Colostrum quality, intestinal microbiota and implications for health in young dairy calves

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Cover: Newborn Calf on Smallholder Dairy Farms in Southern Vietnam
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Abstract

The early life of calves in southern Vietnam and in Sweden were studied in this thesis, with the focus on colostrum quality, gut microbial communities and antimicrobial-resistant *Escherichia coli*. The first of four studies showed that most cows on smallholder farms in Vietnam produced colostrum of good quality and that only around 10% of calves suffered from failure of passive immune transfer. In a second study, bacterial communities in colostrum and in calf faeces samples collected at birth and at 14 days of age were identified by terminal restriction fragment length polymorphism (T-RFLP). The results showed that the microbial communities in colostrum and faeces differed in composition, with greater similarities in composition between colostrum and faeces from newborn calves than faeces from 14-day-old calves. Microbial composition also differed significantly between faeces from newborn and 14-day-old calves. Sequencing of 16S rRNA gene amplicons from a subset of faeces samples from healthy calves and calves treated for diarrhoea with antimicrobial drugs revealed significant differences between the groups, with higher relative abundances of *Faecalibacterium* and *Butyricicoccus* in healthy calves. In a third study, bacterial composition in intestinal contents and in mucosal scrapings collected from various gut segments in two- and seven-day-old calves was characterized by sequencing 16S rRNA gene amplicons using Illumina MiSeq. It was found that microbial community composition was associated with intestinal segment, with major differences in composition between proximal and distal parts of the gastrointestinal tract. However, microbial composition showed high similarity for segments in close proximity within the gut. *Lactobacillus* was present in quite high levels in the abomasum and duodenum, whereas *Escherichia* were more associated with the ileum, caecum, colon and faeces. Microbial composition and diversity altered with age and, although the microbiota in mucosa and gut content remained similar in general, certain microbial groups dominated in either mucosa or gut content. The fourth study revealed high levels of multidrug-resistance in *Escherichia coli* isolated from Vietnamese calves (53% of isolates tested), with $blac_{CTM\text{-}M\text{-}1}$ (2 isolates), $blac_{CTM\text{-}M\text{-}9}$ (1 isolate) and $blac_{CMY\text{-}2}$ (1 isolate) being the main β-lactamase resistance groups detected. There was a high incidence of plasmid-mediated quinolone resistance (PMQR) genes in the *E. coli* isolates (21%). Overall, these results suggest that colostrum feeding and health are key factors affecting gut microbial community in the early life of dairy calves, but that antimicrobial resistance is an emerging problem on smallholder dairy farms in Vietnam.

Keywords: Colostrum, passive immune transfer, antimicrobial resistance, extended spectrum cephalosporinases, plasmid mediated quinolone resistance, gastrointestinal tract, microbial diversity.

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Dedication

To my parents with my respectful gratitude,
To my beloved husband Vo Lam,
and my lovely children:
Vo Huu Trong,
Vo Thuy Thuy Vy.

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 Hang, B.P.T.*, Dicksved, J., Svennersten Sjaunja, K. & Wredle, E. (2017). Colostrum quality, IgG absorption and daily weight gain of calves in small-scale dairy production systems in southern Vietnam. *Tropical Animal Health and Production* 49(6), pp. 1143-1147.

II Hang, B.P.T.*, Wredle, E. & Dicksved, J. (2019). Analysis of the developing gut microbiota in young dairy calves – impact of colostrum microbiota and gut disturbances (manuscript).

III Arapovic, L., Hang, B.P.T., Hernandez C. E., Rustas, B-O., McGuire M.A. & Dicksved, J*. (2019). Development of the microbiota along the gastrointestinal tract of calves in early postnatal life (manuscript).

IV Hang, B.P.T.*, Wredle, E., Börjesson, S., Svennersten Sjaunja, K., Dicksved, J. & Duse, A. (2019). High level of multidrug-resistant *Escherichia coli* in young dairy calves in southern Vietnam. *Tropical Animal Health and Production*. https://doi.org/10.1007/s11250-019-01820-6

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The contribution of Bui Phan Thu Hang to the papers included in this thesis was as follows:

I. Was involved in planning the study. Developed the questionnaire with input from the co-authors and performed the survey with the assistance of Vietnamese colleagues. Performed the majority of the laboratory work, analysed the results in collaboration with the supervisors and wrote the manuscript with regular input from the co-authors. Corresponded with the journal and revised the article under supervision.

II. Was involved in planning the study. Performed some of the laboratory work and analysed the results in collaboration with the supervisors. Wrote the manuscript with regular input from the supervisors.

III. Analysed the results obtained in the practical study and laboratory work in collaboration with the supervisors and the co-authors. Provided input on the manuscript as a co-author.

IV. Was involved in planning the study. Performed some of the laboratory work and analysed the results in collaboration with the supervisors and the co-authors. Wrote the manuscript with regular input from the co-authors. Corresponded with the journal and revised the article under supervision.

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Abbreviations

ANOSIM	Analysis of similarity
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECOFFs	Epidemiological cut-off values
ESC	Extended-spectrum cephalosporinases
FPT	Failure of passive transfer
IgG	Immunoglobulin G
MIC	Minimum inhibitory concentration
pAmpC	Plasmid-mediated AmpC
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PMQR	Plasmid-mediated quinolone resistance
RID	Radial immunodiffusion
rRNA	Ribosomal ribonucleic acid
SCC	Somatic cell count
SE Asia	Southeast Asia
TLR2	Toll-like receptor 2
TPC	Total coliform plate count
TRF	Terminal restriction fragment
T-RFLP	Terminal-restriction fragment length polymorphism
WGS	Whole genome sequencing

1 Introduction

The demand for milk and dairy products in tropical Asia has grown rapidly over the past 30 years (Moran & Morey, 2015). In response, Vietnam has achieved strong dairy development through government support (National Dairy Development Plan, 1990). In 2008, a new National Dairy Development Strategy was implemented, with the aim of encouraging rapid scaling up of dairy production in Vietnam (PM, 2008). Within this strategy, some dairy companies, such as Vinamilk, TH Milk and Dutch Lady, have set up their own large-scale farms (Hostiou *et al.*, 2016). However, smallholder farms dominate dairy production in Vietnam and generate around 90% of total domestic milk production (DLP, 2015). The total number of dairy cattle in Vietnam has increased dramatically in the past few decades, from 35,000 head in 2000 to 275,300 head in 2015. Milk production has also increased significantly, from 12 thousand tons in 1990 to 723.2 thousand tons in 2015 (DLP, 2015; MARD, 2007). However, domestic milk production still only meets around 30% of the total demand in Vietnam (DLP, 2015). The dairy industry, including dairy farms, therefore has great potential to increase, since demand is currently not covered by the sector.

The health and growth of the dairy calf are of key importance for its future production capacity, so appropriate management of newborn calves is an important factor to consider for optimal and sustainable milk production (Krpálková *et al.*, 2014; Indra *et al.*, 2012; Furman-Fratczak *et al.*, 2011; Godden, 2008). The practice of feeding neonatal calves colostrum of high quality in sufficient amounts within a few hours after birth has the ability to decrease calf mortality, promote growth rate and increase future milk production (Indra *et al.*, 2012; Godden, 2008). Moreover, it has been observed that the gut microbiota influences the health (Malmuthuge *et al.*, 2015b; Conroy *et al.*, 2009) and weight gain in newborn calves (Malmuthuge *et al.*, 2015b). Researchers have shown in pigs and humans that neonatal management plays an important role in shaping the early gut microbial communities (Unger *et al.*, 2014; Mulder

et al., 2011). The gut microbiota can be affected by many factors. For example, the type (fresh, heated) and quality of colostrum can influence microbial colonisation of the calf gut (Lima *et al.*, 2017; Malmuthuge *et al.*, 2015a; Fecteau *et al.*, 2002). Moreover, administration of antimicrobials can cause long-term disturbances in the gut microbiota (Panda *et al.*, 2014). Antimicrobial consumption without prescription is very common in countries in Southeast Asia (SE Asia) (Holloway *et al.*, 2017), including Vietnam. This prolific antimicrobial usage has contributed to high levels of antimicrobial-resistant bacteria (Pereira *et al.*, 2014a). For example, it has resulted in high prevalence of antimicrobial-resistant bacteria in chickens in countries in SE Asia (Usui *et al.*, 2014).

Since dairy production is mainly based on smallholder farms and is not a traditional system in Vietnamese agricultural production, farmers and advisory workers need to increase their knowledge about management practices, especially the importance of early-life calf management and of replacement animals (Moran *et al.*, 2016; Moran, 2013). Management of dairy calves on smallholder dairy farm in Vietnam, especially colostrum feeding and its impacts on gut health, has not been fully evaluated.

1.1 Dairy cattle production in Southeast Asia

Dairy production in most countries in SE Asia is still developing to meet the demand arising from increasing consumption in the region. Most dairy animals in the region are crossbreeds (local breeds crossed with breeds from temperate countries) and are kept on small-scale farms in herds of usually less than 10 cows (Ty *et al.*, 2018; Moran, 2013; Lam *et al.*, 2010). Many tropical countries import frozen semen from the Holstein Friesian breed to inseminate local cows, within breeding programmes for improved milk yield (Ty *et al.*, 2018; Konkruea *et al.*, 2017; Lam *et al.*, 2010). However, in the Philippines and southern Vietnam, dairy cows still have low breeding efficiency due to poor breeding management and high environmental temperatures (Lam *et al.*, 2010; Alejandrino *et al.*, 1999; Cavestany *et al.*, 1985).

On smallholder farms in tropical countries, milking is done by machine or hand, depending on the labour available, level of production, technical support and electricity supply (Chantalakhana & Skunmun, 2002). Milking management and udder health are important factors for the production capacity of smallholder dairy farms. For example, Lam et al. (2010) found in a study performed in Vietnam that high milk somatic cell count (SCC) was associated with poor milking management on farms. High SCC in general is undesirable, since it is associated with decreased milk production (Auldist & Hubble, 1998), changes

in milk quality (Lam et al., 2010; Harmon, 1994), reduced welfare (Fregonesi & Leaver, 2001) and impaired food safety (Ruegg, 2003).

