods of time (Mitscherlich and Marth 1984). *Clostridium* spp. grow only under anaerobic conditions.

Most *Bacillus* spp and *Clostridium* spp. are harmless, whereas pathogens such as *Cl. chauvoei*, *Cl. botulinum* and *Bacillus anthracis* can cause serious diseases. Pathogenic clostridia of particular concern for animal health are *Cl. chauvoei*, *Cl. haemolyticum, Cl. perfringens* type C*, Cl. septicum* and *Cl. sordellii*. All these five clostridia are lethal or cause serious clinical diseases in farm animals and can cause severe economic losses for farmers. The route of infection may be oral, or by a wound infection (Timoney *et al.* 1988; Gyles and Thoen 1993). These clostridia can occur in manure and ABP, which are included in substrates to biogas plants (Larsen *et al.* 1994; Bendixen 1996). To our knowledge no well documented case of infection of animals by these clostridia from digested residues has been reported, though a biosecurity risk is evident if infected material is spread on arable land. In earlier studies, *Cl. botulinum* was investigated in composted biowaste (Böhnel and Lube, 2000), and in cattle and pig faeces (Dahlenborg *et al.* 2001, 2003), and inoculated fungi during digestion (Schnürer and Schnürer 2006), but the clostridia mentioned above have not been studied during anaerobic digestion.

Clostridium chauvoei exists in manure and soil (Smith and Holdeman 1968; Gyles and Thoen 1993; Hang'ombe *et al.* 2000); it can cause blackleg in cattle and sheep. Young cattle are more susceptible than old cattle. Infections are most common during the summer and on permanent pasturage and wetlands. The clinical symptoms of blackleg are fever and swollen muscle tissues with entrapped gas; the mortality is high (Timoney *et al.* 1988, Sternberg *et al.* 1999). In some clinical cases of blackleg there is mixed infection with *Cl. chauvoei* and *Cl. septicum* (Sternberg *et al.* 1999).

Clostridium haemolyticum causes icterohaemoglobinuria, or redwater, in cattle. The disease is a frequent complication of liver damage caused by the liver fluke (*Fasciola hepatica*) or from other causes (Gyles and Thoen 1993). The bacterium is a common pathogen in *e.g.* the Rocky Mountains (USA) (Timoney *et al.* 1988) but not in Sweden.

Clostridium perfringens type C causes severe haemorrhagic enterotoxaemia with diarrhea and dysentery in newborn piglets. Piglets, 1-2 weeks old, develop a chronic form of enteritis. They often acquire the infection from the sow´s faeces. Once an infection of *Cl. perfringens* gains access introduced into a pig farm it is very difficult to eradicate the disease, regardless of vaccination programme. In adult sheep *Cl. perfringens* type C can cause sudden death and struck, and in domestic fowl the bacteria can cause necrotic enteritis.

Clostridium perfringens type C can also cause disease in lambs and calves (Timoney *et al.* 1988; Gyles and Thoen 1993; Quinn *et al.* 1994,).

Clostridium septicum causes malignant edema in connection with infected wounds in many animal species. In lambs the organism can cause braxy, an infection of the abomasum. *Clostridium septicum* is common in soil and in the intestinal tract of many animals (Timoney *et al.* 1988; Gyles and Thoen 1993). The clinical symptoms of malignant edema are similar to those of blackleg caused by *Cl. chauvoei* but, unlike in blackleg, the muscle tissue contains little or no gas (Timoney *et al.* 1988).

Clostridium sordellii is a common intestinal tract inhabitant of many animal species and it is also found in soil (Gyles and Thoen 1993). Sometimes *Cl. sordellii* causes gas gangrene in cattle, sheep and horses (Quinn *et al.* 1994).

Clostridium spp. and *Bacillus* spp. are regularly detected after pasteurisation and anaerobic digestion (Larsen *et al.* 1994; Chauret *et al.* 1999; Aitken *et al*, 2005; Bagge *et al.* 2006; Sahlström *et al.* 2008), but less is known about the effect on different pathogenic spore-forming groups during anaerobic digestion. However, it has been shown that the number of colony-forming units (cfu) of inoculated fungi, which are spore-forming micro-organisms, does not decrease during digestion (Schnürer and Schnürer 2006). To reduce the risk of infection from digested residues, it is important to gain an understanding of the effect of the anaerobic digestion process on different spore-forming microorganisms, especially pathogenic bacteria. It is known that different species of clostridia have differing sensitivity to the concentration of carbohydrates and to pH (Volkova *et al.* 1988; Cotter and Hill 2003), factors which vary in the digester tank substrate.

The purpose of the present study was to study the influence of pasteurisation and digestion during the biogas plant process on five pathogenic spore-forming bacteria, by using laboratory-scale biogas reactors.

2 Materials and methods

2.1 Biogas plants

Substrates from four full-scale commercial biogas plants, A-D, were collected from the homogenisation tanks and digesters, and immediately delivered to the National Veterinary Institute (SVA), Uppsala. At all plants, ABP from slaughterhouses and separated biowaste from food industry were used as substrate (Table 1). Two of the plants (B and C) also received manure and slurry from local farms. None of the plants used municipal sewage sludge from

waste-water treatment plants or any other kind of lavatory waste. The anaerobic semi-continuous digestion at the biogas plants was performed under mesophilic (37°C) (plants A and B) or thermophilic (55°C) (plants C and D) conditions. At plant A, hydrochloric acid was added to the digester to increase gas yield, but information about the amount used was not available due to trade secrecy.

Table 1. *Composition of substrate used at biogas plants A-D.*

Biogas plant / Composition of substrate (%)	Plant A	Plant B	Plant C	Plant D
Slaughterhouse waste[a]	43	36	26	47
Cattle manure	0	18	36	0
Pig slurry	0	30	0	0
Kitchen waste	2	5	0	53
Industrial waste	9	6	7	0
Others	46[b]	5	31[c]	0

[a] Animal by-products permitted for biogas plants
[b] Draff, mycelium, discarded foodstuffs from restaurants, whey
[c] Sludge from local slaughterhouse, potato sludge and alcohol

The conditions on the day of sampling were reported from each plant in a questionnaire (Tables 1 and 2). Additionally, some chemical parameters of the substrates were determined at SVA. The pH was measured and the amount of volatile fatty acids (VFA) and ammonium-nitrogen (NH_4^+-N) levels were determined (Table 2).

2.2 Preparation of *Clostridium* spp. strains before inoculation

Five species of clostridia (*Cl. chauvoei, Cl. haemolyticum, Cl. perfringens* type C, *Cl. septicum* and *Cl. sordellii*), were studied (Table 3). One loopful (approximately 5 µL of bacteria collected with a 10 µL loop) of each bacterial species was inoculated into 10 mL Tryptone Glucose Yeast broth (TGY; tryptone, 30 g/L and yeast extract, 10 g/L from Difco, Le Pont de Claix, France, and D-glucose, 20 g/L and L-cysteine HCl 0.1 g/L, from Sigma Aldrich, Steinheim, Germany). The broth was incubated at 37°C for 48 h under anaerobic conditions using anaerobic jars in an anaerobic environment using carbon dioxide (CO_2) (ANAEROgen®, Oxoid, Basingstoke, Hampshire, England).

Table 2. *Specific process parameters in biogas plants A-D according to data from questionnaire and results of the chemical analyses of digested residues performed at the National Veterinary Institute, Uppsala, Sweden.*

Process parameters \ Biogas plant	Plant A	Plant B	Plant C	Plant D
Temperature (°C)	39	38	52	55
Hydraulic retention time (days)	44	30	25-26	50-60
Loading rate per day (VS kg/m^3)	3.63	3	2.2-2.4	1.5-2
NH_4^+-N (g /100g) from questionnaire	0.58	0.4	0.05[b]	0.18
NH_4^+-N, (g /100g)[a] analysed at SVA	0.58	0.33	0.36	0.20
pH from questionnaire	8.2	7.8	8.0	8.2
pH analysed at SVA	8.1	8.2	8.5	8.6
Active volume in the digester (m^3)	7400	6500	1800	2300
Gas production (m^3/year)	$6.8 \cdot 10^6$	$5.5 \cdot 10^6$	$1.2 \cdot 10^6$	$0.75 \cdot 10^6$
Dry matter (%) [c]	5.9	3.8	2.9	1.6

VS = Volatile solids
[a] Direct distillation
[b] Obtained by distillation- and titration (see section 4.7)
[c] Gravimetry

In order to stimulate the production of spores, the broth was then kept at room temperature (approximately 20°C) under anaerobic conditions for 5 days as described by Båverud *et al.* (2003). When the pasteurisation test was performed *Cl. perfringens* type C was kept for 5 days at room temperature under anaerobic conditions followed by 7 days at room temperature in aerobic conditions. The numbers of bacteria were counted in a Bürker chamber (0.01 mm, Assistent, Sondheim/Rhön, Germany) and viable bacteria were quantified by using ten-fold dilution series in peptone saline solution, which was then cultivated (0.1 mL) on Fastidious Anaerobic Agar plates (FAA, LabM, Bury, Lancashire, England), with 5% defibrinated horse blood and incubated at 37°C for 48 h under anaerobic conditions. After incubation the numbers of colonies were estimated. *Clostridium chauvoei, Cl. haemolyticum, Cl. septicum* and *Cl. sordellii* are swarming bacteria that grow over the whole FAA plate. Since it was difficult to

count individual colonies, only $\log_{10}$ resolution could be established. The quantitative dilution experiment was performed in duplicate for each strain.

