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BENEFITS OF FERMENTED LIQUID DIETS FOR SOWS AND THEIR PIGLETS

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**BENEFITS OF FERMEN~~ED~~ED LIQUID DIETS FOR SOWS AND THEIR
PIGLETS**

by

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A thesis submitted to the University of Plymouth

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BENEFITS OF FERMENTED LIQUID DIETS FOR SOWS AND THEIR PIGLETS

A programme of study was undertaken to assess the antimicrobial and potential immunological properties of fermented liquid feed fed to sows in late gestation period.

A liquid feed fermented with *Lactobacillus plantarum* fed to the pregnant sow in late gestation had the potential to beneficially modify the microflora the lactating sows introduces into the environment, which was consequently reflected in beneficial changes in the gut microflora of their litters. In addition, colostrum from sows fed FLF had a significantly greater mitogenic activity on both intestinal cells (79326 ± 3069 CPM) and blood lymphocytes (1903 ± 204 CPM) compared with colostrum from dry feed fed sows (53433 ± 1568 CPM and 1231 ± 61.4 CPM respectively). The combined effects of higher milk quality and the reduction in the level of environmental contamination with faecal pathogens, achieved by FLF, may be important in achieving improved health status for both sows and piglets.

A series of *in vitro* experiments were conducted in order to select a suitable new starter culture from LAB of porcine origin, which would have similar fermentative abilities to *Lactobacillus plantarum*, but could exert possible 'probiotic' effects on the host (pregnant sow). From a total of 87 faecal *Lactobacillus* strains, a homofermentative aggregating *Lactobacillus salivarius*, was selected for further *in vivo* studies as it was able to resist bile and acid conditions, had good adherence abilities to all intestinal compartments (mucus, epithelial cells, collagen), as well as being a potent stimulator of interleukin (IL)-12. In addition, it had comparable fermentation properties to the *Lactobacillus plantarum* that has been widely used to ferment FLF in this laboratory.

In vivo experiments with porcine *Lactobacillus salivarius* strongly indicated that this bacterial strain is an effective inoculant for FLF, with a potential double role: the role of the starter culture and the role of probiotic. This could help sows to overcome disorders due to the stress associated with farrowing, enhance immunity of newborn piglets through better immunological quality of colostrum and control pathogen challenge for both sows and their newborn piglets.

FREQUENTLY USED ABBREVIATIONS

adDMFI (g d ⁻¹)	Average daily dry matter feed intake (grams per day)
ADFI	Average daily feed intake
ClO ₂	Chlorine dioxide
DF	Dry feed
DNA	Deoxyribonucleic acid
FLF	Fermented liquid feed
GI	Gastrointestinal
GLM	General linear model
HPLC	High-performance liquid chromatography
IEC	Intestinal epithelial cells
IL	Interleukin
LAB	Lactic Acid Bacteria
<i>Lactobacillus</i> GG	GG- is named after the first letter of its co-discoverers Sherwood Gorbach and Barry Goldin, who were awarded a patent on this strain in 1989
MRD	Maximum Recovery Diluent
MRS	Mann Rogosa Sharpe (broth/agar)
NFLF	Non-fermented liquid feed
OD	Optical density
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
RBCA	Rose Bengal Chloramphenicol Agar
RNA	Ribonucleic acid
SCFA	Short-chain fatty acids
VRBA	Violet Red Bile agar

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Book chapters

Demečková, V., Moran, C. A., Caveney, C., Campbell, A., Kuri, V. and Brooks, P. H. 2000. The effect of fermentation and/or sanitization of liquid diets on the feeding preferences of newly weaned pigs. In: *Digestive Physiology of Pigs* (eds. Linberg, J.E and Ogle, B), pp. 291-293, CABI Publishing, Nottingham.

Brooks, P.H., Moran, C. A., Beal, J.D., Demečková, V. and Campbell, A. 2001. Liquid feeding for the young piglet. In: *The weaner pig*, (eds Varley, M.A. and Wiseman, J.), pp.153-178, CAB International, Wallingford, UK.