Feeding is another management factor to consider. A study on feeding management in Indonesia found that imbalanced calcium to phosphorus (Ca:P) ratio and an inadequate supply of water to dairy cows are among the key factors explaining the low level of milk production in that country (Kusumanti, 2016). The routine used to offer drinking water has been found to affect herd milk SCC significantly (Lam *et al.*, 2011). In that study, herds in which cows were provided drinking water *ad libitum* had lower milk SCC (around 400,000 cells/mL) than herds in which cows were offered drinking water restrictedly (860,000 cells/mL). In southern Vietnam, around 35% of dairy farmers offer drinking water *ad libitum* to cows, using a separate trough (Lam *et al.*, 2010). Restricted water supply contributes to decreased dry matter intake, reduced milk yield and loss of body weight in dairy cows (Heinrichs & Radostits, 2001).

However, one of the most important management factors for sustainable production is the management of the newborn calf, which is often neglected. It is common practice in southern Vietnam for more than 70% of smallholder dairy farms to feed their calves with milk from buckets, while the remaining farms practice restricted suckling methods (Lam *et al.*, 2010). Heifer calves are kept for replenishing the herd, and are fed two to three times per day at milking time, with a total amount of 4-6 kg milk, while bull calves are sold after birth (Lam *et al.*, 2010). Pre-weaning calf mortality is within the range 15-25% on tropical dairy farms, while it is less than 5% on American farms and around 3% on Australian and Swedish farms (Moran, 2011; Linden *et al.*, 2009; Svensson *et al.*, 2006b). According to Suzuki (2005), calf mortality on Vietnamese farms is 7%. Gastrointestinal disorders are the main cause of death, being responsible for 58% of cases during the pre-weaning period (Azizzadeh *et al.*, 2012). Around 50% of pre-weaning calf mortality in tropical countries is associated with poor calf management (Moran, 2011).

1.2 Colostrum, immunoglobulin G absorption and daily weight gain in calves

1.2.1 Colostrum quality

The first meal the newborn calf is offered is colostrum, which contains high concentrations of nutrients and immunoglobulins (Kehoe *et al.*, 2007). Immunoglobulin G (IgG) is the dominant immunoglobulin, comprising around 85-90% of total immunoglobulin in colostrum (Larson *et al.*, 1980). During colostrogenesis, high concentrations of IgG are transported from the blood into

the mammary glands through receptors on the alveolar epithelial cells (Godden *et al.*, 2009). The concentration of IgG in colostrum can be used for evaluating colostrum quality, *e.g*. an IgG concentration in colostrum of greater than 50 g/L indicates high-quality colostrum (McGuirk & Collins, 2004). The quality of colostrum depends on the volume produced, the time at which the colostrum is collected after calving, the concentration of immunoglobulins and the bacteria levels (Godden, 2008; McGuirk & Collins, 2004).

Calves at birth are essentially agammaglobulinaemic, because immunoglobulins are not transferred from cow to calf *in utero*, and therefore calves depend on absorption of maternal immunoglobulin through colostrum after birth (Godden, 2008). Therefore, feeding colostrum is of vital importance for ensuring adequate passive transfer of immunity, which in the short term reduces the risk of mortality and morbidity in the pre-weaning and post-weaning periods, and improves calf weight gain. Long-term effects such as reduced age at first calving, reduced culling rate during the first lactation and improved milk production in the first and second lactation have also been reported (Faber *et al.*, 2005; Wells *et al.*, 1996; DeNise *et al.*, 1989; Robison *et al.*, 1988). Therefore, feeding sufficiently high-quality colostrum soon after birth is the most important management routine associated with the health and survival of newborn calves (McGuirk & Collins, 2004; Weaver *et al.*, 2000).

1.2.2 Failure of passive transfer of immunity

Calves suffer from failure of passive transfer (FPT) of IgG when the IgG concentration in calf serum is less than 10 mg/mL (Weaver *et al.*, 2000). The mortality rate of calves with low IgG concentration in serum (<10 mg/mL) is twice that in calves with higher concentrations of IgG in serum (Wells *et al.*, 1996). A positive correlation between IgG intake and serum IgG concentration has been reported by Hopkins and Quigley (1997) and Osaka *et al.* (2014). The most important factors influencing transfer of IgG from the colostrum are the time after birth at which the first colostrum feeding takes place, the volume of colostrum consumed and the mass of IgG consumed (Osaka *et al.*, 2014; Chigerwe *et al.*, 2009; Chigerwe *et al.*, 2008; Godden, 2008).

Timing and volume of colostrum intake

Intestinal epithelial cells in newborn calves quickly lose their ability to absorb intact large molecules, such as immunoglobulins, and this pathway becomes completely closed at approximately 24 h of life (Weaver *et al.*, 2000). Therefore, newborn calves should receive clean and adequate good-quality colostrum soon after birth (NRC, 2001). However, the efficiency of absorption of

immunoglobulins depends not only on the time to first colostrum feeding, but also on the method of colostrum feeding.

Colostrum should be fed within the first 4 h after birth, since this is the optimum time for IgG absorption. The efficiency of IgG absorption in neonatal calves declines during the first 6 h of life (Davis & Drackley, 1998). In a study by Beam *et al.* (2009), east and west American dairy farmers who fed the first colostrum within 4 h of life had 2.7 times fewer calves with FPT than farmers who fed colostrum more than 4 h after birth. For every 60 minutes that consumption of first colostrum is delayed, IgG absorption decreases by less than 0.3% from calving to 12 h after birth, but decreases by more than 2.5% to at least 18 h after birth (Osaka *et al.*, 2014). It has also been observed that serum IgG concentration declines by 2 mg/mL for every 30-minute delay in colostrum feeding (Rajala & Castrén, 1995).

The method of colostrum feeding can also contribute to development of FPT and impaired calf health (Godden *et al.*, 2009; Rajala & Castrén, 1995). Calves removed from their dam within 3 h after birth are reported to have a significantly lower prevalence of FPT than calves removed after more than 3 h (Trotz-Williams *et al.*, 2008). The reason could be that the voluntary ingestion of colostrum is insufficient when the calf is left with the dam to suckle (McGuirk & Collins, 2004). Feeding colostrum through an oesophageal tube and artificial nipple feeding (*e.g.* bottle) are common practices in industrial dairy production. It has been observed that nipple feeding results in higher passive transfer than tube feeding (Godden *et al.*, 2009). Furthermore, the neonatal calf should ingest a critical mass of 100-200 g of immunoglobulins to avoid FPT (Osaka *et al.*, 2014; Chigerwe *et al.*, 2008; McGuirk & Collins, 2004).

Source and storage of colostrum

Most dairy farms in east and west American and in Europe (*e.g*. France, Finland and Sweden) generally pool colostrum from several cows before feeding neonatal calves (Delafosse *et al.*, 2015; Beam *et al.*, 2009; Michanek *et al.*, 1990). However, pooling colostrum decreases the IgG concentration, by decreasing the overall quality (Weaver *et al.*, 2000). In a survey of US dairy farms, Morrill *et al.* (2012) found indications that pooled colostrum had significantly lower IgG concentrations, higher SCC and higher total coliform plate count (TPC) than non-pooled colostrum. USDA (2018) reports that 6% of dairy heifers die during the milk feeding period.

Fresh, frozen and refrigerated colostrum show no difference in terms of the concentration of IgG they contain, but refrigerated colostrum has a significantly higher TPC (Morrill *et al.*, 2012). It has been observed that calves fed either fresh or frozen-thawed colostrum show no difference in IgG concentration of colostrum or in serum IgG concentration (Holloway *et al.*, 2001). Klobasa *et al.* (1998) also demonstrated that freezing good-quality colostrum is an appropriate storage method. It has been reported that in SE Asia, calves on many farms are bottle-fed fresh colostrum from their mothers (Muhid *et al.*, 2011).

Pasteurisation of colostrum for 30 or 60 minutes at 60 °C is reported to be adequate to reduce total bacterial count and maintain the concentration of IgG (Elizondo-Salazar & Heinrichs, 2009; Godden *et al.*, 2006). In fact, calves fed heat-treated colostrum have been shown to have significantly higher serum IgG concentrations than calves fed unpasteurised colostrum (Elizondo-Salazar & Heinrichs, 2009; Johnson *et al.*, 2007). Calves fed heat-treated colostrum may absorb a higher proportion of the total IgG presented to the small intestine, which may be explained by the lactogenic immunity effect provided by colostrum (Saif & Smith, 1985). Colostrum also plays an important role in gastrointestinal epithelial maturation, preventing attack by disease (Cortese, 2009). However, the impact of colostrum on the process of gastrointestinal maturation is poorly understood.

1.3 Role of microbiota in the gut

1.3.1 Gut microbiota influence on host health

The microbial community living in the gut is important in health and disease conditions (Owyang & Wu, 2014). Moreover, the microbiota is key in development of the mucosal immune system (Sommer & Bäckhed, 2013), metabolism (Graessler *et al.*, 2013) and colonisation resistance (*e.g*. restricting establishment of pathogens in the gut) (Lawley & Walker, 2013). It has been demonstrated that *Faecalibacterium* plays a crucial role in maintaining gastrointestinal homeostasis and protecting against enteric infections (Minamoto *et al.*, 2015; Oikonomou *et al.*, 2013; Sokol *et al.*, 2008). The risk of disease development is enhanced when the gastrointestinal microbiota is disturbed, for example by gut inflammation or antimicrobial treatment (Antharam *et al.*, 2013). Diarrhoea is the most common cause of disease in calves, with most cases occurring below one month of age (Svensson *et al.*, 2006a). Diarrhoea can be caused by viruses (*e.g*. coronavirus, rotavirus), bacteria (*e.g*. enterotoxigenic *Escherichia coli* and salmonella) and protozoa (*e.g*. *Cryptosporidium parvum*) (El-Seedy *et al.*, 2016; Izzo *et al.*, 2011). Diarrhoeal diseases inhibit the growth rate of calves and result in higher age at first parturition (Stanton *et al.*, 2013). Kyle (2007) observed that 25% of dairy calves in the age range 1-60 days studied on smallholder farms in southern Vietnam suffered from diarrhoea.