Table 3. *Concentrations* [a] *of the test species of bacteria in the inoculated Tryptone Glucose Yeast broth.*

		Clostridium spp.				
Trial ↓	SVA identity of strain →	*Clostridium chauvoei* AN 2548/02	*Clostridium haemolyticum* LP 2361/89	*Clostridium perfringens* type C CCUG [b] 2036	*Clostridium septicum* AN 3062/04	*Clostridium sordellii* AN 1562/05
Pre trial: Detection level		5	2	5	6	7
Pasteurisation plants A-D		5	2	5	6	7
Digestion plant A		5	2	5	5	6
Digestion plant B		5	<1	6	5	6
Digestion plant C		5	<1	3	6	7
Digestion plant D		5	2	3	5	6

[a] $\log_{10}$ cfu/ml
[b] CCUG = Culture Collection University of Gothenburg

2.3 Analysis of *Clostridium* spp. in inoculated samples before and after pasteurisation

At all biogas plants, substrates were collected from the homogenisation tanks before pasteurisation and stored at -20°C until analysis. For each of the four plants, 32 samples of 5 g substrate were transferred into test tubes. Then the five previously prepared strains were, for each strain, inoculated in six tubes with one mL broth/tube of clostridia. This left two uninoculated samples for control purposes. In the next step, half of each group (three tubes/strain) and one uninoculated tube used as control were pasteurised at 70°C for 60 min. After pasteurisation, 9 mL of sterile physiological saline was added to all tubes and shaken thoroughly. The tubes were then left for 15 min to allow the content settle, and then one loopful from each tube was spread on one FAA plate. The FAA plates were incubated at 37°C for 48 h under anaerobic conditions, under which inoculated bacteria will grow in mixed culture with the substrate flora. After incubation, one loopful was taken by a streak from each FAA plate and then used for DNA preparation.

2.4 Analysis of *Clostridium* spp. in inoculated samples during anaerobic digestion

The same five species of clostridia as were used in the pasteurisation study were prepared as above, but in 50 mL TGY-broth (Table 3). Quantitative analysis of

the number of viable bacteria was made by culture. Diluted enrichment broth was cultured on FAA plates (0.1 mL) and mixed in melted Tryptose Sulphite Cycloserine agar (TSC, perfringens agar base, Oxoid) (1 mL). Incubation and quantification were performed as described above.

To avoid problems due to this and to ensure that results were not dependent on plate type, both FAA plates and TSC plates were used. As cultivation on FAA plates proved better than on TSC plates, only FAA plates were used in the pasteurisation part of the study.

Substrates from all four biogas plants were collected from the digester tanks and inoculated with the test strains (Table 3) within 1 to 16 h of sampling. Five millilitres of the digester substrate from each plant were added to separate vials (30 mL) during flushing with N_2/CO_2 (80/20%) atmosphere. The vials were sealed with a butyl rubber stopper and using an aluminium cap and then 1 mL of the bacterial suspension was inoculated using a needle and syringe. For each plant, the digester substrate was transferred into 195 inoculated vials and 13 uninoculated vials, giving three inoculated vials per strain for five strains, and one uninoculated vial per sampling for 13 sampling occasions. The vials with digester substrate from plants with mesophilic digestion (plants A and B) were incubated at 37°C and the incubation was interrupted regularly over 30 days (on days 0, 1, 2, 3, 4, 6, 8, 10, 12, 15, 20, 25 and 30). The vials with digester substrate from plants with thermophilic digestion (C and D) were incubated at 55°C and the incubation was interrupted regularly over 20 days (on days 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18 and 20). At every interruption, three vials per clostridia and one uninoculated vial were removed for analysis.

When the digestion was interrupted, the vials were removed from the incubator and the seal was broken. Nine millilitres of sterile physiological saline was added to each vial, shaken thoroughly and then left for 15 min to settle. From each vial 0.1 mL was then cultivated by spreading the inoculum over the surface of one FAA plate. Further, from each vial 1 mL was cultivated in TSC by mixing the sample with melted TSC-agar. All agar plates were then incubated at 37°C for 48 h under anaerobic conditions. After incubation, one loopful was taken by a streak from each FAA plate and used for DNA preparation.

The colonies on each TSC plate were counted and suspected colonies were subcultivated anaerobically on horse blood agar at 37°C for 24 h. After subcultivation one loopful of suspected colonies from the horse blood agar plate was used for DNA preparation.

The background flora was counted at the beginning, in the middle and at the end of the anaerobic digestion from both inoculated and uninoculated vials. Viable bacteria were quantified by using ten-fold dilution series in peptone

saline solution and then cultured (0.1 mL) on FAA plates. One millilitre from each dilution was mixed in melted TSC agar. All agar plates were then incubated at 37°C for 48 h under anaerobic conditions and colonies were counted.

2.5 Detection level of PCR for *Clostridium* spp. in biogas plant substrate

In order to establish the detection level of the PCR-method, samples were spiked with the investigated clostridia. The five species of clostridia (*Cl. chauvoei, Cl. haemolyticum, Cl. perfringens* type C, *Cl. septicum* and *Cl. sordellii*), were prepared as described above in 10 mL TGY-broth (2.2 and Table 3).

Substrates sampled at homogenisation tanks from all four biogas plants were mixed. Ten bottles containing 10 g of homogenisation tank substrate in each bottle were each inoculated with 1 mL bacterial suspension of different dilutions from the ten-fold dilution series from the preparation of the strains (see 2.2). The diluted bacterial suspensions were mixed thoroughly with substrates. Half of the homogenisation tank bottles were left at room temperature for 1 h to simulate the situation before pasteurisation, and the other half was heated to 70°C for 60 min to mimic pasteurisation. All bottles were agitated and then left to allow the content to settle for 15 min.

Samples from the digesters tanks from all four biogas plants were mixed and five bottles containing 10 g of mixed digester substrate were prepared. Each bottle was inoculated with 1 mL bacterial suspension of different dilutions from the ten-fold dilution series from the preparation of the strains. The diluted bacterial suspensions were mixed thoroughly with substrates and left at room temperature for 1 h.

From each bottle 0.1 mL was cultivated on FAA plates and incubated at 37°C for 48 h under anaerobic conditions. To confirm the identity of the strains after incubation, one loopful was taken by a streak from each FAA plate and used for DNA preparation. The method is illustrated in Fig. 1.

Both the background flora and the spiked samples, except *Cl. perfringens*, were swarming and it was difficult to count separate colonies. Therefore, the number of spiked bacteria was estimated by using a ten-fold dilution series. Lowest detection concentration was established subsequently by PCR. The method gives $\log_{10}$ resolution. The detection levels were obtained by observing positive and negative PCR results from the different dilutions of each bacterial species. The procedure was performed with all five clostridia. The detection levels were established by repeating the trial in duplicate.

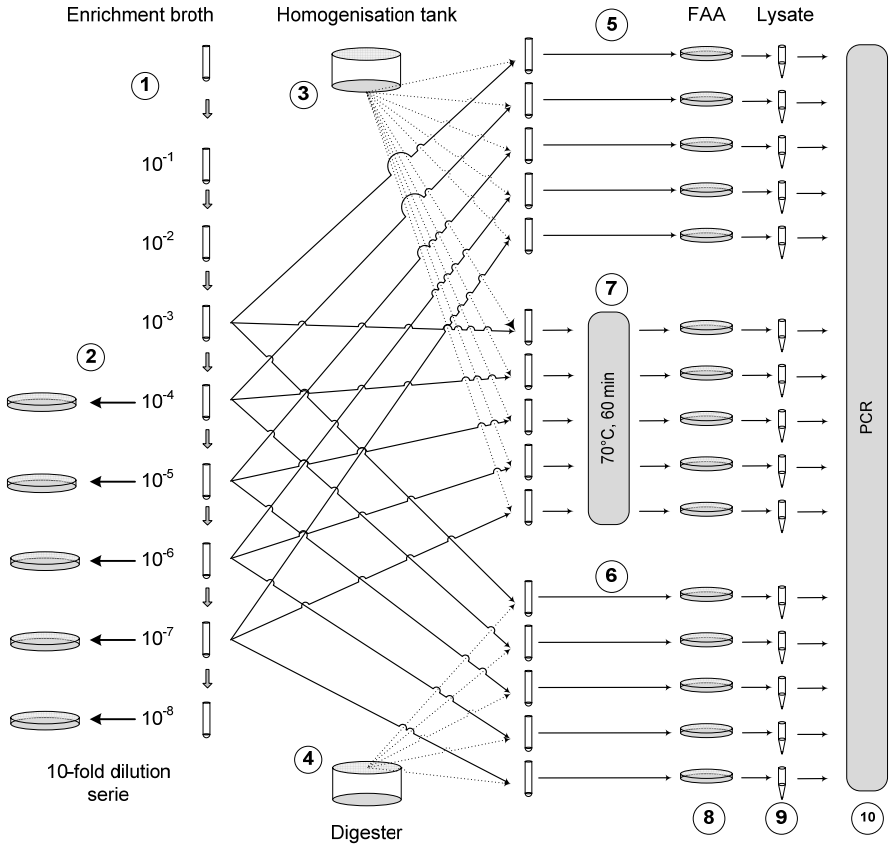

Figure 1. Determination by PCR of detection level for *Clostridium* spp. in biogas plant substrate. Different dilutions of bacteria were inoculated into substrate from the homogenisation tank and the digester. These steps were repeated for each *Clostridium* sp. included in this study.