Journal publication

Štyriak, I., Demečková, V., and Nemcová, R. 1999. Collagen (Cn-I) binding by gut lactobacilli. *Berliner und Münchener Tierärztliche Wochenschrift* 112, 301-304.

Demečková, V., Štyriak, I., Zatkovič, B. and Nemcová, R. 2000. Collagen binding by lactobacilli. *Czech Journal of Animal Science* 45, Suppl.1, 1.

Štyriak, I. Demečková, V., Žatkovič, B. and Kmet', V. 2001. Collagen binding by vaginal aggregative lactobacilli. *Veterinary Medicine-Czech* 46, 89-94.

Demečková, V., Kelly, D., Coutts, A.G. and Brooks, P.H. 2001. Effect of fermented liquid feed (FLF) on the quality of sow colostrum. *Recent Advances in Animal Nutrition in Australia* 13, 17A

Demečková, V., Brooks, P.H., Campbell, A.C. and Caveney, C. 2001. The effect of fermented liquid feed (FLF) on the faecal bacterial flora of lactating sows. *Recent Advances in Animal Nutrition in Australia* 13, 18A

Demečková, V., Kelly, D., Coutts, A.G.P., Brooks, P.H. and Campbell, A. 2002. The effect of fermented liquid feeding on the faecal microbiology and colostrum quality of farrowing sows. *International Journal of Food Microbiology* 79, 85-97.

Brooks, P.H., Beal, J.D., Demečková, V. and Niven, S. 2003. Probiotics for pigs – and beyond. In: *Role of probiotics in animal nutrition in view of demand of European consumers*, (eds. van Vuuren, A.M. and Rochet, B.), pp. 49-89, ID-Lelystad, Netherlands.

Brooks, P.H., Beal, J.D., Niven, S., Demečková, V. 2003. Liquid feeding of pigs. II. Potential for improving pig health and food safety. *Animal Science Papers and Reports* 21, (Supp. 1), 23-39.

Papers presented at conferences

Moran, C.A., Demečková, V. and P.H. Brooks. 2000. Fermented liquid feed as a potential replacement of growth promoting antibiotics for newly weaned pigs. International Probiotic Conference 2000, High Tatras, Slovak Republic. 11-14 October.

Brooks, P.H., Beal, J.D., Niven, S. and Demečková, V. 2003. Liquid feeding of pigs 2: potential for improving pig health and food safety. International conference on 'Effect of genetic and non-genetic factors on carcass and meat quality of pigs.' Siedlce, Poland, April 24-25.

Demečková, V., Tsourgiannis, C.A. and Brooks, P.H. 2003. Feeding fermented liquid feed to the gestating sow can reduce pathogen challenge of the neonatal environment. 5th International symposium on the epidemiology and control of foodborne pathogens in pork, Heraklion, Crete, Greece, October 1-4.

Posters presented at conferences

Kmet', V., Kmet'ová M., Žatkovič B., Demečková V., Kozarová M., Toth D., Polek B. 1999. The role of aggregation promoting factor in lactobacilli isolated from living organism and environment. BAGECO-6, Florence, Italy, June 20-24.

Demečková, V., Moran, C.A. and Brooks, P.H. 2000. The potential of chlorine dioxide (ClO₂) to produce a 'biosafe' feed for the newly weaned pigs. BSAS Occasional Meeting: The weaner pig, Nottingham, 5-7 September.

Demečková, V., Kelly, D., Coutts, A.G. and Brooks, P.H. 2001. Effect of fermented liquid feed (FLF) on the quality of sow colostrum. 16th Symposium in Recent Advances in Animal Nutrition in Australia, Armidale, Australia, July 13-16.

Demečková, V., Brooks, P.H., Campbell, A.C. and Caveney, C. 2001. The effect of fermented liquid feed (FLF) on the faecal bacterial flora of lactating sows. 16th Symposium in Recent Advances in Animal Nutrition in Australia, Armidale, Australia, July 13-16.

Demečková, V., Kelly, D., Brooks, P.H. and Campbell, A. 2002. The potential of fermented liquid feed (FLF) to affect the health status of newborn piglets. SfAM January meeting, *Frontiers in microbial fermentation and preservation*, Wageningen, Netherlands, 9-11 January.