The microbial diversity (variety of living organisms) in calves suffering from diarrhoea has been reported to be lower than that in healthy calves, and an association between increased faecal microbial diversity and increased growth rate in calves has been observed (Oikonomou *et al.*, 2013; Rada *et al.*, 2006). Measurements of microbial diversity reflect the differences between species and the relative abundance of species (Leinster & Cobbold, 2012). Few research

studies with the focus on gut microbiota have been conducted in SE Asia in general, or in Vietnam in particular. To better understand how microbiota composition and function impacts newborn dairy calves, more studies are needed.

1.3.2 Development of gut microbiota and factors affecting gut health in dairy calves

The microbial content of the mammalian gut changes primarily in two different phases, from birth to weaning and from weaning to adulthood (Wopereis *et al.*, 2014). Neonatal calves are non-ruminant and the anatomy and physiology of the gastrointestinal tract in the newborn calf change during the first 2-3 weeks. Calves are exposed to the greatest stress and metabolic and immunological challenges from birth to 6-8 weeks of life.

Calves acquire microbes, primarily the dominant microbes in the vaginal canal, during calving (Laguardia-Nascimento *et al.*, 2015; Zambrano-Nava *et al.*, 2011). The vaginal microbiota in cattle contain mainly Firmicutes, Bacteroidetes and Proteobacteria (Laguardia-Nascimento *et al.*, 2015), and these phyla dominate in the gastrointestinal tract of calves at three weeks of age (Malmuthuge & Griebel, 2014). Calves are also exposed to microbes from the dam's skin and surrounding environment during suckling (Doré *et al.*, 2012).

The development and establishment of the gut microbiota from birth to weaning is a complex process. During the first hours of life, calves should be fed colostrum, which is dominated by *Lactobacillus, Bifidobacterium* and *Escherichia coli* (Malmuthuge *et al.*, 2015a), *Staphylococcus* spp., coliforms and *Streptococcus* spp. (Lima *et al.*, 2017). Coliforms and lactobacilli dominate in the faeces of calves at three days after birth, while bifidobacteria is more abundant at seven days of age and declines slightly during the first seven weeks of life (Vlková *et al.*, 2006). Mayer *et al.* (2012) demonstrated the presence of *Citrobacter* spp. and *Leuconostoc* spp. in faeces of healthy calves after the first six hours of life, but these species were not detected at 24 h after birth. At 20 weeks of age, the microbial concentrations are highest in the calf rumen, caecum and colon, and lowest in the abomasum and duodenum (Vlková *et al.*, 2008). Microbial colonisation may be influenced by age of the animal and by the specific part of the gastrointestinal tract.

The type (*e.g.* fresh, heat-treated) of colostrum fed to calves can affect the development of the intestinal microbiota in calves within the first hours of life. The prevalence of *Bifidobacterium* is reported to be lower in the small intestine of calves fed fresh colostrum or no colostrum than in that of calves fed heat-treated colostrum (60 °C for 60 min) (Malmuthuge *et al.*, 2015a). In that study, the dominance of *E. coli* in the small intestine was found to be higher in calves

fed fresh colostrum or no colostrum than in those fed heat-treated colostrum, while calves fed either fresh colostrum or heat-treated colostrum were associated with a lower prevalence of *Lactobacillus* groups than calves fed no colostrum (Malmuthuge *et al.*, 2015a). Therefore, type of colostrum influences the establishment of gut microbiota in calves.

The faecal microbial communities in newborn calves become more diverse with increasing age of the calf (Klein-Jöbstl *et al.*, 2014). This development of the microbiota is dependent on the type of diet (Malmuthuge *et al.*, 2013; Edrington *et al.*, 2012), microbial load in the environment (Fecteau *et al.*, 2002), housing (Pereira *et al.*, 2014b) and administration of prebiotics or probiotics (Foditsch *et al.*, 2015). Administration of antimicrobials has also been shown to have an impact on gut microbial communities and the occurrence of diarrhoea in calves (Oultram *et al.*, 2015; Uyeno *et al.*, 2010; Berge *et al.*, 2009). Faeces samples from one-week-old calves treated with oxytetracycline have been shown to have lower microbial diversity than faeces from untreated calves (Oultram *et al.*, 2015). Uyeno *et al.* (2010) investigated the gut microbiota in calves that were fed colostrum containing antimicrobials (polymixin B and bacitracin) and found that in the first three weeks after birth, Bacteroidetes and Firmicutes were present in similar concentrations and accounted for 40% of the total sequences for every phylum. These findings indicate that antimicrobials can alter gut microbial composition and also indicate the presence of genes for antimicrobial resistance in calf gastrointestinal microbiota (Maynou *et al.*, 2017; Maynou *et al.*, 2016; Thames *et al.*, 2012).

1.3.3 Antimicrobials, impact of resistance development and microbiota

The emergence and spread of antimicrobial-resistant microorganisms is a rising problem and a threat to global public health (WHO, 2017). Overuse and misuse of antimicrobial drugs is problematic, since it contributes to the spread of antimicrobial-resistant bacteria (Kim *et al.*, 2013). In livestock production, antimicrobials are used to prevent and treat animal diseases and, in some countries, are still used as growth promoters (Page & Gautier, 2012). In countries in SE Asia, antimicrobials are widely used inappropriately in livestock production (Usui *et al.*, 2014; Getachew *et al.*, 2013). Vietnam, Thailand and China consume over 30% of all antimicrobials used in livestock production worldwide (Van Boeckel *et al.*, 2015). In Vietnam, 70% of the drug products used in animal production are antimicrobials (An, 2009). Antimicrobial agents are administered in high doses to livestock and some antimicrobial drugs can be used without a prescription in Vietnam (Chuc *et al.*, 2014; Kim *et al.*, 2013; Thai *et al.*, 2012) and in other countries in SE Asia (Holloway *et al.*, 2017). Therefore, antimicrobial resistance is widespread in the region (Usui *et al.*, 2014). In

Sweden, use of antimicrobials is a key tool in mastitis control and intramammary antimicrobial therapy is recommended for treatment of subclinical mastitis (Persson *et al.*, 2011). Unfortunately, the prevalence of subclinical mastitis of dairy cows is high in Vietnam and the use of antimicrobials is necessary in such cases (Östensson *et al.*, 2013). Moreover, antimicrobials are used worldwide for the treatment of diarrhoea in neonatal calves (Azizzadeh *et al.*, 2012).

Antimicrobial use has been reported to have an influence on the gut microbiota in various species, including humans (Claesson *et al.*, 2011; Sekirov *et al.*, 2010), pigs (Schokker *et al.*, 2015; Looft *et al.*, 2014) and horses (Costa *et al.*, 2015). Penicillin treatment has been found to significantly alter the microbiota in calves less six months old (Grønvold *et al.*, 2011). For example, faecal microbial diversity in one-week-old calves treated with oxytetracycline is lower than that in untreated calves (Oultram *et al.*, 2015). Moreover, Pereira *et al.* (2016) reported that the raw milk calves receive may contain antimicrobial residues (*e.g.* ceftiofur, penicillin, ampicillin and oxytetracycline). The relative abundance of microbiota displays significant differences between calves fed milk with antimicrobial residues and non-antimicrobial residues, but only at the genus level. Environmental contamination with antimicrobial residues can lead to development of resistance in the environment and emergence of antimicrobial-resistant strains (McKinney *et al.*, 2018). In this thesis, the incidence of antimicrobial-resistant *Escherichia coli* in young dairy calves was studied.

2 Objectives of the thesis

The overall aim of the thesis was to generate science-based knowledge regarding the early life of dairy calves in relation to colostrum quality, gut microbial colonisation and antimicrobial-resistant bacteria.

Specific objectives were to:

- Evaluate colostrum quality, Immunoglobulin G (IgG) absorption and weight gain of calves on smallholder dairy farms in Vietnam
- Determine the composition of microbiota in colostrum and calf faeces and assess the microbiota composition in relation to colostrum quality parameters, diarrhoea and antimicrobial treatment
- Characterise the microbiota along the gastrointestinal tract of calves in early postnatal life
- Study the occurrence of antimicrobial resistance in *Escherichia coli* isolated from young calves in Vietnam.

3 Materials and methods

This section briefly summarises the materials and methods used in the studies described in Papers I-IV in this thesis. Detailed descriptions can be found in the individual papers. Sampling of materials for Papers I, II and IV was conducted from August to December 2014 on smallholder dairy farms in Dong Nai province, southern Vietnam (Figure 1). Collection of samples for Paper III was conducted at the Swedish Livestock Research Centre, Swedish University of Agricultural Sciences, Uppsala.

3.1 Study area and designs

Southern Vietnam has a tropical monsoonal climate characterised by high temperatures and high humidity for most of the year. This area has distinct rainy (April to November) and dry seasons (December to March). Annual rainfall ranges from 1500 to 2000 mm (Sterling & Hurley, 2008). In Dong Nai province, to the west of Ho Chi Minh City, the average temperature is 29 °C, while the humidity varies from 75 to 82%. Minimum and maximum annual rainfall in the province is 900 mm and 1300 mm, respectively.

An overview of the number of farms, cows and calves included in the different studies is provided in Table 1. The farmers were interviewed to obtain data on colostrum feeding, calf rearing, animal health and medical treatment. Additional observations on calf management were made by the same interviewer throughout the study. When a cow gave birth on the study farms, the date of birth, sex, birth weight, time of separation from mother, time from birth until feeding colostrum, health of the calf and antimicrobial use were recorded (Papers I, II and IV). The calving cows in Papers I, II and IV were crossbred Holstein. All calves recruited to the study at birth were followed until 14 days of age in Papers I, II and IV.