1. *Clostridium* sp. was inoculated into enrichment broth and incubated. After incubation the broth was diluted in a ten-fold series.
2. From the ten-fold dilution series, 0.1 mL was cultured. After incubation the colonies were counted.
3. Substrates sampled at homogenisation tanks were mixed with diluted bacterial suspensions in bottles.
4. Substrates sampled at digester tanks were mixed with diluted bacterial suspensions.
5. Homogenisation tank bottles were left at room temperature for 1 h to simulate the situation before pasteurisation.
6. Digester tank bottles were left at room temperature for 1 h.
7. Homogenisation tank bottles were heated to 70ºC for 60 min to mimic the pasteurisation step.
8. From each bottle 0.1 mL was cultivated on FAA plates and incubated.
9. After incubation a streak was taken for DNA preparation.
10. The DNA preparations were analysed by PCR, using specific primer pairs.

2.6 DNA preparation

One loopful of colony material was suspended in 500 µL of phosphate-buffered saline (PBS, Merck, Darmstadt, Germany) and centrifuged for 10 min at 7,200 x *g*. The supernatant was discarded and the pellet was washed again by the same procedure, then re-suspended in 250 µL of sterile H_2O. The bacterial cells were lysed by boiling the suspension for 12 min before storage at -20°C until further analysis. This lysate was used as template in PCR.

2.7 PCR for detection and identification of clostridia

The background bacteria were often growing as a swarming flora on agar plates and it was difficult to obtain specific pathogen bacteria in pure culture. Therefore, this study was based on PCR methods with designated primer pairs for each clostridia studied. These PCR methods are more sensitive to the inoculated clostridia in background flora than culturing only.

Identification of *Cl. perfringens* type C was performed according to Engström *et al.* (2003). Briefly, the samples were analysed by PCR in order to establish the presence of *alfa*, *beta*, *epsilon* or *iota* toxin genes. For detection and identification of *Cl. chauvoei*, *Cl. haemolyticum* and *Cl. septicum* in the samples, specific PCR primer pairs complementary to the spacer region of the 16S 23S rRNA gene were used as described by Sasaki *et al.* (2000 and 2002). For detection and identification of *Cl. sordellii* in the samples, specific primer pairs based on 16S rRNA gene sequences were used as described by Kikuchi *et al.* (2002).

Pure cultures of each strain were used as positive control in the PCR. On all six occasions when the strains were cultivated, a new positive control was made (Table 3). All primer pairs were tested against all five *Clostridium* spp. used in this study and all primer pairs were tested against the uninoculated samples as negative controls. Positive and negative controls for the PCR reaction were always analysed at the same time as the samples.

The amplicons were analysed by electrophoresis in agarose gels (1.5% Agarose NA, from GE Healthcare, Uppsala, Sweden) which were stained with ethidium bromide. The PCR-products were visualized under UV-light.

3 Results

3.1 Chemical analysis

The results from chemical analysis of the digested residues are presented in Table 2.

3.2 Number of inoculated *Clostridium* spp.

The numbers of inoculated bacteria analysed quantitatively by using ten-fold dilution series are presented in Table 3. *Clostridium* spp. were counted in a Bürker chamber to 6-8 $\log_{10}$ /ml in TGY broth. Spores were observed in the chamber but were difficult to count.

3.3 Detection of *Clostridium* spp. in samples before and after pasteurisation

3.3.1 Uninoculated samples

The quantity of bacteria in all uninoculated samples was below the detection limit for *Cl. chauvoei*, *Cl. haemolyticum* and *Cl. perfringens* type C. Uninoculated substrate samples from two plants (A and C) were above the detection limit for *Cl. septicum* and samples from three plants (A, B and C) were above the limit for *Cl. sordellii*.

3.3.2 Inoculated samples

Clostridium chauvoei, *Cl. perfringens* type C, *Cl. septicum* and *Cl. sordellii* were detected both before and after pasteurisation (Table 4). *Clostridium haemolyticum* was not detected except in samples collected from plant A before pasteurisation and plant D after pasteurisation (Table 4).

Table 4. *Number of positive samples out of three tests per strain before and after pasteurisation of biogas plant substrate.*

Plant / Strain	A		B		C		D	
	BP	AP	BP	AP	BP	AP	BP	AP
Clostridium chauvoei	3	2	3	3	2	3	3	3
Clostridium haemolyticum	1	0	0	0	0	0	0	2
Clostridium perfringens C	2	3	3	3	3	3	2	3
Clostridium septicum	3	3	3	2	3	3	3	2
Clostridium sordellii	3	3	3	1	3	3	3	2

BP = Before pasteurisation
AP = After pasteurisation

3.4 Detection of *Clostridium* spp. in samples during anaerobic digestion

3.4.1 Uninoculated samples

The quantity of bacteria in all uninoculated samples was below the detection limit for *Cl. chauvoei, Cl. haemolyticum* and *Cl. perfringens* type C. Some of the uninoculated substrates sampled from all four plants were above the detection limit for *Cl. septicum* (Fig. 2). One uninoculated sample from plant B was above the detection limit for *Cl. sordellii* on day 3.

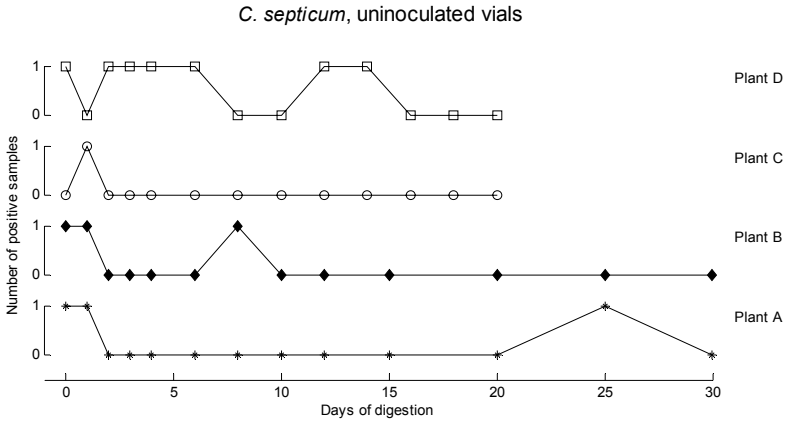

Figure 2. Analysis of samples for *Clostridium septicum* in uninoculated samples during digestion, as a function of time and biogas plant.

3.4.2 Inoculated samples

Clostridium septicum and *Cl. sordellii* were detected in all samples except two during the anaerobic digestion process. In substrate from biogas plant A, *Cl. chauvoei* was detected throughout the whole digestion process, but in substrate from plants B, C and D, the presence of *Cl. chauvoei* varied and could not be detected in the last samples from the digestion (Fig. 3). *Clostridium perfringens* type C was detected only during the first few days in substrate from all four plants (Fig. 4), *Cl. haemolyticum* could only be detected during the first week of digestion (Fig. 5).

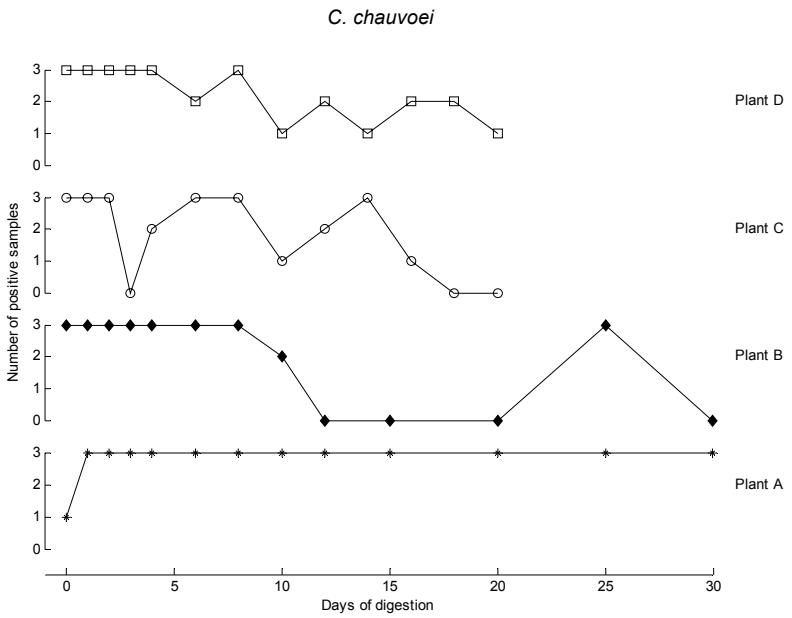

Figure 3. Number of positive vial samples (out of three) during digestion as a function of time and biogas plant for digester substrate inoculated with *Clostridium chauvoei*.