Demečková, V., Kelly, D., Brooks, P.H. and Campbell, A. 2002. Influence of feeding fermented liquid feed on faecal bacterial flora and selected colostrum parameters of lactating sows. *BSAS annual meeting*, University of York, 8-10 April 2002.

Demečková, V., Tsourgiannis, C.A. Brooks, P.H. and Campbell, A. 2003. Fermented liquid feed (FLF) can reduce *E. coli* bloom at farrowing and prevent constipation problems during lactation. *BSAS annual meeting*, University of York, 24-26 March 2003.

Demečková, V., Tsourgiannis, C.A. and Brooks, P.H. 2003. Effect on average daily feed intake during lactation and piglet growth during the first 2 weeks of life of feeding sows fermented liquid feed (FLF), non-fermented liquid feed (NFLF) or dry feed (DF). *BSAS annual meeting*, University of York, 24-26 March 2003.

Tsourgiannis, C.A., Demečková, V., Edison, J. and Brooks, P.H. 2003. Effect of dietary salt (NaCl) level on the growth performance of liquid fed growing-finishing pigs. *BSAS annual meeting*, University of York, 24-26 March 2003.

Tsourgiannis, C.A., Demečková, V., Edison, J. and Brooks, P.H. 2003. The effect on litter performance at birth of feeding gilts fermented liquid feed (FLF), non-fermented liquid feed (NFLF) or dry feed (DF) for 14 days pre-farrowing. *BSAS annual meeting*, University of York, 24-26 March 2003.

Demečková, V., Tsourgiannis, C.A. and Brooks, P.H. 2003. The effect of fermented liquid feed, fed in late pregnancy, on the protein and immunoglobulin contents of porcine colostrum. 9th International symposium on Digestive Physiology in Pigs (DPP), Banff, Alberta, Canada, May 14-17.

Demečková, V., Tsourgiannis, C.A. and Brooks, P.H. 2003. Beneficial changes of *Lactobacilli*, coliforms and *E. coli* numbers in the faeces of farrowing primiparous sows, achieved by fermented liquid feed, positively affect subsequent neonatal colonisation. 9th International symposium on Digestive Physiology in Pigs (DPP), Banff, Alberta, Canada, May 14-17.

Tsourgiannis, C.A., Demečková, V., Eddison, J. and Brooks, P.H. 2003. Effect of dietary salt (NaCl) level on chewing behaviour by liquid fed growing-finishing pigs. 9th International symposium on Digestive Physiology in Pigs (DPP), Banff, Alberta, Canada, May 14-17.

Tsourgiannis, C.A., Demečková, V., Brooks, P.H. and Eddison, J. 2003. Effect of dietary salt (NaCl) level on drinking behavior by liquid fed growing-finishing pigs. 9th International symposium on Digestive Physiology in Pigs (DPP), Banff, Alberta, Canada, May 14-17.

Tsourgiannis, C.A., Demečková, V., Brooks, P.H. and Eddison, J. 2003. Effect of dietary salt (NaCl) level on biting by liquid fed growing-finishing pigs. 37th International Congress of the ISAE, Abano Terme, Italy, June 24-28.

Signed: Wasta Demečková
Date: October 6th, 2003

Chapter 1

Literature review

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1.1 The neonatal pig

The neonatal period is a particularly critical stage during which the long-term development of the pig can be affected. It is the period of the pig's life that can be regarded as a transition from the protected existence of the foetus to the vulnerable life of the free-living organism (Reeds *et al.*, 2000). The newborn must overcome respiratory, immunological, digestive, nutritional and thermoregulatory challenges if it is to survive. Indeed, recent UK data showed that an average of 10 % of potential growing pigs die before weaning (Meat and Livestock Commission, 2001). In the European Union (EU) one in six piglets is stillborn or does not survive until weaning, which means that approximately 50 million piglets, of which 33 million are born alive, are lost each year (Le Dividich, 2002). In addition to a serious economic impact, these high preweaning mortality rates are also unacceptable from an animal welfare point of view. One of the major achievements in pig production over the last 30 years has been the improvement in sow productivity from about 16 to 22 piglets reared per sow per year (Close and Cole, 2000). Individual attention from the producers at this point of the pig's life pays off with more live pigs at birth and consequently larger litters at weaning, which are two key factors for a good profitable swine herd.