Table 1. *Experimental design of the studies described in Papers I-IV*

	Paper I	Paper II	Paper III	Paper IV
Number of farms	40	38	1	41
Number of cows	80	76	-	-
Number of calves	80	76	25	84

The study performed at SLU (Paper III) included 25 bull calves (13 calves at two days old and 12 calves at seven days old) of the breeds Swedish Holstein and Swedish Red. The criteria for selection of neonatal calves were birth weight greater than or equal to 30 kg and that the calf had been allowed to stay with its mother for 2 h after birth. The calves were offered colostrum, with the criterion that it should have IgG concentration (Brix value) greater than or equal to 20%.

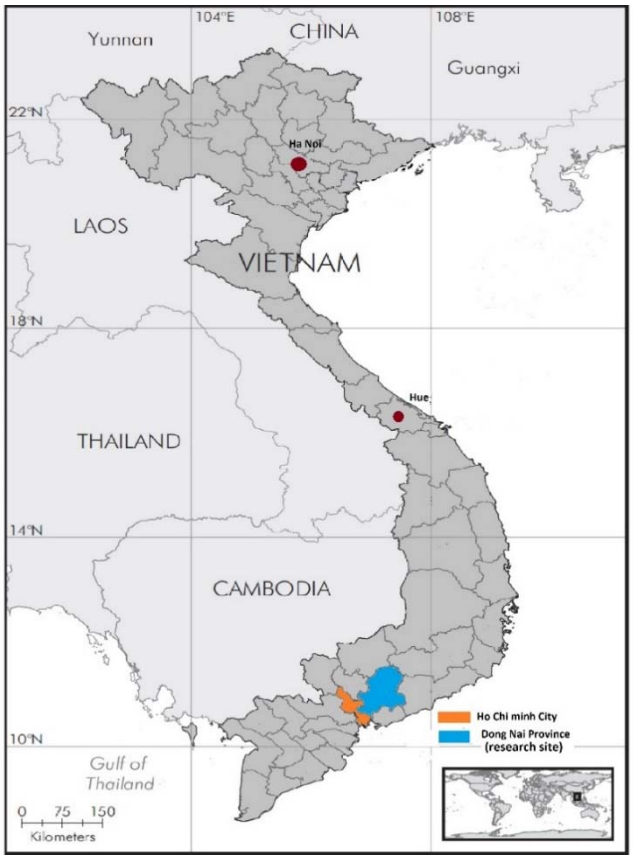

Figure 1. Administrative map of Vietnam showing the study area, Dong Nai Province (source: Sterling and Hurley (2008)).

3.2 Sampling

Colostrum samples (Papers I and II) were collected within 4 h after calving, by hand milking into a bucket. The colostrum was then mixed thoroughly and samples were transferred to sterile tubes. Blood samples were collected via jugular venepuncture into serum separator tubes from the calves at seven days of age, for evaluation of IgG absorption. The body weight of calves was measured at birth and at 14 days of age.

Faeces samples (Papers II and IV) were collected directly from the rectum of calves at birth (D0) and at 14 days old (D14). The faeces samples were transferred to sterile tubes, immediately placed on ice and transported to the laboratory for isolation of *Escherichia coli* (*E. coli*). Isolated *E. coli* were transferred to 2-mL microtubes (SARSTEDT, Nümbrecht, Germany) containing 0.5 mL serum broth supplemented with 15% glycerol and placed in a freezer at -80 °C. One of five frozen isolates of *E. coli* from each calf sample was selected at random and sent frozen (on dry ice) to the National Veterinary Institute, Uppsala, Sweden, for testing of antimicrobial susceptibility. The remaining faeces and colostrum samples were kept in a freezer at -20 °C and were then sent frozen (on dry ice) to the Department of Animal Nutrition and Management, Swedish University Agriculture and Sciences, Uppsala, Sweden, for identification of microbiota composition.

In Paper III, the bull calves were euthanised with a lethal dose of pentobarbital at two (n=13) and seven (n=12) days of age. Samples were collected from several parts of the gastrointestinal tract (rumen, abomasum, proximal duodenum, distal ileum, caecum, distal colon and faeces). Faeces samples were collected directly from the rectum of the calves. Gut content and mucosal scrapings were collected separately from each intestinal segment. The mucosal scrapings were collected after washing the gut mucosa with sterile saline solution. Immediately after collection, all samples were placed on ice and within four hours the samples were transferred to a freezer at -80 °C and stored until analysis.

3.3 Analysis

Colostrum quality, Ig absorption and weight gain

Colostrum IgG and serum IgG were estimated using a digital Brix refractometer (PAL-1, Atago Co. Ltd., Tokyo 173-0001 Japan). Protein and fat content in

colostrum were analysed using the mid-infrared spectroscopy method (Milk Analyser, Miris AB, Uppsala, Sweden). The body weight of calves was measured using a digital scale (TANITA HD-380, Tanita Corporation, Japan).

Analysis of the microbiota

For the intestinal and faeces samples, deoxyribonucleic acid (DNA) was extracted using a QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) with a modified protocol that included bead beating-based lysis of the bacterial cell walls. DNA from the colostrum samples was isolated using a Power Food Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA 92010, USA), after careful removal of the fat layer. No-template controls were processed in parallel with the DNA isolations, to check for potential contamination of samples during the DNA isolation steps.

Duplicate terminal restriction fragment length polymorphism (T-RFLP) analyses were conducted for each sample included in Paper II. In the T-RFLP analyses, 16S rRNA gene PCR amplicons were generated with the broad-range bacterial primers Bact 8F and 926R, the PCR products were digested with the HaeIII restriction enzyme (GE Healthcare, Uppsala, Sweden) and the digested fragments were separated on an ABI 3730 capillary sequencer (ABI Applied Biosystems, Foster City, CA, USA), as described previously (Dicksved *et al.*, 2008). The size of the fluorescently labelled fragments was determined by comparison with the internal GS ROX-500 size standard (ABI). T-RFLP electropherograms were imaged using Peakscanner software (Applied Biosystems). Relative peak area of each terminal restriction fragment (TRF) was determined by dividing the area of the peak of interest by the total area of peaks within the following threshold values: lower threshold at 50 bp and upper threshold at 500 bp.

For the 16S amplicon sequencing analyses used for a subset of the samples in Paper II and all samples in Paper III, broad-range 16S rRNA gene primers targeting the V3-V4 region of 16S rRNA gene were used in PCR. The PCR primers used were constructed to contain unique tags, allowing pooling of multiple samples prior to sequence analysis. After successful amplification, the PCR products were purified, the DNA concentration was measured and the purified PCR products were pooled in equimolar amounts. The PCR amplicons were sequenced using Illumina sequencing.

The raw reads generated were demultiplexed and assigned to different samples according to the respective barcode. The paired-end sequence reads were merged and quality filtered. Sequences were processed using QIIME (Version 1.7.0) (Caporaso *et al.*, 2010) in Paper II and according to the procedure described by Sinclair *et al.* (2015) in Paper III.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of 12 common antimicrobials was determined for each isolate using broth microdilution according to the Clinical and Laboratory Institute (CLSI, 2013). These tests were performed in VetMIC panels (National Veterinary Institute, Uppsala, Sweden).

Isolates with cefotaxime resistance were phenotypically tested for production of extended-spectrum cephalosporinases (ESC), by broth microdilution using EUVSEC2 panels (Trek Diagnostic System, Oakwood Village, OH, USA) and cation-adjusted Mueller Hinton broth (Becton Dickinson, Cockeysville, MD, USA). All isolates showing cefotaxime or ceftazidime resistance on the EUVSEC2 panels were further screened by multiplex-PCR for detection of the following gene groups: plasmid-mediated AmpC (pAmpC) (Pérez-Pérez & Hanson, 2002) and $bla_{CTX\text{-}M}$ (Woodford *et al.*, 2005). Isolates with ertapenem or meropenem resistance were further screened by whole genome sequencing (WGS) for genes encoding for carbapenem resistance.

Isolates with ciprofloxacin resistance and nalidixic acid MIC <32 μg/mL were selected for PCR detection of plasmid-mediated quinolone resistance (PMQR) genes. The screening for PMQR genes included *qnrA*, *qnrB*, *qnrS* and *aac(6')-1b-cr*, using PCR assays described elsewhere (Cavaco *et al.*, 2008; Cattoir *et al.*, 2007; Park *et al.*, 2006).

3.4 Statistical analysis

Mixed statistical models were used for the analyses in Paper I (Littell *et al.*, 2006), since the data contained many observations for each farm. Protein, fat, and IgG concentration in colostrum, IgG in calf serum and average daily weight gain were analysed using mixed linear models, with farm as a random factor. The 'Mixed' procedure in the SAS package (SAS, 2014) was used for this purpose. Assumptions underlying the analyses were checked using diagnostic plots of model residuals. Descriptive statistics were obtained using the SAS 'Corr' procedure. In all analyses, $P<0.05$ was regarded as significant.

Microbial community structures were compared using multivariate and univariate statistical models (Papers II and III). The multivariate models involved principal coordinate analysis (PCoA) based on Bray Curtis distance, and were used to identify clustering patterns among samples. Analysis of similarity (ANOSIM) with Bray Curtis distance matrices was used to evaluate whether there were differences between groups/clusters. Univariate statistical methods were used to test for differences in alpha diversity and relative abundance between groups. The Mann-Whitney test was used when comparing the median between two groups, while the Kruskal-Wallis test was used in when

comparing more than two groups. The Wilcoxon test was used for the paired analyses. Only microbial taxa with average relative abundance >1% were included in the univariate analysis (Papers II and III). All statistical tests were performed using the statistical software Past (Hammer *et al.*, 2001)

Fisher's exact test was used to compare the observed proportions of resistance to each antimicrobial compound in isolates from samples obtained from calves aged 0 and 14 days (Paper IV). The significance level was set to $P<0.05$. All statistical analyses were conducted in Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX, USA).