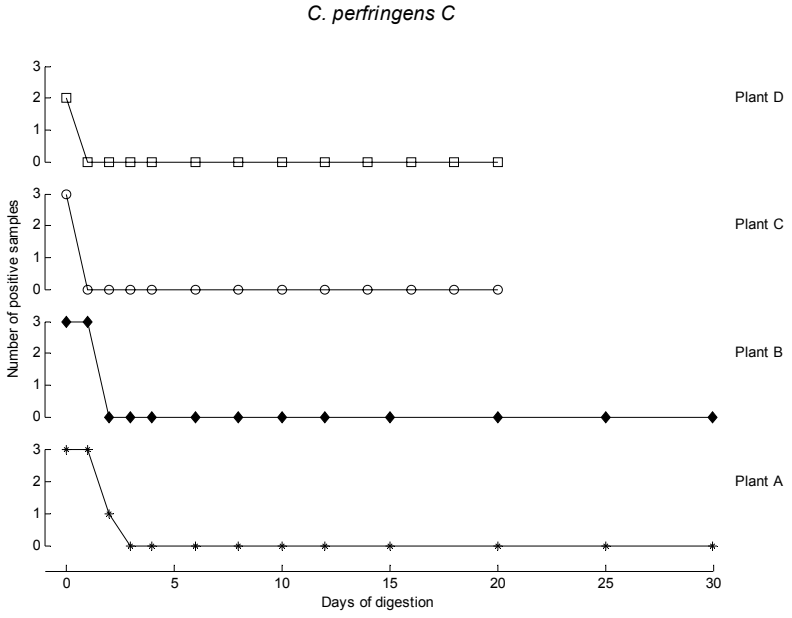

Figure 4. Number of positive vial samples (out of three) during digestion as a function of time and biogas plant for digester substrate inoculated with *Clostridium perfringens* type C.

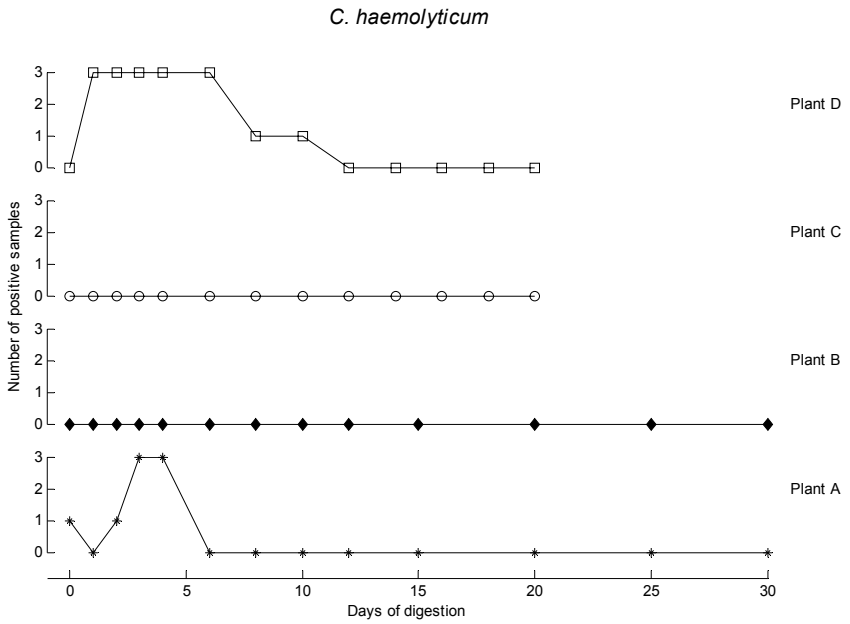

Figure 5. Number of positive vial samples (out of three) during digestion as a function of time and biogas plant for digester substrate inoculated with *Clostridium haemolyticum*.

3.5 Detection levels of PCR for *Clostridium* spp. in biogas plant substrate

The detection level of the method used was 100-200 cfu/g for *Cl. chauvoei*, *Cl. haemolyticum* and *Cl. perfringens* type C in substrates before pasteurisation, after pasteurisation and in digester substrate. For *Cl. septicum* and *Cl. sordellii* the detection level was 1 cfu/g in these substrates.

3.6 PCR

All reference strains used in this study tested positive by PCR with their specific primer pairs and negative with the other primers.

4 Discussion

Full-scale biogas plants received different substrates from day to day, but the composition of carbohydrates, fats and proteins were the same as far as possible in order not to disturb the digester flora. The latter differs between biogas

plants and changes over time. Consequently, bacterial experiments using full-scale biogas plants are difficult to perform due to many uncontrollable parameters. This study was a laboratory-scale investigation, although using substrates from full-scale biogas plants, which can make results difficult to interpret. Furthermore, different species of clostridia have differing prerequisites regarding the conditions for sporulation and germination, *e.g.* temperature, pressure, access to carbohydrates and pH (Volkova *et al.* 1988; Cotter and Hill 2003; Margosch *et al.* 2006). Therefore, it is important to increase our knowledge about the effects of anaerobic digestion on different pathogenic clostridial species. The results of this study showed that the impact of anaerobic digestion does differ between pathogenic clostridial species.

4.1 *Clostridium chauvoei* and *Clostridium perfringens* type C

Clostridium chauvoei could not be detected at the end of the anaerobic digestion for all biogas plants except plant A, at which hydrochloric acid was added to the digester. At plant A *Cl. chauvoei* was detected throughout the digestion. There were no significant differences in pH that could explain the difference, though it could be the effect of a different type of flora in the digester favouring *Cl. chauvoei* in plant A. Bacterial competition in the digester may inhibit *Cl. chauvoei* growth. However, if the condition in the digester does inhibit *Cl. chauvoei,* the risk of spreading the bacteria via biogas plant residues is minimized, except when hydrochloric acid is used during digestion. This is especially true if the digested residues are to be spread in areas free from an endemic problem with blackleg.

Clostridium perfringens type C was detected after pasteurisation, but only during the first few days of anaerobic digestion in substrates from all four plants, irrespective of mesophilic or thermophilic digestion. In the digestion study, we could not establish if *Cl. perfringens* type C existed as spores or as vegetative cells. However, the experiment cannot be repeated with the same substrate due to the live culture of digester substrate. Nor did the number of bacteria used for inoculation of the samples have any influence (Table 3). In this study, *Cl. perfringens* type C apparently does not survive more than a few days during digestion. These results indicate that digestive residues may be spread in the field with respect to *Cl. perfringens* type C with a low risk of disease in pigs. In one other study, *Cl. perfringens* type C was not inactivated by digestion at 35°C or at 53°C in small-scale digesters (Olsen and Larsen 1987). However, more studies need to be done.

4.2 *Clostridium septicum* and *Clostridium sordellii*

Clostridium septicum and *Cl. sordellii* survived the pasteurisation step and thrived in the digester and could be detected throughout the digestion (data not shown). In uninoculated samples, *Cl. septicum* and *Cl. sordellii* were detected both before and after pasteurisation and during anaerobic digestion in all four plants. Since these bacteria occur in the intestinal tract (Timoney *et al.* 1988; Gyles and Thoen 1993) and sometimes in clinical samples from cattle and sheep (Gyles and Thoen 1993), the most probable explanation for the positive uninoculated samples is that *Cl. septicum* and *Cl. sordellii* originated from manure and ABP used in the biogas plants. The presence of *Cl. septicum* in uninoculated vials seemed to be unpredictable (Fig. 2).

4.3 *Clostridium haemolyticum*

Clostridium haemolyticum was detected in only a few samples before and after pasteurisation and only for a short period of days during anaerobic digestion. The quantity bacteria were probably too few in the enrichment broth used for inoculation, particularly when the digestion of samples from plants B and C started. The detection level for *Cl. haemolyticum* was 100 cfu/g and the quantity of bacteria added to the substrate was probably below the detection level (Table 3). This might explain why all samples proved negative. However, the positive control from the FAA proved positive by PCR. The enrichment broth might have been suboptimal. Before this study was started, several enrichment broths were tested (Fastidious anaerobic broth, horse serum, beef broth and TGY). From this pre-testing TGY was found to be the most suitable broth for *Cl. haemolyticum*.

For optimal growth, *Cl. haemolyticum* requires strict anaerobic conditions and tryptophan (Timoney *et al.* 1988). Maybe shortage of tryptophan in the enrichment broth could explain the poor growth of *Cl. haemolyticum*. Due to the lack of *Cl. haemolyticum* in the digestion study, the results from plants B and C should be interpreted with caution, because of the non-detectable quantities of bacteria inoculated and on which the results were based. Unfortunately, the study cannot be repeated with the same substrate, but the results from plants A and D showed that *Cl. haemolyticum* did not survive in the digester. A possible hypothesis is that *Cl. haemolyticum* germinates more easily than other clostridia and therefore they are more easily outnumbered.