The nutrition and management of the newborn pig begins prior to birth. In fact, it is impossible to separate completely prenatal and postnatal development because the foetal environment influences postnatal characteristics and development. One objective of feeding the gestating sow during the last 3-4 weeks of gestation is to supply sufficient nutrients to the foetuses for optimal development and birth weight. The majority of foetal development occurs in the last trimester of gestation (Bazer *et al.*, 2001). Proper nutrition

and correct feed management of the lactation diet in the last four weeks of gestation will ensure sufficient nutrients available to the foetuses for optimal development.

1.1.1 Nutrition during pregnancy and foetal development

The average gestation period for sows is 112-116 days, depending on the breed, size of litter, and season (Richard *et al.*, 1976). Appropriate feeding during this time is important because it determines the size and subsequent viability of piglets at birth as well as the amount of mammary tissue at parturition. Daily energy requirements during pregnancy correspond to the sum of requirements for maintenance, uterine growth and maternal gain (Noblet *et al.*, 1990). It has been reported that high levels of feed intake during the first 3 weeks of gestation may have a negative impact on embryo survival (Trottier and Johnston, 2001). Therefore, the safe practice is to restrict feed intake to 2.0 kg per day during the first 21 days post-breeding (Burrin, 2001). However, some other studies contradict the view that low level feeding in early pregnancy may somehow be beneficial to subsequent litter size (Kirkwood *et al.*, 1990; Hughes, 1993). Undernutrition in early gestation has to be very severe ($< 1 \text{ kg day}^{-1}$) to reduce embryo survival (Speer, 1990). Mid gestation (21 to 75 days) represents the most appropriate time period, in which to get sows back into the correct body condition. A body condition score of 3 is desirable at this time. The daily energy needs for growth of the gestating sow are about 1.29 Mcal, of which approximately 15% is deposited in the foetus (Verstegen *et al.*, 1987). Feeding *ad libitum* during mid to late gestation (day 75 till 90) should also be avoided. Weldon *et al.*, (1991) demonstrated that excess energy intake during this time-period reduced secretory cell numbers in mammary glands. In summary, it can be stated that overfat sows during gestation are more likely to experience (Trottier and Johnston, 2001):

- Increased embryonic mortality
- Increased farrowing difficulty
- More crushed pigs
- Decreased feed intake during lactation
- Lower milk production
- Increased susceptibility to heat stress

On the other hand, insufficient gestating diet may results in:

- Failure to return to oestrus
- Lower conception rates
- Smaller subsequent litter size

It has been suggested that sows will have minimum weaning-to-service intervals when they weigh 150 kg or more at weaning (Aherne *et al.*, 1992). According to Noblet *et al.*, (1990) a single value cannot be recommended as the energy requirement of pregnant sows. They concluded that ME requirements during pregnancy differ with body weight (BW) of the animals, environment, previous lactation BW loss and the desired maternal weight gain. The nutrient requirements to support lactation are considerably greater than those of gestation. The daily energy needs for lactation are 3 to 4 times greater than for gestation, ranging from 15-20 Mcal day⁻¹ depending on sow BW (Burrin, 2001). The nutrient requirements of the lactating sow are a function of the sow's needs for growth and maintenance and the needs for milk production. Milk production requires a large supply of substrates. It has been shown that 65-70% of the total energy requirement of the lactating

sow is to support milk production but very little is known about mammary uptake and metabolisms of specific nutrients in sows (Klopfenstein *et al.*, 1999).