4 Main results

4.1 Practical farming

According to the responses to the questionnaire, smallholder dairy farmers well understood the importance of feeding colostrum in time for calf health, but they did not check colostrum quality. Calves were immediately separated from their mothers at birth and offered colostrum early in life by bucket feeding (Paper I). Farmers administered antimicrobials to most dairy cows during the dry period, to prevent intra-mammary infections, and in most cases antimicrobials were used without the advice of a veterinarian or a prescription (Papers II and IV).

4.2 Colostrum quality, IgG absorption and average daily gain in calves

Of the 76 maternal colostrum samples analysed (four had to be discarded due to condensed colostrum), 91% had good quality (Brix value >22%). Failure of passive immune transfer to calves was low, affecting only around 10% of the calves. The average daily weight gain of female calves (0.75 kg) was higher than that of bull calves (0.54 kg). There was a positive correlation between serum IgG and colostrum total protein content, and a negative correlation between daily weight gain and diarrhoea in calves (Paper I).

4.3 Development of the gut microbiota in calves (Papers II and III)

The colostrum microbiota had a different composition than the faecal microbiota, although there was a higher overlap between colostrum microbiota and faecal microbiota samples in newborn calves (Paper II). Facultative

anaerobic bacteria such as *Streptococcus*, *Acinetobacter*, *Enterobacter* and *Corynebacterium* mainly dominated in colostrum. Strict anaerobic bacteria, such as *Faecalibacterium*, were also detected in colostrum. However, the composition of microbiota displayed large individual variation between different colostrum samples, based on the sequence data for eight colostrum samples. The microbiota in colostrum fed to a particular calf and in the corresponding faeces sample collected from that calf were not correlated, although there was overlap in composition of microbiota between faeces and colostrum samples (Paper II).

The composition of the gut microbiota differed significantly between newborn calves and calves at 14 days old (P=0.0001) (Paper II). It was more difficult to obtain a PCR product from a large fraction of the D0 samples, despite several attempts, which indicates that these D0 samples contained lower levels of bacteria than the detection limit of the method used. Overall, the microbiota composition assessments by T-RFLP did not show any associations between the microbiota composition and metadata variables examined in the study, with no differences in microbiota between male and female calves and no correlations to serum Brix values or average daily weight gain of the calves.

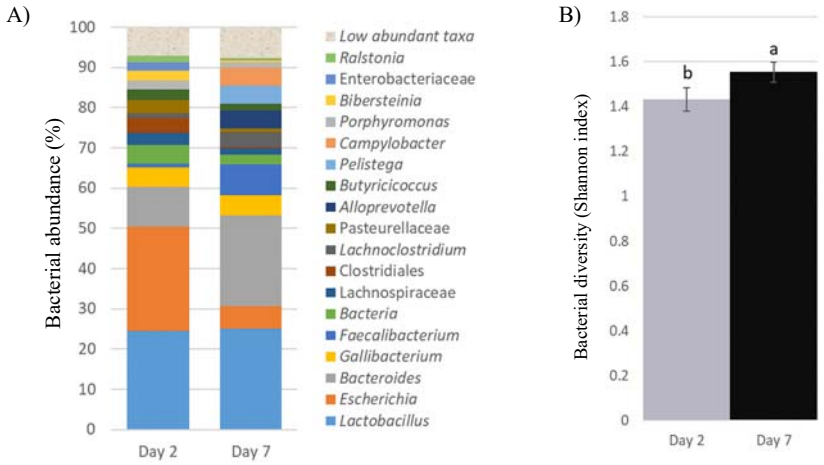

Figure 2. The microbiota in the gut of neonatal calves at Day 2 and Day 7 of age. A) Distribution (mean relative abundance of each group) of the main bacterial taxa found in the samples. B) Bacterial diversity (mean ± standard error) of each group. Different letters indicate significant difference at P<0.05 (Paper III).

Age-related variation in the composition and diversity of the microbiota in calves was observed. Comparisons of the faeces samples collected at birth and at 14 days age revealed that the microbiota differed substantially between these

samples (Paper II). Differences in composition of the microbiota were also seen when comparing samples collected within a shorter age span (Figure 2). The microbiota in two-day-old calves was dominated by *Escherichia* and this group was significantly more prevalent than in seven-day-old calves. Conversely, *Bacteroides* and *Faecalibacterium* dominated in seven-day-old calves ($P<0.01$). *Lactobacillus* was also one of the dominant genera (Figure 2A), but it showed similar relative abundances in both two- and seven-day-old calves.

The composition of the microbiota differed substantially between gut segments, in particular between the proximal and distal parts of the gut (Figure 3A). The microbiota in the abomasum and duodenum had greater relative abundance of *Lactobacillus* than the ileum, caecum, colon and faeces ($P<0.01$), whereas *Escherichia* were more associated with colonisation of the distal intestinal tract ($P<0.01$). In addition, *Faecalibacterium*, *Butyricicoccus*, Clostridiales and Lachnospiraceae dominated in the large intestine and faeces of calves, but not in other segments of the gut. These are strictly anaerobic bacteria and are commonly found in the distal parts of the gut, including in older individuals. The two- and seven-day-old calves had similar bacterial diversity in the different segments of the gut (Paper III), but with significantly lower bacterial diversity in the ileum compared with the other gut segments (Figure 3B).

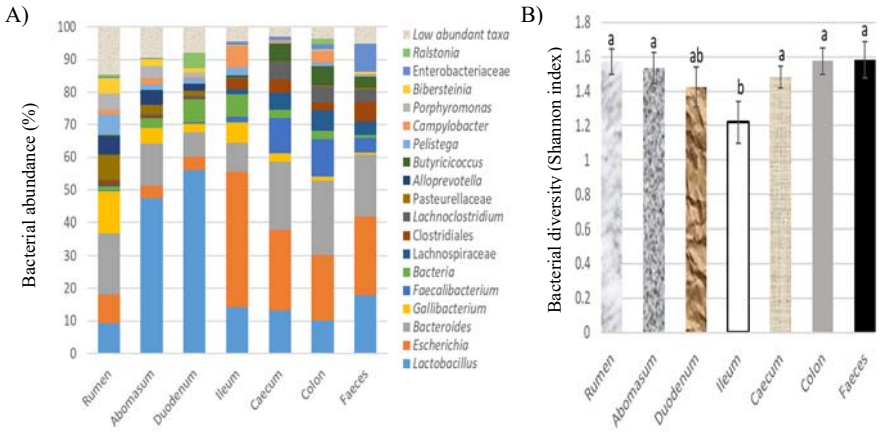

Figure 3. The microbiota in different segments of the gastrointestinal tract and in faeces samples from neonatal calves. A) Distribution (mean relative abundance of each group) of the main bacterial taxa found in the samples. B) Bacterial diversity (mean ± standard error) of each group. Different letters indicate significant difference at $P<0.05$ (Paper III).

The overall composition of the microbiota did not differ greatly between the intestinal mucosa and gut content (Paper III). However, the bacterial diversity was higher in the mucosa than in the gut content (Figure 4B). In addition, there were statistically significant differences in relative abundance of *Lactobacillus* ($P<0.01$), *Escherichia* and *Ralstonia* ($P<0.05$) between the gut content and mucosa of calves. *Lactobacillus* was present in significantly higher relative abundance in the gut content, while *Escherichia* and *Ralstonia* were present in higher proportions in the gut mucosa (Figure 4A).

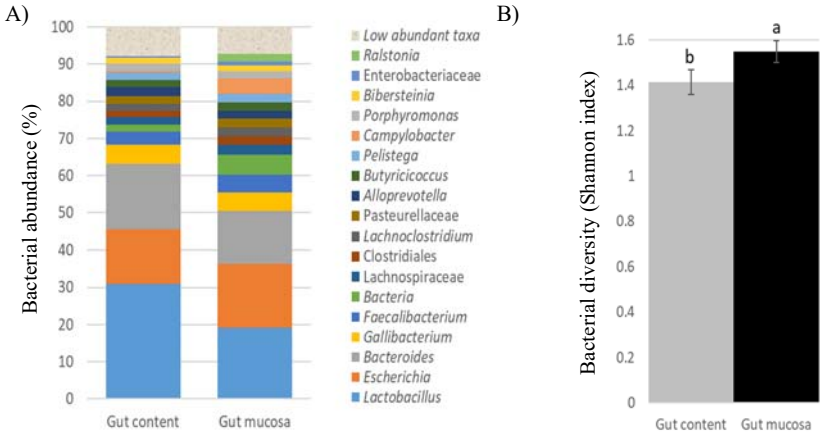

Figure 4. The microbiota in the gut content and mucosa of neonatal calves. A) Distribution (mean relative abundance of each group) of the main bacterial taxa found in the samples. B) Bacterial diversity (mean ± standard error) of each group. Different letters indicate significant difference at $P<0.05$ (Paper III).

In Paper II, *Lactobacillus*, *Escherichia-Shigella* and *Bacteroides* dominated in the gut of all 14-day-old calves. However, several of the calves included in Paper II had been treated with antimicrobials, due to diarrhoea, inflammation of the umbilicus or swollen joints. Comparisons of healthy calves and antimicrobial-treated calves by ANOSIM revealed no differences between the groups (Figure 5). However, when the antimicrobial-treated calves were further divided into calves with and without diarrhoea differences were found, and were linked to the relative abundance of *Faecalibacterium* ($P=0.005$) and *Butyricicoccus* ($P=0.014$). Both these genera were associated with healthy calves, with significantly higher relative abundance in healthy calves than in those with diarrhoea.

Antimicrobials are commonly used in the treatment of bacterial infections in all animal species (*e.g.* pigs, poultry, cattle *etc*.). Most of the smallholder

Vietnamese farmer surveyed in this thesis reported that they frequently treat their calves with antimicrobials for diarrhoea, inflammation of the umbilicus or swollen joints. In Paper II, 20 out of 76 calves analysed had been treated with antimicrobials before 14 days of age. The healthy calves showed numerically higher microbial diversity than the treated calves, but the difference was not statistically significant (P>0.05) (Figure 5B).