4.4 Background flora

In heavily contaminated samples, such as substrates from biogas plants, a method whereby the background flora does not interfere with the result is necessary for detection of specific pathogens. For some pathogenic bacteria, selective media for culturing are available, but unfortunately not for the pathogenic clostridia used in this study, excepting *Cl. perfringens*. PCR primer pairs selected to be specific for each of these clostridia were chosen. Even when the background flora is growing as swarming colonies on the agar plates it is possible to identify the pathogenic bacteria of interest by PCR. Furthermore, the samples can be heated to reduce non spore-forming bacteria.

Clostridium chauvoei, Cl. haemolyticum, Cl. septicum and *Cl. sordellii* are bacteria which usually grow as swarming bacteria on FAA plates. On TSC plates these bacteria will not swarm, but TSC is designed for *Cl. perfringens*. The other four clostridia in this study did grow, but not all thrived on TSC.

The background flora was counted on FAA plates and TSC in both uninoculated and inoculated vials in the beginning, the middle and at the end of the anaerobic digestion and the results were in the range 5-8 $\log_{10}$ cfu/g (data not shown).

4.5 Biogas plants

The mesophilic plants (A and B) in this study had a hydraulic retention time between 30 and 44 days and the thermophilic plants (C and D) had between 25 and 60 days. The average retention time for anaerobic digestion in commercial full-scale plants is 25-30 days. During the study, plant D increased the active volume in the digester, and consequently had a longer retention time and a low gas production on the sampling occasion. However, differences in results could not be observed between the mesophilic plants (A and B) and the thermophilic plants (C and D). Furthermore, in this laboratory study the different kinds of biowaste received by the plants did not affect the results or the survival of the pathogenic spore-forming bacteria.

Digester substrate is a living microbial culture and when performing laboratory studies it is important not to destroy the methane-producing bacterial flora in the material. To avoid interruption of the anaerobic digestion process, the digested material was delivered immediately from the plants and inoculated with the bacterial suspension. That is why the experiment cannot be repeated a second time with the same material.

4.6 PCR for detection and identification of clostridia

From pure cultures DNA is easy to amplify, but problems arise with contaminated samples, such as samples from biowaste. Isolation of pathogenic clostridia based on PCR-specific primer pairs unique for different clostridia is desirable. Detection of DNA by PCR can be hampered by numerous substances, including humic acids, VFA, fats and proteins (Rossen *et al.*1992). However, the cultivation step before PCR reduces the influence of inhibitory substances (Burtscher and Wuertz 2003). When using PCR, the DNA of both viable and non-viable cells is amplified. If only viable cells are to be detected an enrichment step can be applied (Burtscher and Wuertz 2003). In this study, culture on FAA plates before PCR was used to avoid detection of non-viable bacteria and false-positive PCR reactions. DNA from the background flora could inhibit the PCR reaction (Rossen *et al.* 1992). Therefore, an investigation to determine the lowest number of these five pathogenic clostridia that could be detected by PCR in heavily contaminated samples was performed in the detection limit study.

The *Cl. sordellii* primers can give positive signals for species other than *Cl. sordellii*, but these species can be distinguished from *Cl. sordellii* itself by the different size of the PCR products (Kikuchi *et al.* 2002). In this study all PCR products were compared with the size of the PCR product from the positive control when interpreting the results.

4.7 Chemical analysis

The responses to the questionnaire tallied with the results of our chemical analysis, with one exception. Different concentrations of ammonium-nitrogen (NH_4^+-N) (0.36 g/100 g and 0.05 g/100 respectively) were found in the substrate from plant C. These differences may have arisen from the use of differing analytical methods (Tables 2). Both values could have been anticipated and it was concluded that the two different values did not have an influence on the results of this study. The amount of VFA was between <0.01 and 0.21 g/100 g and evaluated as normal for respective plant (data not shown), even if the value 0.21 g/100 g seemed to be high.

4.8 Pathogen survival and environmental aspects

It was expected that at least some of the clostridia tested would survive pasteurisation and anaerobic digestion, since *Cl. perfringens* was detected after pasteurisation and digestion in earlier studies (Larsen *et al.* 1994; Sahlström *et al.* 2008). Even if some clostridia seemed to pass unaffected through the biogas

process, the pasteurisation step before digestion has advantages, as pathogens other than spore-forming micro-organisms, for example *Salmonella* spp., *E. coli* O157 and parasites, are reduced (Bendixen 1996; Sahlström *et al.* 2008). Furthermore, pasteurisation before digestion increases the digestibility of sludge (Skiadas *et al.* 2005).

Three of the inoculated clostridia investigated in this study seemed to be reduced by the biogas plant process. The results may mean that the disease risks associated with spreading of *Cl. chauvoei*, *Cl. haemolyticum* and *Cl. perfringens* type C are negligible.

During anaerobic digestion of inoculated fungi, the number of total fungal cfu does not decrease; rather the diversity of fungal species decreases (Schnürer and Schnürer 2006). This decrease in species diversity was observed in the present study for *Cl. septicum* and *Cl. sordellii*, as both could be recovered in almost pure culture at the end of digestion (data not shown).

In all four plants, the pH was about 8 in the digester. No relation between pH, raw biowaste composition, thermophilic and mesophilic digestion could be established from the results, except for hydrochloric acid and *Cl. chauvoei*. For non spore-forming bacteria, such as *Salmonella* spp., acidic conditions and VFA are inhibitory in mesophilic digestion (Salsali *et al.* 2006).

Many pathogenic clostridia are soil bacteria, including *Cl. botulinum, Cl. chauvoei, Cl. haemolyticum, Cl. perfringens*, *Cl. septicum*, and *Cl. sordellii* (Gyles and Thoen 1993; del Mar Gamboa *et al.* 2005). If digested residues are spread on soil already contaminated with clostridia the risk increase is negligible. But areas with uncontaminated soil, caution should be taken before spreading digested residues. In Sweden there are endemic areas with clostridial diseases in cattle and sheep. To protect grazing animals in these regions from clostridial infections annual vaccination routines are undertaken first. However, *Cl. botulinum* had been detected in manure (Dahlenborg *et al.* 2001, 2003) and can survive composting (Böhnel and Lube 2000). Under certain conditions *Cl. botulinum* produces toxins and further study of the effect on clostridia in biowaste are therefore necessary.

Some spore-forming bacteria appeared to pass through the biogas process unaffected and thus digested residues should be spread with consideration of the risk of spreading diseases. This risk should be compared with the disadvantages of using artificial fertiliser, *i.e.* the limited natural resources of phosphorus (Muga and Mihelcic 2008). In this view the advantage of using pasteurised digested residues as fertiliser may outweigh the risk of spreading diseases.

5 Conclusions

This study has shown that anaerobic digestion has varying effects, depending on the species of pathogenic *Clostridium* spp. However, the results are uncertain and must be confirmed by new studies before further conclusions can be drawn. Species of clostridia can survive the pasteurisation step at biogas plants. Consequently, precautions should be taken before spreading digested residues on farmland. From an ecological viewpoint the spreading of digested residues, view with the risk of causing animal diseases, is more environmentally acceptable than using artificial fertilisers.

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Detection and identification by PCR of *Clostridium chauvoei* in clinical isolates, bovine faeces and substrates from biogas plant

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Abstract

Background: *Clostridium chauvoei* causes blackleg, an acute disease associated with high mortality in ruminants. The apparent primary port of entry is oral, during grazing on pasture contaminated by spores. Cases of blackleg can occur year after year on contaminated pastures. A method to determine the prevalence of *C. chauvoei* spores on pasture would be useful.

The standard method for *C. chauvoei* detection is culture and biochemical identification, which requires a pure culture. In most muscle samples from cattle dead from blackleg the amount of *C. chauvoei* in samples is high and the bacterium can easily be cultured, although some samples may be contaminated. Detection by PCR would be faster and independent of contaminating flora.

Digested residues from biogas plants provide an excellent fertiliser, but it is known that spore-forming baeria such as *Clostridium* spp. are not reduced by pasteurisation. The use of digested residues as fertiliser may contribute to the spread of *C. chauvoei*. Soil, manure and substrate from biogas plants are contaminated with other anaerobic bacteria which outgrow *C. chauvoei*. Therefore, detection by PCR is would be useful. This study applied a PCR-based method to detect of *C. chauvoei* in 25 muscle and blood samples, 114 manure samples, 84 soil samples and 33 samples from the biogas process.

Methods: Muscle tissues from suspected cases of blackleg were analysed both by the standard culture method followed by biochemical identification and by PCR, with and without preculture. To investigate whether muscle tissue samples are necessary, samples taken by swabs were also investigated. Samples from a biogas plant and manure and soil from farms were analysed by culture followed by PCR. The farms had proven cases of blackleg. For detection of *C. chauvoei* in the samples, a specific PCR primer pair complementary to the spacer region of the 16S-23S rRNA gene was used.

Results: *Clostridium chauvoei* was detected in 32% of muscle samples analysed by culture with identification by biochemical methods and in 56% of cases by culture in combination with PCR. *Clostridium chauvoei* was detected in 3 (out of 11) samples from the biogas plants collected before pasteurisation, but samples taken after pasteurisation and after digestion all tested negative. *Clostridium chauvoei* was not detected in any soil or silage samples and only one manure samples tested positive.