1.1.2 Postnatal care

The newborn pig has three basic requirements:

- A warm, draught free and clean environment
- Adequate and regular nutrition
- Safety from disease and crushing

1.1.2.1 Neonatal environment

Environment is a very broad term relating to all factors that affect the animal. In the neonatal environment factors such as warmth and hygiene play pivotal roles (Cutler *et al.*, 1999). At birth, the neonate must begin to regulate its own body temperature to survive. The neonatal pig contains less than 2% body fat and almost no subcutaneous fat (Makkink and Schrama, 1998). These small fat reserves as well as glycogen represent the major source of readily utilised energy substrates for heat production within the first 12 to 24 hours of life (Close, 1992). Because of the low body-fat covering, the newborn pigs are extremely cold sensitive and therefore they must have supplemental heat to prevent chilling and to maintain a normal body temperature. Cold, wet conditions will rapidly deplete energy reserves in the neonate and will severely compromise its survival. Chilling is probably responsible for more piglet deaths than any other cause (Le Dividich and Noblet, 1981; Close, 1992). It has also been shown that cold stress of the neonate reduces

its acquisition of colostral immunoglobulins, which is reflected in increased mortality (Blecha and Kelley, 1981). During the first day of life, colostrum intake was reduced from 290 g in piglets exposed to 30 to 32°C to 212 g in those exposed to 18 to 20°C (Le Dividich and Noblet, 1981). In order to avoid hypoglycemia and hypothermia, the piglet needs an exogenous source of energy. In the first days of life, colostrum provides as much as 60% of the newborn pigs' energy needs for heat production due to presence of lactose and fat (Burrin, 2001). In addition, it also has an enhancing effect on the hormonal and metabolic mechanisms controlling blood glucose levels. Piglets consume approximately 15 ml of milk per feeding providing approximately 7 g of lactose and 16 g of fat (Cutler *et al.*, 1999). It has been suggested (Noblet and Le Dividich, 1981) that piglets need at least 150 g colostrum per kg body weight in order to maintain a normal nutrient profile in the blood.

The lower critical temperature of the lactating sow differs greatly from that of piglets. At birth, piglets require a very warm environmental temperature of 35°C, whereas lactating sows prefer 15-20°C (McGlone *et al.*, 2001). Excessive farrowing house temperatures depress sow appetite, with subsequent weight loss in lactation and reduced suckling ability. On the other hand, a daily increase of 50-60 g feed is required for each degree below the sow's critical temperature (Aherne *et al.*, 1999). Therefore, if the floor and room temperature are below the piglet's comfort zone, it is essential to ensure that there are adequate heating lamps or under-floor heating in order to provide the right temperature in the creep area (Walton, 1991). It was suggested that at this stage of piglet's life, especially during the first 3 days, it is better to move heat to the piglets in some way rather than hope to draw the piglets away with warmth. Svendsen *et al.*, (1986) reduced piglet mortality in the first week from 7% to 1.1% by providing a movable heat source. This is based on the fact that the newborn piglet has a strong drive to move towards maternal odors

(Morrowtesch and McGlone, 1990). Piglets will learn their mother's odor signature within the first 12h of life. This odor drive is much stronger than drive to seek a warm air temperature and it is actually this olfactory attraction that is the root cause of crushing in the first 72h of life (Morrowtesch and McGlone, 1990).

The second environmental factor, which can have a big influence on newborn health and performance, is hygiene of the farrowing house. Implementation of a good sanitation program is usually much less expensive than any disease treatment. It has been shown that the physical environment may be a significant source of bacterial strains that colonize the gut of piglets. Many studies have demonstrated that dust in pig buildings is biologically active and it contains viable bacteria, fungi, endotoxins, toxic gases, and other hazardous agents (Arbuckle, 1968; Butera *et al.*, 1991; Donham, 1991; Martin *et al.*, 1996; Nowak, 1998; Zucker and Muller, 2002). The airborne Gram-negative bacterial flora was dominated by *Enterobacteriaceae* with *E. coli* and *Enterobacter agglomerans* as predominant species (Zucker and Muller, 2002). Animals, such as neonatal piglets, are particularly vulnerable to microbial invasion for the first few weeks of life and unless immunological assistance is provided, they may be killed by microorganisms that represent little threat to an adult. Strict hygiene, thorough cleaning and disinfection of the farrowing environment can help to reduce environmental microbial challenge and more specifically can reduce the incidence of pathogens that may be exclusive to newborn piglets. However, the sow remains a source of potentially pathogenic bacteria. One of the most common source of pathogens is the faeces of the dam and/or other pigs. It has been shown that suckling piglets ingest a large quantity of sow's faeces (18-25 g day⁻¹) and bedding material (Sansom and Glead, 1981). In addition, the sow's teats could be heavily contaminated with microbes from faeces and the environment. Gastrointestinal infections