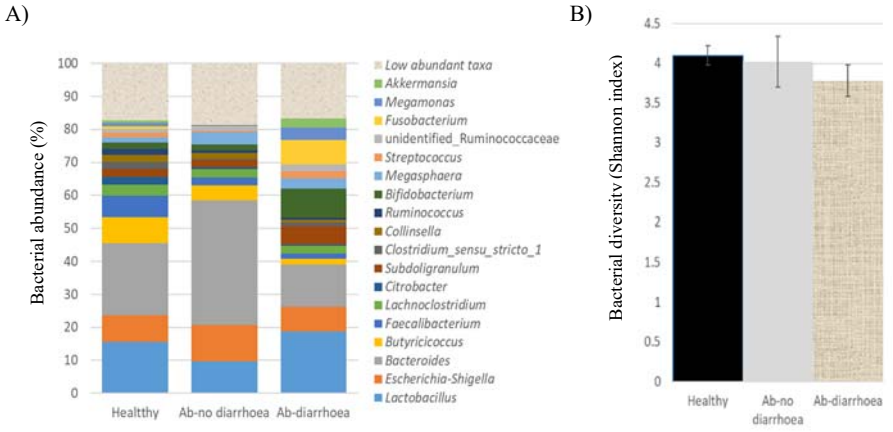

Figure 5. The microbiota in faeces samples from 14-day-old healthy calves, from 14-day-old calves treated with antimicrobials (Ab-no diarrhoea) and from 14-day-old calves treated with antimicrobials for diarrhoea (Ab-diarrhoea). A) Distribution (mean relative abundance of each group) of the main bacterial taxa found in the samples. B) Bacterial diversity (mean ± standard error) of each group (Paper II).

4.4 Occurrence of antimicrobial resistance in *E. coli* isolated from faeces samples (Paper IV)

Escherichia coli strains were isolated from 144 of the 168 faeces samples tested in Paper IV. Forty percent of all *E. coli* isolates were found to be susceptible to all antimicrobial drugs tested. The remaining 60% of the isolated strains had one or several types of antimicrobial resistance. Comparison of samples taken at birth and at 14 days of age revealed that the calves were colonised with antimicrobial-resistant *E. coli* already on the day of their birth. In analyses of all resistances, resistance to tetracycline was most common (57% of isolates), followed by resistance to sulfamethoxazole (49%), ampicillin (48%), trimethoprim (43%) and ciprofloxacin (40%). The proportion of multi-resistant isolates was high and 53% of *E. coli* isolates were resistant to at least three antimicrobials. The proportion of antimicrobial resistance to ampicillin, chloramphenicol, ciprofloxacin, streptomycin, sulfamethoxazole, tetracycline

and trimethoprim was significantly higher ($P<0.05$) in *E. coli* isolated from faeces samples taken from calves at 14 days of age than in *E. coli* isolated from the faeces of newborn calves (Figure 6). Four isolates carried a gene encoding for extended-spectrum cephalosporinases, and these genes belonged to $bla_{\text{CTX-M}}$ group 1 (2 isolates), $bla_{\text{CTX-M}}$ group 9 (1 isolate) and bla_{CMY-2} (1 isolate). Thirty-three isolates showed plasmid-mediated quinolone resistance (PMQR) phenotype, and 30 of these carried the *qnr*S gene.

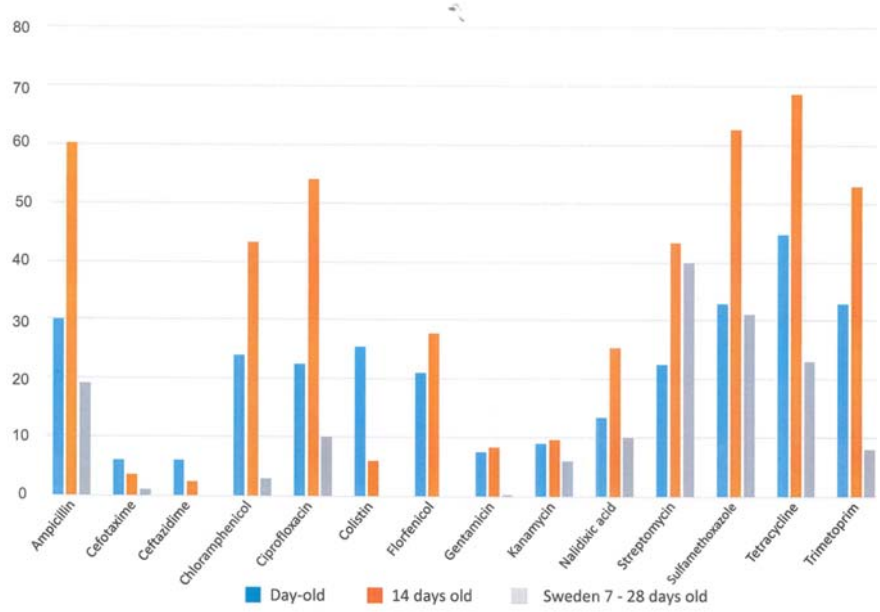

Figure 6. Proportion (%) of antimicrobial-resistant *Escherichia coli* among isolates from faeces of newborn calves and calves at 14 days of age on farms in Vietnam and in calves aged from 7 to 28 days at a research centre in Sweden (Swedish data from Duse *et al.*, 2015).

5 General discussion

5.1 Colostrum feeding and its influence on immunoglobulin G absorption in calves on smallholder farms in southern Vietnam

Dairy production in Vietnam is mainly performed by smallholder farmers, and Holstein Friesian crosses dominate in the region. Management practices on these smallholder farms regarding housing, feeding and water regimes are similar throughout the country. This means that, although only cows and calves from 40 smallholder dairy farms were studied in this thesis, the results can be valid for other farms in Vietnam.

The farmers surveyed milked colostrum by hand within 4 h after calving and this practice might be one explanation for the observation that 91% of colostrum samples were of good quality, *i.e*. above the threshold value of 22% Brix value (IgG >50 g/L colostrum) (Paper I). Angulo *et al.* (2015) also found that colostrum IgG concentration is high and stable within 4 h after parity. Colostrum should be offered to calves during the first 4 h of life in order to optimise the passive transfer of IgG across the small intestinal epithelium (Angulo *et al.*, 2015; Weaver *et al.*, 2000). The farmers surveyed in this thesis were well aware of the importance of the timing for colostrum feeding. Thus newborn calves were fed colostrum within the first 4 h of life and a high proportion (90%) of the calves had serum IgG levels greater than 8.3% Brix value (IgG >10 g/L serum) (Paper I). Shivley *et al.* (2018) found that 8.1% of calves in 12 states in the USA suffer from failure of passive transfer (FPT), which is a somewhat lower level than observed in this thesis. Calves with serum IgG concentrations less 10 g/L are considered to have FPT (Shivley *et al.*, 2018; Weaver *et al.*, 2000), the consequence of which is an increased risk of calf mortality (Wells *et al.*, 1996). Previous studies have reported a positive correlation between IgG intake and

serum IgG concentration (Shivley *et al.*, 2018; Osaka *et al.*, 2014; Hopkins & Quigley, 1997), as also observed in this thesis. Good colostrum quality and high absorption of IgG in calves can contribute to improved health, high growth rate, high milk production at the first lactation and a decreased risk of being culled (Furman-Fratczak *et al.*, 2011; Godden, 2008).

Not surprisingly, none of the farmers surveyed tested the quality of colostrum, so it could be worthwhile developing an inexpensive, easy to use, accurate and reliable method for measuring colostrum quality on smallholder farms. A Brix refractometer, as used in the present study (Paper I), is an indirect measurement method that successfully provides estimates of bovine colostrum IgG (Løkke *et al.*, 2016; Bartier *et al.*, 2015; Quigley *et al.*, 2013; Bielmann *et al.*, 2010). It is also practical on the farm (Cuttance *et al.*, 2017; Elsohaby *et al.*, 2015; Deelen *et al.*, 2014). The Brix value of 22% is an appropriate threshold for both optical and digital refractometers, based on sensitivity and specificity, compared with 50 mg IgG/mL colostrum for radial immunodiffusion (RID) measurements (Bielmann *et al.*, 2010).

Colostrum is not only rich in nutrients and IgG antibodies, but also in bioactive molecules (*e.g.* growth factors, hormones and prebiotics) (Kehoe *et al.*, 2007; Blum & Hammon, 2000). These molecules play a crucial role by stimulating animal growth, intestinal epithelial maturation and defence from disease, and by activating local immune system development (Maldonado-Gomez *et al.*, 2015; Cortese, 2009). These molecular functions contribute to creating an appropriate ecological environment for establishment of the initial composition of the gut microbiota. In Paper II, the microbiota in many of the colostrum samples had a similar composition to that in the faeces samples taken at birth. Early colonisation by bacteria may affect gut barrier function (Malmuthuge *et al.*, 2015b). Therefore, the associations between microbial composition and serum IgG concentration were examined in Paper II. However, there were no apparent associations between the dominant microbial taxa and passive immunity, estimated by Brix value.

In Paper II, two out of eight of the colostrum samples were dominated by *Streptococcus*. Based on an earlier T-RFLP data analysis in which TRF were linked to bacterial species (Dicksved *et al.*, 2009), it was also evident that the TRF corresponding to streptococci was high in many of the colostrum samples in the T-RFLP data generated in Paper II. These results might be a consequence of high prevalence of *Streptococcus agalactiae* in infected udders in southern Vietnam (Östensson *et al.*, 2013). Lima *et al.* (2017) also found differences in the microbial taxonomic structure of colostrum of cows with and without clinical mastitis. The incidence of subclinical mastitis is high in this geographical region (Östensson *et al.*, 2013), which is possibly the reason for the large variation in

microbiota composition between different colostrum samples in our study. During the dry period, intra-mammary antibiotic therapy had been administered to all cows included in the present study in order to prevent clinical mastitis, and this may also have influenced the microbial composition of colostrum.