Conclusion: The diagnostic method used for *C. chauvoei* was not applicable in estimating the risk of blackleg on particular pastures from manure or soil samples, but found to be highly useful for clinical samples.

Background

Clostridium chauvoei belongs to the histiotoxic clostridia and causes blackleg, a disease associated with high mortality in cattle and sheep. Animals other than ruminants are rarely infected [1-3]. Young growing ruminants on pasture are especially sensitive to *C. chauvoei*. In endemic areas *C. chauvoei* may be present in soil and faeces [2,4,5]. Once pastures have become heavily contaminated, cases of the disease usually occur year after year in susceptible animals [2]. The infection appears to be transmitted by the oral route during grazing or when eating silage or hay contaminated by spores.

Isolation of *C. chauvoei* is difficult, since it must be cultured under strict anaerobic conditions, and samples are frequently contaminated with other anaerobic bacteria that outgrow *C. chauvoei* [6]. The standard method for detection of *C. chauvoei* is based on culture and in most muscle samples from cattle dead from blackleg the amount of *C. chauvoei* is high. However, this is not always the case and contaminant flora may hamper culture. Before biochemical identification, it is important to have a pure culture of the strain. A fast and reliable detection system for *C. chauvoei* is desirable in samples containing a mixed flora. Samples from blackleg cases are sent to the laboratory as muscle tissue, since this is the easiest material from which to extract pure culture. A method not requiring pure culture, samples could be sent to the laboratory as swabs from infected muscle tissues.

Manure can contain bacterial spores and possibly also vegetative bacteria that pass through the digestive systems of animals grazing on contaminated pasture. Manure is part of the substrate for biogas plants, and spore-forming bacteria may survive pasteurisation and digestion [7,8]. An anaerobic digestion process is commonly used for energy production in the form of biogas. The digested residues produced are an excellent fertiliser, rich in plant nutrients, and application of digested residues to agricultural land reduces the need for artificial fertilisers. However, organic wastes contain many different types of biological contaminants. To reduce the risk of spreading pathogens, the recommended biowaste treatment method before anaerobic digestion is heating to 70°C for 60 min if manure and animal by-products are present in the substrate, as regulated by EU Commission regulation EC no 1774/2002 and 208/2006. Heating at 70°C for 60 min reduces *Salmonella* spp. [8,9]. However, some spore-forming bacteria such as *Clostridium* spp. and *Bacillus* spp. are not eradicated [7-10].

Manure and animal by-products from slaughterhouses are sent to local biogas plants. If cattle from high-risk areas shed *C. chauvoei* in faeces or if animal by-products are contaminated with *C. chauvoei*, it is possible to spread the bacteria to previously unaffected areas via biogas plants if contaminated digested residues are spread in areas free from *C. chauvoei*. In soil, spores can survive for many years [11].

In certain areas of south-east Sweden, blackleg is regarded as an endemic disease [12]. A method to determine the prevalence of *C. chauvoei* spores on pasture could also serve as an indication of the risk of blackleg, enabling farmers to design herd-specific vaccination programmes. In areas where blackleg is endemic, it is both an economic problem for farmers and an animal health problem. Affected farms usually have vaccination routines, but farmers seek to minimise the use of vaccine [3,12]. It would help farmers to know where on their farm the prevalence of *C. chauvoei* is high, in order to focus vaccination routines on animals grazing high-risk pastures.

The aim of this study was to investigate the suitability of using PCR for detecting *C. chauvoei* in muscle tissue taken at autopsy and investigate the prevalence of *C. chauvoei* in faeces, soil, biogas substrate and digested residues samples.

Methods

Collection of samples from suspected cases of blackleg

One blood sample from 2005, five muscle samples from 2006, six muscle samples from 2007 and 13 muscle samples from 2008 were included in the study. Muscle tissues and a blood sample from cattle with clinical symptoms of suspected blackleg were sent by ordinary postal services to the bacteriological laboratory at the National Veterinary Institute (SVA). Muscle tissue samples were transported in sealed plastic bags and blood sample in a Vacutainer® tube. These samples were cultured anaerobically at 37°C for 48 h on Fastidious Anaerobic Agar plates (FAA, LabM, Bury, Lancashire, England), with 5% defibrinated horse blood. The bacteria isolated were identified by biochemical methods according to standard methods at SVA, which comprise fermentation of glucose, maltose, lactose, sucrose, starch, mannitol and fructose, and production of lecithinase, tryptophanase, urease and hydrolysis of aesculin. One loopful (approximately 5 μL of bacteria collected with a 10 μL loop) was collected from each FAA plate by a streak over the swarming flora (mixed, non-discrete colonies). The loopful was used for DNA preparation followed by PCR detection. After routine analysis for *C. chauvoei*, the muscle samples were stored at -20°C and the blood sample was taken for DNA preparation directly.

The following samples was used for DNA preparation followed by PCR detection

a) Muscle piece, approximately 1 g.

b) A muscle piece, approximately 1 g, added to 500 μL of sterile physiological saline, macerated and left in the water for 5 min. The muscle piece was then removed from the water, and the remaining water was analysed.

c) Meat juice (500 μL), collected from the bottom of the muscle tissue storage bottle.

d) Blood sample (500 μL).

Muscle samples collected by swabs

Specimens were taken from most of the muscle tissues (22) by swabs (Amies' medium with charcoal, Nordic Biolabs, Täby Sweden) and a postal service comprising 1, 3 and 6 days (3 swabs/-muscle tissue) was simulated. The samples were taken in the centre of the muscle tissue and after sampling the swabs were left on a bench at room temperature (approximately 20°C) during the simulated postal service. After 1, 3 and 6 days the swabs were streaked onto FAA plates and incubated at 37°C for 48 h under anaerobic conditions. One loopful of colony material was used for DNA preparation.

Collection of samples from biogas plants

Samples from a biogas plant located in south-east Sweden were taken weekly at 11 different occasions before pasteurisation, after pasteurisation and after digestion. None of farms A-K tested (see below) sent manure to the biogas plant, but these farms did send their animals to the local slaughterhouse. This slaughterhouse sent animal by-products to the biogas plant. Approximately 200 g of samples were collected in clean pots on each sampling occasion. The samples were stored at -20°C until analysis.

Five g of each biogas plant sample were placed in a separate bottle and 10 mL of sterile physiological saline were added. The bottles were shaken thoroughly and left for 30 min., allowing the material to settle. Two mL of the supernatant were heated at 65°C for 10 min and two mL were enriched in Fastidious Anaerobic Broth (FAB, LabM, Bury, Lancashire, England), and heated at 65°C for 10 min. An aliquot from each one was then streaked onto FAA plates and incubated in an anaerobic jar at 37°C for 48 h. One loopful of colony material was collected for DNA preparation.

Collection of samples from farms

Manure, soil and silage

Eleven dairy farms (A-K) from the island of Öland of south-east Sweden were included in this study (Table 1). Blackleg is endemic on this island [12]. All samples from farms A-I were collected at one occasion during a visit to the island. Farms J and K reported cases of blackleg after the visit and were subsequently included in the study. *Clostridium chauvoei* had been detected in muscle samples from these two farms collected at necropsy. On farms G, H, I and J, individual faecal samples were taken from the floor behind housed cows. On all other farms, the faeces samples were collected outdoors as cowpats on grassland. Soil samples were collected from all farms. Depending on the ground, grassland or soil, between three to eleven samples were collected on each farm. Two farms (A and B) grazed their animals on a common pasture by the seashore. For these farms, only three soil samples were taken around the cattle house and eleven soil samples were taken from the common pasture. All samples were collected during the grazing period. In total, 114 faecal samples, 84 soil samples and four silage samples were collected for analysis. The weight of each the sample was approximately 100–200 g and they were collected in clean pots. The samples were stored at -20°C until further analysis.

Table 1: Description of farms A-K from which samples were obtained for the study

Farm	A	B	C	D	E	F	G	H	I	J	K
No. cows	130	72	50	24	120	40	70	50	130	120	nk
No. heifers	150	140	80	30	130	80	100	45	260	nk	nk
Cases of blackleg	14	nk	1	2	5–6	5–6	10	10	6–8	1–2	3
Year of occurrence	2002	2002	2001	2001	2003	2001	2000 2001 2002	2004	2003 2004	2005 2006	2006
Affected animals	heifers	calves, heifers	heifers	heifers	heifers	1 cow 4–5 heifers	heifers	calves	heifers	heifers	nk
Vaccination performed	yes	yes	yes	yes	yes	yes	yes	no	no	no	no
No. of faeces samples	10	10	10	10	10	7	10	10	10	20	7
No. of soil samples	3*	3*	3	9	5	8	10	6	11	11	4
No. of silage samples	-	-	-	-	-	-	-	-	-	-	4

nk = Not known

* For farms A and B, 11 soil samples were taken from a common pasture by the seashore plus three soil samples were taken around each cattle house.

Five g of each sample of manure, soil and silage were placed in a separate bottle, 10 mL of sterile physiological saline were added and these samples were prepared by the same procedure as the biogas samples (See previous chapter).