associated with *E. coli* represent a serious problem for neonatal pigs. These bacteria are present in the sow's intestine in large numbers but increase dramatically just prior to farrowing due to stress occasioned by movement and parturition (Arbuckle, 1968; Maclean and Thomas, 1974).

Stress in general, has been shown to modulate an individual's immune system through the release of certain signal molecules such as catecholamines, cytokines and glucocorticoids (stress hormones), but the exact mechanisms is not yet fully understood (Sheridan *et al.*, 1994; Sainz *et al.*, 2001). There are complex bi-directional interactions among the central nervous system (CNS), the endocrine system, and the immune system (Moberg, 2000). In the dairy industry, substantial evidence indicates that innate and acquired defence mechanisms are lowest from 3wk precalving to 3wk postcalving, which is associated with the physical and metabolic stresses of pregnancy, calving and lactation (Mallard *et al.*, 1998). Moreover, very recent study with pigs provides first experimental evidence that prenatal maternal stress during late gestation is able to impair both humoral and cellular immune function in suckling piglets and that gestational stress in pigs may affect the ontogeny of the foetal immune system with consequences on the susceptibility to diseases and immune responsiveness to stressful stimuli of the offspring (Tuchscherer *et al.*, 2002). Many studies in the field of stress immunology have demonstrated that stress can suppress the immune system, primarily at the level of T lymphocytes and natural killer (NK) cells (La Via and Workman, 1991; Apanius, 1998; Raberg *et al.*, 1998; Alverdy and Rocha, 1999; Sapolsky *et al.*, 2000; Manteuffel, 2001; Tuchscherer *et al.*, 2002; Wonnacott and Bonneau, 2002; Yang and Glaser, 2002). In addition, it is also well established that stress decreases mucosal IgA function leading to increased susceptibility to mucosal infections (Alverdy and Rocha, 1999).

Specific signal molecules such as epinephrine (adrenalin), norepinephrine (noradrenalin), dopamine, interleukin-6 (IL-6), cyclic adenosine monophosphate (cAMP), glucocorticoids and prostaglandins are upregulated during episodes of acute and chronic stress and have been implicated as effectors of viral reactivation and recurrent disease (Sainz *et al.*, 2001). Support for direct effects of catecholamines on the immune system comes from the demonstration of adrenergic receptors on immune cells, namely T and B lymphocytes, macrophages, neutrophils and natural killer (NK) cells (Sheridan *et al.*, 1994). In terms of glucocorticoids (GC) it has been demonstrated that GC inhibit MHC class II antigen expression, tumor necrosis factor (TNF) production by macrophages, interferon gamma (IFN- γ) synthesis, interleukin-2 (IL-2) gene expression by lymphocytes as well as IL-1 gene expression by macrophages (Sheridan *et al.*, 1994). In pigs, GC concentrations increase sharply a few days prior to parturition and decrease during early lactation (Anderson, 2000). The study of Alverdy *et al.*, (1994) demonstrated that administration of glucocorticoids to rats resulted in a marked decrease of sIgA level in bile associated with bacterial overgrowth, adherence to and translocation from the caecum. Recently, it has been shown that catecholamines can enhance the growth of pathogenic bacteria. Supplementation of minimal medium inoculated with bacterial cultures with norepinephrine, epinephrine, dopamine, or synthetic catecholamine (isoproterenol) resulted in marked increases in bacterial growth compared to controls. From the catecholamines tested, norepinephrine had the greatest enhancing effects on the growth of *E. coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (Belay and Sonnenfeld, 2002). Growth of *Staphylococcus aureus* was also enhanced in the presence of norepinephrine, but not to the same degree as was the growth of Gram-negative bacteria. Nietfeld *et al.*, (1999) obtained similar results in their study by demonstrating *in vitro* growth enhancing activity of norepinephrine on the swine pathogen *Salmonella choleraesuis*. More interestingly,