Apart from being detected in colostrum samples, *Escherichia-Shigella*, *Faecalibacterium*, *Bacteroides* and *Streptococcus* were also found in faeces samples from calves that had been fed the colostrum (Paper II). However, there were no associations between the abundance of the dominant genera in colostrum samples and in faeces samples collected from calves at 14 days of age.

5.2 The microbiota in the gastrointestinal tract of calves

The relative abundance and diversity of the microbiota in calves differed between two and seven days of age (see Figure 2). *Escherichia* dominated and were present in significantly higher abundance in two-day-old calves compared with seven-day-old calves. The microbiota in young calves is commonly dominated by certain genera, which was evident in both Papers II and III. *Lactobacillus*, *Escherichia*, *Bacteroides*, *Faecalibacterum* and *Butyricicoccus* were found in high relative abundance both in calves studied in Vietnam and calves in Sweden (Figure 7), which also agrees with previous findings (Oikonomou *et al.*, 2013; Uyeno *et al.*, 2010). When data from the two different studies described in Papers II and III were analysed in parallel, it was found that *Bacteroides* and *Faecalibacterum* increased when comparing two-day-old and 14-day-old calves. *Bacteroides* has been shown to play an important role in the development of immunological tolerance to commensal microbiota (Mazmanian *et al.*, 2008). The abundance of *Lactobacillus* in the gut of young calves has been correlated with an increase in total serum IgG concentration (Al-Saiady, 2010), increased weight gain and feed conversion ratio, and reduced diarrhoea incidence (Abe *et al.*, 1995). These effects are stronger in pre-weaned calves than weaned calves (Abe *et al.*, 1995), suggesting that probiotic supplements are more effective when the gut microbiota is being established and less effective when the microbiota has stabilised. In a study on humans, Karczewski *et al.* (2010) found that *Lactobacillus plantarum* could stimulate Toll-like receptor 2 (TLR2), which may regulate gut permeability through tight junction proteins. A higher proportion of *Lactobacillus* in young dairy calves may be important for regulation of epithelial tight junctions and intestinal homeostasis (Malmuthuge *et al.*, 2012). Thus, early gut colonisation by microbiota is likely an important stage for the developing gut and naïve immune system (Fouhy *et al.*, 2012; Hansen *et al.*, 2012) and may have long-term health effects (Conroy *et al.*, 2009). Several studies have reported that facultative anaerobes (*e.g.* *Escherichia*,

Staphylococcus spp., *Streptococcus* spp.) are the first to colonise newborn animals. The increase in bacterial numbers helps to create an anaerobic environment in the gut that is more appropriate for obligate anaerobes (*e.g.* *Bacteroides* spp., *Clostridium* spp.), which expand dramatically in numbers thereafter and outnumber the facultative anaerobes (Round *et al.*, 2010; Bourlioux *et al.*, 2003; Falk *et al.*, 1998). The high variation in microbial diversity and relative abundance in the gastrointestinal tract of calves during early life suggests that the composition of the gut microbiota may be easier to influence in this period than in adults.

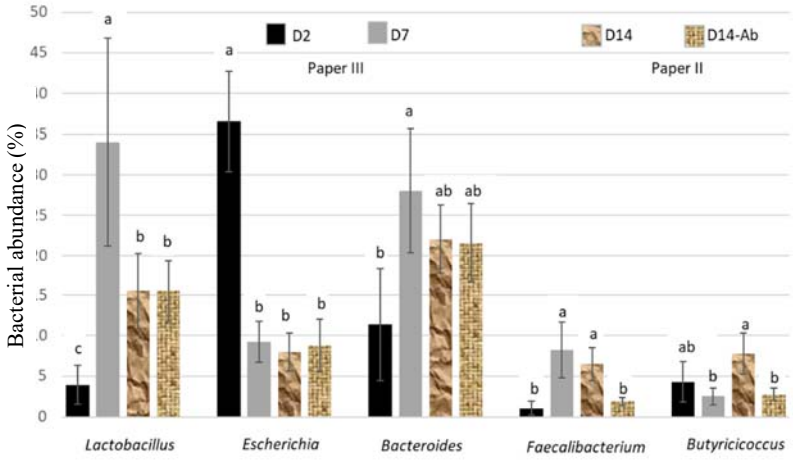

Figure 7. Relative abundance (mean ± standard error) of dominant genera in faeces samples from calves at two (D2) and seven (D7) days of age (Paper III), and of healthy (D14) and antibiotic-treated (D14-Ab) calves at 14 days of age (Paper II). Different letters indicate significant difference at $P<0.05$.

The bacterial populations identified in Paper III displayed a clear gut segment association, with high similarity in composition in the abomasum and duodenum and high similarity in composition between the distal gut segments (Figure 3A). These differences in composition between the gut segments may be due to differences in available substrates for the bacteria to utilise in the different segments. They could also be due to differences in pH, host secretions and transit time of the digesta. The faecal microbiota in calves in Paper III had a similar composition to that in the distal gut (*e.g.* caecum and colon) and ileum, but was clearly different from that in the proximal gut (*e.g.* rumen, abomasum and duodenum) (Figure 3A). Thus, faeces samples represent only a part of the total gut microbiota and only resemble the distal parts of the gut. The calves had similar microbial diversity in the different segments of the gut, but there was significantly lower microbial diversity in the ileum compared with the other gut

segments (Figure 3B). This might be a consequence of the high degree of colonisation by *Escherichia* (Figure 3A). On the other hand, the levels of *Lactobacillus* were quite high in the duodenum and abomasum, which might be due to colostrum having a higher concentration of oligosaccharides than mature milk (McGrath *et al.*, 2016). In an *in vitro* study by Champagne *et al.* (2014), it was found that oligosaccharides in colostrum stimulate *Lactobacillus* growth rates. In this thesis, it was found that the microbiota in the rumen had different proportions of microbial composition compared with the other gut segments. The early colonisation of microbial populations in the rumen may help the calf to develop the capability to adapt to solid feed particle after weaning, according to findings by Malmuthuge *et al.* (2012). *Faecalibacterium* and *Butyricicoccus* abundance was higher in the faeces and the large intestine than in the small intestine of the calves studied in this thesis (Figure 3A). Alipour *et al.* (2018) reported that *Butyricicoccus* and *Faecalibacterium* species are commonly correlated in relative abundance in the gut. One shared feature of *Faecalibacterium* and *Butyricicoccus* is the ability to produce butyrate. Butyrate is not only a nutrient source for the gut colonocytes, but is also thought to be beneficial for immunological maturation of the gut mucosa (Furusawa *et al.*, 2013). Presence of sufficient amounts of butyrate early in life could be an important factor in preventing gut disorders caused by bacterial pathogens and in increasing growth of the animal (Guilloteau *et al.*, 2010). This increasing growth may be due to either butyrate serving as a nutrient or to more healthy calves.

The presence of gut microbiota is necessary for the development of the intestinal epithelium and the mucosal immune system (Sommer & Bäckhed, 2013). The data generated in Paper III showed that microbial diversity was higher in the gut mucosa than in the gut content (Figure 4B). Although there was a large overlap in microbial community composition between gut mucosa and gut content, specific microbial groups were also found in significantly different relative proportions in mucosa and gut content (Paper III). *Escherichia* and *Ralstonia* were more over-represented in gut mucosa, while *Lactobacillus* was more over-represented in gut content (Figure 4A). Similarly, Malmuthuge and Griebel (2014) showed that microbial composition varies depending on gut segment and sample type used (mucosa or gut content). A study in mice has found that the inner stratified and firmly attached mucus layer inhibits intestinal bacteria from entering into direct contact with the colonic epithelial cells (Johansson *et al.*, 2008). It was also shown in that study that the mucus layers vary in organisation and composition in different segments of the gut. Differences in mucus layer organisation and composition are most likely associated with variations in the mucosa-associated microbiota along the gut.

Thus, microbiota in closer proximity to epithelium cells may be more relevant from an infection perspective and from a host interaction perspective.

5.3 Factors affecting the microbiota in newborn calves

When the microbiota composition was compared between healthy calves and calves treated with antibiotics due to diarrhoea, significant differences were found between the groups ($P<0.05$). Two genera were significantly lower in calves treated with antibiotics for diarrhoea, namely *Faecalibacterium* and *Butyricicoccus* (Paper II). *Faecalibacterium prausnitzii* has been shown to be important for maintaining intestinal homeostasis by promoting secretion of anti-inflammatory cytokines, reducing production of pro-inflammatory cytokines and promoting production of butyrate (Minamoto *et al.*, 2015; Oikonomou *et al.*, 2013; Sokol *et al.*, 2008). High relative abundance of *Faecalibacterium* spp. in calf faeces has been correlated with a lower incidence of diarrhoea (Oikonomou *et al.*, 2013), and oral administration of *F. prausnitzii* to calves is also reported to improve weight gain and lower the incidence of diarrhoea during the pre-weaning period (Foditsch *et al.*, 2015). The findings presented in this thesis provide further insights regarding the potentially beneficial effect of *Faecalibacterium* in newborn calves and its association with health. *Butyricicoccus* has also been associated with health (Tomassini, 2015) and has been evaluated for probiotic use (Eeckhaut *et al.*, 2013). Early establishment in sufficient levels of *Faecalibacterium* and *Butyricicoccus* may be important for resilience to gut disturbances.