Data collection from farms

On each sampling occasion, data concerning herd size, vaccination routines, number of samples and history of blackleg were collected using a questionnaire (Table 1). Data were also collected on other aspects such as feeding, manure-spreading practices and grazing routines (data not shown).

DNA preparation

During preparations for this project, four different DNA preparation methods were evaluated. The DNA preparation methods were phenol/chloroform, boiled lysate and two commercial kits (UtraClean™ Soil DNA Isolation Kit, Mo Bio labs, Solana Beach, California, USA and Genomic DNA purification Kit, Fermentas, Burlington, Canada) for which the manufactures' recommendations were followed.

The colony material from FAA plates, blood, muscle pieces, muscle pieces macerated in sterile physiological saline and meat juice were suspended in 500 μL of phosphate-buffered saline (PBS, Merck, Darmstadt, Germany) and centrifuged for 10 min at 7,200 × g. The supernatant was discarded and the pellet was washed again by the same procedure. The pellet was thereafter re-suspended in 250 μL of sterile H_2O. The bacterial cells were lysed by boiling the suspension for 15 min before storage at -20°C until further analysis. This lysate was used as template in PCR.

PCR

For detection and identification of *C. chauvoei* in the samples, a specific PCR primer pair complementary to the spacer region of the 16S-23S rRNA gene was used as described by Sasaki *et al*. [13,14].

The amplicons were analysed by electrophoresis in agarose gels (1.5% Agarose NA, from GE Healthcare, Uppsala, Sweden) which were stained with ethidium bromide. The PCR products were visualised under UV-light. The size of the amplicon was 509 bp [13]. Boiled lysate of *C. chauvoei* AN 2548/02 was used as a positive control. Closely related clostridia such as *Clostridium septicum* and *Clostridium perfringens*, which can occur as contaminants in clinical infections of *C. chauvoei*, were used as negative controls.

To confirm that the PCR product of *C. chauvoei* AN 2548/02 originated from *C. chauvoei*, further analysis was performed by 16S rRNA gene sequencing. The PCR product was diluted and cycle sequencing reactions were carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The nucleotide sequences were determined with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences were merged into one contig using the programme ContigExpress, Vector NTI suite ver. 9.0. To compare the sequences, similarity searches were done in GenBank [15].

Detection threshold of the PCR method

Preparation of the strain

One loopful of *C. chauvoei* (AN 2548/02) was inoculated into 10 mL of serum broth. The broth was incubated at 37°C for 48 h under anaerobic conditions. In order to stimulate the production of spores, the broth was then kept at room temperature for further 5 days as described by Båverud *et al*. [16]. The concentration of bacteria was checked by the viable count method. The numbers of bacteria were quantitatively analysed using a ten-fold dilution series in peptone saline solution, which was then streaked (0.1 mL) onto FAA plates and incubated anaerobically at 37°C for 48 h. After incubation the number of colonies was counted. The quantitative dilution experiment was performed twice.

Clostridium chauvoei *spiked samples*

In order to determine the detection level of the PCR method spiked samples of cattle manure, soil, silage and substrate from a biogas plant were analysed. The samples from the biogas plant were from both before and after pasteurisation and from digested residues. Soil and manure were taken from an area free of blackleg as well as the biogas plant samples.

From the bacterial suspensions, 10-fold dilutions were made in peptone saline solution. One mL from each dilution step between 1 and 7 was thoroughly mixed with 5 g of from faeces, soil or silage substrate. Five g of biogas substrate before and after pasteurisation were mixed with 1 mL of the bacterial suspension from each dilution step between 1–5 and digested residue was mixed with bacterial suspension from dilution steps between 1 and 6. All bottles were agitated and then left to settle for 30 min. The detection thresholds for *C. chauvoei* for each stage in the biogas process were estimated using ten-fold dilutions in peptone saline solution followed by total viable counts onto FAA plates (0.1 mL) incubated at 37°C for 48 h under anaerobic conditions. For confirmation of the strains, one loopful from each agar plate was used for DNA preparation after incubation. All dilution series were made in duplicate.

Results

Samples from suspected cases of blackleg

Clostridium chauvoei was detected in 8 out of 25 (32%) of the samples from the suspected cases of blackleg analysed by culture methods and identification by biochemical methods. For PCR in combination with culture on FAA plates, 14 out of 25 (56%) of samples were above the detection threshold. In muscle pieces or blood without any preculture 3 out of 24 (12%) samples were above the detection threshold of PCR, while in meat juice 2 out of 23 (9%) samples were above the detection threshold and in muscle pieces macerated in sterile physiological saline 6 out of 23 (26%) samples were above the detection threshold (Table 2).

Muscle samples collected by swabs

In 22 muscle tissues cultured on FAA plates, 14 (64%) were above the detection threshold of PCR, while 11 (50%) swabs from the same 22 muscle tissues were above the detection threshold (Table 3).

Samples from biogas plants

Three samples from the biogas plants taken before pasteurisation were above the detection threshold of PCR for *C. chauvoei*, but samples taken after pasteurisation and after digestion were all below the detection threshold (Table 4).

Samples from farms

All field samples of faeces (114) except one, soil (84) and silage (4) were below the detection threshold of the PCR method for *C. chauvoei*. One faecal sample was above the detection threshold (Table 4).

PCR

From the evaluated DNA preparation methods the most reliable method was boiled lysate with previous culture on FAA plates. For phenol/chloroform and the two commercial kits, the detection threshold was approximately ten times higher than for boiled lysate (data not shown). DNA concentrations were not measured in the DNA template of boiled lysate because of the mixed flora.

Table 2: Comparisons of *Clostridium chauvoei* detected by different methods

Sample	Material	Culture followed by biochemical identification	PCR on culture colonies	PCR on muscle piece or blood	PCR on meat juice	PCR on muscle piece macerated in sterile physiological saline
AN 3656/05	blood	+	+	-	nd	nd
AN 165/06	muscle	+	+	+	-	+
AN 1106/06	muscle	+	+	-	-	+
AN 1717/06	muscle	+	+	-	-	-
AN 1818/06	muscle	-	-	-	-	-
AN 2091/06	muscle	-	+	-	-	-
B 16476/07	muscle	-	+	+	-	-
B 17520/07	muscle	-	-	-	-	-
B 19034/07	muscle	-	-	-	-	-
P 2500/07	muscle	+	+	nd	nd	nd
P 2855/07	muscle	-	+	-	+	+
P 4822/07	muscle	-	-	-	-	-
P 2446/08	muscle	-	+	-	-	-
B 29245/08	muscle	-	+	-	-	+
B 32286/08	muscle	-	-	-	-	-
P 3086/08	muscle	+	+	-	-	-
P 3240/08	muscle	-	-	-	-	-
B 41901/08	muscle	-	-	-	-	-
B 41902/08	muscle	-	-	-	-	-
B 43014/08	muscle	-	-	-	-	-
B 43531/08	muscle	-	-	-	-	+
P 3837/08	muscle	-	-	+	-	+
B 46320/08	muscle	+	+	-	-	-
P 4324/08	muscle	-	+	-	+	-
P 4325/08	muscle	+	+	-	-	-
Summary		8+/25	14+/25	3+/24	2+/23	6+/23

nd = Not determined

Comparisons of *Clostridium chauvoei* detected by culture followed by biochemical identification culture followed by PCR and direct PCR on muscle tissue from clinical samples of blackleg.

Table 3: Comparison between muscle tissue samples and swab samples

Sample	Culture followed by biochemical identification	Summary of PCR results from Table 2	1 day	3 days	6 days
AN 165/06	+	+	-	-	-
AN 1106/06	+	+	+	+	+
AN 1717/06	+	+	-	+	-
AN 2091/06	-	+	-	-	-
B 16476/07	-	+	+	+	+
B 17520/07	-	-	-	-	-
B 19034/07	-	-	-	-	-
P 2855/07	-	+	+	+	+
P 4822/07	-	-	-	-	-
P 2446/08	-	+	-	-	-
B 29245/08	-	+	+	+	+
B 32286/08	-	-	-	-	-
P 3086/08	+	+	+	+	+
P 3240/08	-	-	-	-	-
B 41901/08	-	-	-	-	nd
B 41902/08	-	-	-	-	nd
B 43014/08	-	-	-	-	nd
B 43531/08	-	+	-	+	-
P 3837/08	-	+	-	-	+
B 46320/08	+	+	+	+	+
P 4324/08	-	+	+	-	+
P 4325/08	+	+	+	-	+
Summary	6+/22	14+/22	8+/22	8+/22	9+/19

nd = Not determined

Muscle tissue samples from suspected cases of blackleg. *Clostridium chauvoei* were analysed by culture and biochemical identification, culture and PCR and swab samples from the same muscle samples were analysed by culture and PCR. The swabs were used to simulate a postal service simulation by keeping them on the bench for 1, 3 and 6 days.

The positive control, *C. chauvoei*, strain AN 2548/02, was included in all PCR analyses and it always proved positive as evaluated from electrophoresis in agarose gels. PCR products from closely related clostridia such as *C. septicum* and *C. perfringens* were not detected by the specific primer pair used for detecting of *C. chauvoei*.