Alverdy *et al.*, (1999) reported that stress can significantly affect not only host epithelial defense function but also bacterial virulence phenotypes. Via discreet and specific sensory input signals to bacteria (alterations in pH, redox state, nutrient composition, water content (hydrophobicity), norepinephrine concentration) the molecular machinery of otherwise commensal strains of bacteria can shift the phenotypes of residential colonizers to more virulent and invasive strains (Alverdy and Rocha, 1999; Hendrickson *et al.*, 1999). Hendrickson *et al.*, (1999) demonstrated that catabolic stress in the mouse was associated with more abundant presence of surface type 1 fimbriae and consequently a 7,500-fold increase in the numbers of indigenous *E. coli* bacteria adhering to the caecum. Furthermore, evidence from several studies, (reviewed by Soderholm and Perdue, 2001), indicates that stress can also adversely increase the epithelial permeability of the small intestine and colon. The stress-induced barrier dysfunction results in enhanced passage into the mucosa of both small molecules, including chemotactic peptides derived from bacteria, and macromolecules, such as intact proteins, with antigenic potential. This lowered immune responsiveness during stress together with endocrine changes around parturition and stress-induced intestinal barrier defects, can explain, at least in part, the dramatic increase of coliforms in the gut of farrowing sows as well as increased incidence of peripartum diseases.

From the foregoing discussion it can be concluded that control of the pathogen load within the pigs' environment is a critical part of management as the relative proportion of different organisms in the sow's faeces and the extent to which the piglet comes into contact with the faeces may affect colonisation of the gut.

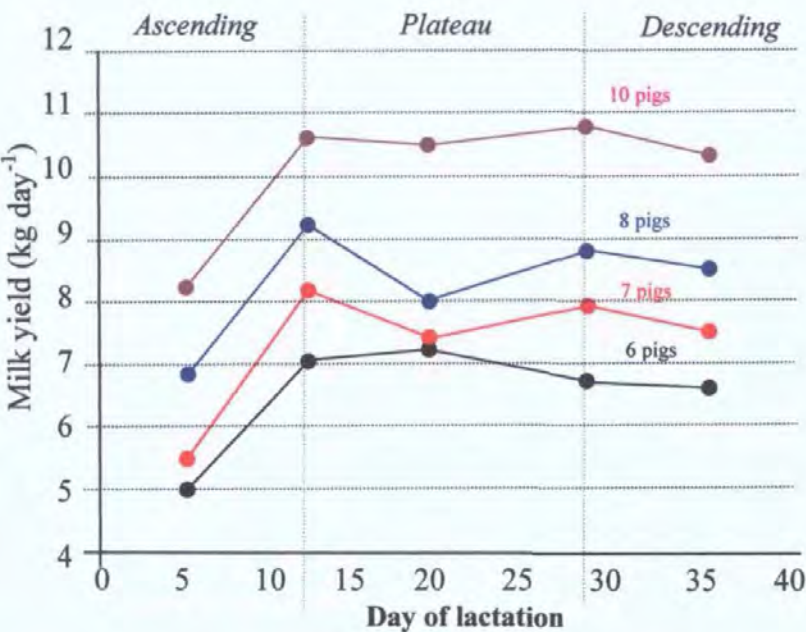
1.1.2.2. Neonatal nutrition

Once suckling is established, nutrition and therefore growth of the neonatal piglet are almost entirely dependent on colostrum and milk availability (Louveau *et al.*, 2000). Efficient swine production faces a number of challenges which relate directly to the sow's ability to produce sufficient milk to meet the needs of the rapidly growing litter (Hurley, 2001). Studies indicate that quantity and composition of milk produced by the sows accounts for 44% of the growth rate of the piglets. In sows, milk production peaks between 3-4 weeks postpartum (King, 2000). The availability of energy, macronutrients and other factors from colostrum / milk immediately after birth is critical for piglet survival and development. Therefore it is essential that newborn pigs achieve adequate colostrum intake immediately after birth, not only for nutritional, but also for immunological benefits.