Antibiotic treatment of calves suffering from diarrhoea or non-diarrhoea may cause changes in microbial abundance at 14 days of age (Figure 5A). This probably implies an effect of antibiotics on the microbial communities in the gastrointestinal tract of the newborn calf. However, the results obtained in this thesis did not indicate the same effect when looking separately at the calves without diarrhoea, but treated with similar antibiotics to those with diarrhoea. Oultram *et al.* (2015) also found that antibiotic treatment for calf diarrhoea and respiratory diseases results in variations in the faecal microbial composition of pre-weaned calves. Bacterial diversity is reported to be lower in calves with diarrhoea and pneumonia than in healthy calves (Oikonomou *et al.*, 2013). The relative abundance of certain bacteria in the calf gut has been found to be significantly different between calves fed raw milk containing ceftiofur, penicillin, ampicillin and oxytetracycline and non-antibiotic residues (Pereira *et al.*, 2016). Similar antibiotic usage has been shown to decrease the diversity of gastrointestinal microbiota profiles in humans (Claesson *et al.*, 2011) and in pigs (Looft *et al.*, 2014). The study in Paper II was a short-term assessment of the

microbial biodiversity, but a study in canines has found that depression of some taxa following antibiotic treatment persists for several months (Suchodolski *et al.*, 2009). Feeding waste milk containing β-lactam antibiotic residues is reported to increase the presence of β-lactamase resistance genes in the *Escherichia coli* population of pre-weaned calves compared with calves fed milk replacer (Maynou *et al.*, 2016).

In this thesis, associations between microbiota profile and other variables, such as gender, weight gain, birth weight, transfer of passive immunity and antibiotic treatment were also assessed in the calves (Paper II). However, none of these variables was linked to the composition of the faecal microbiota in either newborn calves (D0) or 14-day-old calves.

5.4 Incidence of antibiotic-resistant *Escherichia coli* in pre-weaned calves

Farmers reported unrestricted access to antibiotics and many farmers bought antibiotics without a prescription from a veterinarian or an animal health worker, as is common practice in the region. Antibiotic use mainly to treat or prevent mastitis was primarily based on farmers' previous experience and on drug sellers' advice based on symptoms described by the farmer, rather than being based on veterinary diagnostics. The farmer commonly adjusted the dose depending on the origin of the drug. Unsurprisingly, a high level (53%) of multidrug-resistant *Escherichia coli* was detected in newborn dairy calves in the study area in southern Vietnam. Arbitrary use of antibiotics in pig, poultry, fish and shrimp production has also been found in other studies conducted in SE Asia in general and in the Mekong river delta, Vietnam, in particular (Nhung *et al.*, 2016; Carrique-Mas *et al.*, 2015).

A high prevalence of resistance to ampicillin, tetracycline and chloramphenicol was detected in Paper IV (Figure 6). This probably reflects a long tradition of use of these antibiotics in livestock production and is in line with findings in recent studies in SE Asia (Changkaew *et al.*, 2015; Nhung *et al.*, 2015; Lay *et al.*, 2012). The antibiotic resistance detected in Paper IV was significantly higher in *E. coli* strains isolated from calves at 14 days of age than in strains isolated from newborn calves (Figure 6). Resistant strains dramatically colonise dairy calves after birth, but the prevalence of antimicrobial-resistant strains commonly peaks in calves at 14 days of age (Donaldson *et al.*, 2006) and thereafter decreases in calves aged 4-6 weeks (Berge *et al.*, 2005). The reason is a transition from susceptible to resistant strains, and then back to susceptible strains, in the gut of the developing calf, and there is no re-emergence of susceptible strains that dominate when the calves are younger (Hinton *et al.*,

1985). The incidence of antibiotic-resistant *Escherichia coli* was higher in calves on smallholder farms in Vietnam study than in calves in Sweden (Paper IV), as also found in a study by Duse *et al.* (2015). This may be the consequence of overuse and misuse of antibiotics, and of a lack of proper legislation on the manufacturing and distribution of pharmaceutical products. In addition, smallholder dairy farms in Vietnam are very close to each other geographically, which may pose a risk of antimicrobial resistance spreading between farms.

The farmers surveyed in this thesis often applied livestock wastewater and manure directly to grass crops. Livestock manure can harbour a large numbers of pathogenic bacteria (*e.g. Escherichia coli*, *Salmonella* spp., *Campylobacter* spp.), parasites (*e.g. Ascaris suum*, *Cryptosporidium* spp.) and viruses (*e.g.* rotavirus) (Christou & Kosmidou, 2013; Milinovich & Klieve, 2011). Animal waste represents a critical link in the spread of antimicrobial resistance, because it harbours antimicrobial-resistant bacteria (Sivagami *et al.*, 2018). These findings probably explain the increasing dominance of antibiotic resistance genes detected in Paper IV. There was a high incidence of quinolone-resistant *Escherichia coli* (21%) and a large proportion (30 out of 33 isolates in plasmid-mediated quinolone resistance (PMQR) screening) of *qnrS* gene on the farms studied. The high level of plasmid-borne resistance genes could contribute greatly to further spread and sharing of genes. San Millan (2018) found that plasmids drive the horizontal transfer of antibiotic resistance genes. Moreover, interactions between plasmids and the bacterial chromosome impact the spread of antibiotic resistance (Gama *et al.*, 2018).

5.5 Considerations on the methods used in this thesis

The methodological approach selected and the number of samples analysed play an important role in obtaining data from which it is possible to draw powerful conclusions. For example, the extraction and purification of DNA from samples are important in molecular studies. In this thesis, DNA was isolated from gut samples using the QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) (Papers II and III), while DNA was isolated from colostrum using a Power Food Microbial DNA Kit (MO BIO Laboratories Inc., Carlsbad, CA 92010, USA) according to the manufacturer's protocol (Paper II). These different kits were used to ensure that the extracted genetic content accurately represented the population of microbiota in the different types of sample matrix (colostrum and intestinal samples). However, the different DNA isolation methods used for colostrum and intestinal samples could have affected the observed species composition, so comparisons of data for these samples matrices should be made with caution. Moreover, in Paper II the 16S rRNA gene was sequenced in only

eight colostrum samples and 40 faeces samples from the 14-day-old calves when assessing the microbiota taxa present in colostrum and faeces, which is a limited number of samples. Although the T-RFLP method that was used for all samples in Paper II gives an overview of the microbial community profile, the Illumina-based sequencing method provides a higher resolution of the community composition and also reveals the microbial taxa present in the samples. Moreover, 50% out of the D0 faeces samples did not produce a PCR product, despite several attempts. This was most likely due to the number of bacteria in these samples being too low, *i.e.* below the detection limit of the PCR method used.

6 Conclusions

- A majority (91%) of cows on Vietnamese smallholder dairy farms surveyed in this thesis produced colostrum of high quality. As a result, only 10% of calves suffered from failure of passive immune transfer.
- The composition of the microbiota in calves clearly changed during the first two weeks of life. The microbiota in colostrum was more similar to the faecal microbiota in newborns than in 14-day-old calves. *Lactobacillus*, *Escherichia*, *Bacteroides*, *Faecalibacterium* and *Butyricicoccus* were found in high relative abundance in the early life of dairy calves. *Escherichia* dominated and was present in significantly high abundance in two-day-old calves.
- The composition of the microbiota in the gastrointestinal tract differed between gut segments and between mucosa and intestinal content. Thus studies of microbial communities based on analysis of faeces samples do not reflect the entire gastrointestinal tract, but only the distal part.
- At 14 days of age, *Faecalibacterium* and *Butyricicoccus* were present in significantly higher relative abundances in healthy calves than in those with diarrhoea.
- Calves were colonised with antimicrobial-resistant *Escherichia coli* already in the first day of life.
- A high proportion of tetracycline and ampicillin resistance was found and was carried by 50% of isolates.
- Around 53% of the isolates were resistant to at least three antimicrobials, 3% carried a gene encoding for extended-spectrum cephalosporinases and 21% had a plasmid-mediated quinolone resistance phenotype.

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Popular science summary

Calf health has a marked effect on the profitability of dairy production, due to the direct costs of calf losses, treatment of diseases and the long-term impact on their performance. Digestive and respiratory problems are two major causes of calf losses. Digestive problems, such as ruminal and intestinal diseases, have serious influences on the health of dairy calves. In particular, calf diarrhoea is a major problem during the pre-weaning period and results in high mortality rates for dairy calves in many countries worldwide. Diarrhoea in newborns is also a main reason for antibiotic treatment in dairy calves. The use of antibiotics in livestock production has clear implications for public and animal health, due to the increasing problem of antibiotic resistance. Improving calf health by better management and feeding practices plays an important role in decreasing antibiotic use. Antibiotics are also an important factor that can change the gut microbiota composition.

The thesis investigated the early life of dairy calves in relation to colostrum quality offered, gut microbial colonisation and presence of antimicrobial-resistant bacteria (*Escherichia coli*). The microbiota along the gastrointestinal tract of young calves was analysed and the impact of colostrum quality, diarrhoea and antibiotic treatment on microbial composition and immunoglobulin G (IgG) absorption was assessed. In addition, antimicrobial resistance in calves was studied.

The analyses revealed that 91% of colostrum samples from newly calved cows on smallholder dairy farms in Vietnam was of good quality. Calves on these farms were fed colostrum with high levels of IgG within the first 4 h of life, a practice associated with good IgG absorption in calves. The microbial composition of colostrum showed large variations and was more similar to the faecal microbiota in newborn calves than in 14-day-old calves.

The microbial composition, based on the analysis of faeces samples, was found to differ between different gut segments. The gastrointestinal tract bacterial composition also differed between mucosa and gut content in newborn

calves. Diarrhoea and antibiotics treatment were key factors affecting the gut microbiota in young calves.

Antibiotic resistance was found to be higher in *Escherichia coli* strains isolated from Vietnamese calves at 14 days of age than in strains isolated from newborn calves. Of the isolates obtained, 53% were classified as multidrug-resistant (*i.e.* resistant to three or more antibiotic classes) and 21% of isolates had plasmid-mediated quinolone resistance (PMQR) phenotype, carried the *qnrS* gene. In Vietnam, humans live in close proximity to calves and thus there is a high risk of these genes spreading to the human population.

Overall, the results in this thesis show that cows on smallholder farms in southern Vietnam produce good-quality colostrum and that farmers feed colostrum to calves within the first 4 h after birth, giving the calves a good start in life. Good calf health was shown to correlate to the gut microbiota and increase growth rate in young dairy calves. Arbitrary use of antibiotics was observed in the study area and is worrying, because it leads to increased antibiotic resistance on farms, from where it can spread to humans.

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