Sequence analysis of the 16S rRNA gene of strain AN 2548/02 and similarity searches in GenBank showed that it was identical to *C. chauvoei*, strain ATCC 10092^T [GenBank: U51843].

Table 4: Detection of *Clostridium chauvoei* by PCR in manure, soil, silage and biogas plant substrate samples

Material	No. of samples	No. of positives
Manure	114	1
Soil	84	0
Silage	4	0
Before pasteurisation	11	3
After pasteurisation	11	0
After digestion	11	0

Detection threshold of the PCR method

The detection thresholds for samples consisting of manure, soil, silage and biogas substrate before and after pasteurisation and digested residues were 200 colony forming units (cfu)/g.

Discussion

Samples from suspected cases of blackleg

For muscle samples from suspected cases of blackleg, the results from culture on FAA plates followed by PCR from colony material were in agreement with, or performed even better, than the biochemical identification method (Table 2). These results are consistent with Uzal *et al.* [17]. This study demonstrated that DNA preparation from culture before PCR gave better results than PCR applied directly on biomass.

Culture followed by biochemical identification methods is complicated in samples with a high content of contaminating flora because of the need for pure culture and the sensitivity was substantially lower than for culture and PCR. In eight cases it was impossible to detect *C. chauvoei* by culture and biochemical identification but the organism was detected by PCR (Table 2). For making a diagnosis of blackleg, muscle tissue is taken at autopsy since the

amount of *C. chauvoei* probably is higher in muscle tissue than in blood. The single blood sample was incorrectly taken, but the question was blackleg.

Likewise, in a previous study of a blackleg outbreak, culture and detection by PCR gave better results than biochemical analysis [18]. In one of the cases, primary culture of one muscle on a blood agar plate was overgrown by *C. septicum* but gave clear positive results for *C. chauvoei* in the PCR.

In most clinical cases of blackleg the amount of *C. chauvoei* in infected tissue is high and can easily be detected by the PCR method independent of contaminating flora. Moreover, the PCR method is much faster than the traditional biochemical detection method, which takes at least five days, and it is important to have a pure culture of the strain, which might require extra time for culture. To avoid contaminating flora, the muscle tissue should not be too small; approximately 100–200 g would be adequate. Without pure culture no strains can be for subsequent studies in the future, which is a disadvantage of using PCR detection as the sole method.

In cattle experimentally infected with *C. chauvoei*, PCR analyses were carried out on minced muscle and other organs and *C. chauvoei* was detected in all organs tested [19]. Minced muscle pieces from suspected cases of blackleg were directly tested by PCR and compared with culture. The detection by PCR gave better results than culture and biochemical identification [19], which is in disagreement with the results in the present study where only 12% (3 out of 24) of the blood or muscle pieces were above the detection threshold of PCR, while 9% of the meat juice samples were above the detection threshold (Table 2). Inhibitory substances are probably present in muscle tissues. Inhibitors for the enzymatic reaction of PCR amplification have been identified in liver, spleen and kidney [20] and there may be similar inhibitors present in muscle tissue. The results from muscle pieces macerated in sterile physiological saline samples (26%) did not correspond well to those of the biochemical test. Direct PCR on muscle tissue or other similar samples is probably not suitable for replacing culture steps followed by biochemical tests or by PCR.

Muscle samples collected by swabs

To investigate whether it is necessary to send muscle tissue for analysis of *C. chauvoei* or whether it is sufficient to use swabs for sample collection, a postal service was simulated. Of the 22 muscle tissues investigated, 64% were above the detection threshold of PCR. For swabs from the same samples after the postal service simulation, 36% to 47% swabs were above the detection threshold (Table 3). It would be more practical to take samples with swabs instead of muscle tissues if swabs are sufficient for analysis of *C. chauvoei*. However, muscle tissues gave more positive samples than swabs when both were analysed by culture and PCR (Table 3). The incidence of *C. chauvoei* on swabs from the positive muscle tissue cases seemed to be random. However, it appeared to make little or no difference whether the swabs were stored for 1, 3 or 6 days.

Samples from biogas plants

Clostridium chauvoei was detected in 3 samples out of 11 in biogas substrate before pasteurisation but it could not be detected after pasteurisation or after digestion (Table 4). The bacteria in these kinds of samples could originate from manure or from animal by-products from the slaughterhouse, as both these sources are used for biogas production. None of the farms sampled in this study send their manure to the biogas plants due to the geographical distance, but animals are sent to the local slaughterhouse. Cases of blackleg have also been reported in the catchment area of the biogas plant.

After biogas processing, the digested residues are used as fertiliser. Spore-forming bacteria can survive pasteurisation and digestion in biogas plants [7,8] and digested residues from biogas plants may therefore be capable of spreading blackleg to new areas. However, no *C. chauvoei* was detected in processed product so perhaps the environment in the digester is not suitable for *C. chauvoei*, or the flora in the digester outgrows *C. chauvoei*. Clostridial spores can survive for long periods in soil [11], which has to be taken into account in further studies on *C. chauvoei* in digester.

Samples from farms

In spite of the fact that all farms investigated had reported suspected cases of blackleg in recent years, all samples from faeces, soil and silage tested negative for *C. chauvoei*. There could be several explanations for this. Spores could still be present in the material, but below the detection level. Moreover, PCR inhibitors may be present in the samples and the PCR can be hampered [21]. Cattle faeces contain volatile fatty acids (VFA) and the soil samples may contain traces of metal, both of which are known to interfere with the PCR reaction. However, the culture step before PCR reduces the influence of inhibitory substances.

Since *C. chauvoei* is a soil bacterium some seasonal variation may occur, for example heavy rainfall may contribute to spreading of the spores [22] and the amount of spores in the soil can be more accessible to cattle. Most sampling was carried out in early autumn when the weather was sunny and thus the concentration of spores in the samples would be expected to be low. The number of samples may have been insufficient for detection of environmental contamination. However, for practical reasons, very high

numbers of environmental samples are rarely available from the field.

Suspected cases of blackleg on the farms had primarily occurred in calves and heifers but one farm reported a case in an adult cow. Seven of the eleven farms A-K had vaccination routines in place at the time of sampling (Table 1).

Two of the farms stored manure before spreading and the others did not, but none of the farms spread manure on pasture. Local veterinarians have reportedly seen more cases of blackleg after shrubbery clearing on the island of Öland, especially on Alvaret (a barren limestone plain, almost 40 km long and 10 km wide, with special flora depending on the thin soil and high pH). Alvaret is usually used as pasture by neighbouring farms. Not much shrubbery clearing had been done in the year of sampling.

PCR

Cultural procedures are expensive and time-consuming, and contamination with other anaerobic bacteria that outgrow *C. chauvoei* frequently causes problems [17,19]. When using PCR, the DNA of both viable and non-viable cells are amplified. If only viable cells are to be detected an enrichment step can be applied [17,23]. In this study culture on FAA plates before PCR was used to avoid detection of non-viable bacteria and false positive PCR reactions. DNA is easy to amplify from pure cultures, but problems arise with contaminated samples, such as samples from biowaste. Detection of DNA by PCR can be hampered by numerous substances including humic acids, VFA, fats and proteins [21]. However, the culture step before PCR was used as an enrichment step and, therefore, reduces the influence of inhibitory substances. Due to the swarming flora, no purification from the FAA plates could be done.

Detection threshold of the PCR method

The detection level was 200 cfu/g. This may seem to be a poor detection level but it can be explained by the fact that samples such as manure, soil and biogas substrate are heavily contaminated by the surrounding flora. Sasaki [19] reported a detection level of 10 cfu/g, apparently in cleaner samples. However, since the detection level in our study was 200 cfu/g and since such samples were expected to contain a low numbers of *C. chauvoei*. Thus, the method used in the present study is not practically applicable.

Environmental aspects

The detection threshold of the PCR- method after preculture is hardly at current stage suitable for guaranteeing that animals are free from infection or for determining the status of pastures. Instead the currently applied recommendations have to be used, since the method cannot be used as a basis for vaccination routines. In areas where blackleg is endemic, annual vaccination before grazing is recommended, in spite of the costs and the time required.

This study gave some indication that *C. chauvoei* does not pass through the biogas process. The number of *C. chauvoei* in biowaste perhaps decreased in the digester to below the detection threshold of the method and the risk of spreading digested residues is thus negligible. More studies about clostridial survival through the biogas process are needed before definite conclusions can be drawn. However, the advantages of digested residues as a fertiliser perhaps outweigh the risk of spreading blackleg.

Conclusion

In samples of affected muscular tissue taken at autopsy culture in combination by PCR seemed to be faster, simpler and safer than the conventional analysis by culture and biochemical identification of *C. chauvoei*. However, the corresponding use of PCR on manure and soil samples is not practically applicable as a possible tool for detecting *C. chauvoei* before moving or selling animals from contaminated areas to disease-free areas or for determining the risk of blackleg on particular pastures.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EB and SSL designed the study and EB performed the practical analyses and writing the manuscript, with some assistance from SSL. KEJ was responsible for the PCR and sequence methods, and discussed and approved the final manuscript.

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