The physiology underlying the formation of colostrum and the development of the lactation function in the mammary gland of the sow is in close synchrony with the birth of the piglet. The number of mammary glands per sow varies between 8 and 18, with an average of 12 (Cutler *et al.*, 1999). Growth of the porcine mammary gland occurs during gestation and continues into the 3rd or 4th week of lactation (Kim *et al.*, 1999). Early gestation is associated with elongation and branching of mammary ducts, mid gestation with formation of lobule-alveolus system, and after day 105 of gestation, metabolic activity of mammary gland is stimulated which results in synthesis and secretion of the milk constituents (Pond and Houpt, 1978). Growth of the gland is histologically complete by day 90 (Klopfenstein *et al.*, 1999). Secretion into the lumen of the ducts and alveoli begins by day 75 of gestation. By parturition, the gland is full of colostrum, and lactation begins under the influence of oxytocin, which is released to the blood stream (Pond and Houpt,

1978). Unlike the human, the sow has an exceptionally strong control of milk ejection. Some studies have shown that duration of milk ejection is only 10-20 seconds with average nursing interval less than 1 hour, which means that suckling piglet normally receives more than 24 feedings daily (Ellendorff and Poulain, 1984; Atwood and Hartmann, 1992; Auldist *et al.*, 2000; Hurley, 2001). Milk removal from the lactating mammary gland is the major factor in maintaining milk secretion (Hurley, 2001) and frequency of suckling plays an important role in regulating mammary gland development as well as milk production (Spinka *et al.*, 1997; Auldist *et al.*, 2000). Lacteal phase of lactation can be further divided into three parts - ascending, plateau, and descending (Figure 1.1).

Figure 1.1. Pattern of milk production of first-litter sows nursing litters of different sizes (adapted from Klopfenstein *et al.*, 1999).



Different publications report different time frames for these parts of lacteal phase, which are related to differences in breeds, nutrition, and parities of the sow as well as methods

used to estimate milk production (Harkins *et al.*, 1989; Grun *et al.*, 1993; Toner *et al.*, 1996). According to later publications, the end of the ascending phase is around day 14 postpartum (Figure 1.1). Plateau phase lasts from day 14 up to 28, which means that in intensive swine production units, sows never reach the descending phase as piglets are weaned at an early age. Milk composition varies dramatically among species and even within a species. However, for most species the major components of milk are water, fat, proteins, lactose, and minerals (Klopfenstein *et al.*, 1999).

Sow lactation could be broken down into two major parts: the colostral phase, which last for 18-24 hours, and the lacteal phase, which last for the rest of lactation. The composition of sow's colostrum and milk have been described in numerous publications (Perrin, 1955; Aumaitre and Seve, 1978; Klobasa *et al.*, 1987; Wu and Knabe, 1994; Darragh and Moughan, 1998; Klopfenstein *et al.*, 1999) and are briefly outlined in Table 1.1. Colostrum is a specialized pre-milk secretion of the mammary gland over the last few weeks of pregnancy together with proteins actively transported from the bloodstream. It represents the first food available to the newborn. This secretion of colostrum does not last for days, but it is created for the newborn to give it vital nutrients and antibodies the piglet needs immediately after birth. Unlike some mammals, which receive virtually complete passive immunity in utero, newborn pigs are born devoid of any immune proteins (agammaglobulinemic) (Wagstrom *et al.*, 2000). This is due to differences in the histology of porcine placenta. Histologically, the porcine placenta consists of six tissue layers and is classified as a diffuse epitheliochorial, which means that there is no transport of immunoglobulins from the dam to the foetus (Pond and Houpt, 1978). Therefore, sow colostrum is the only source of immunoglobulins and nonspecific immunity available to the newborn pig for disease-defense capabilities (Porter, 1969). The critical role of

colostrum ingestion in developmental regulation during the immediate neonatal period was emphasized in the study of Burrin (1997). They showed that skeletal, cardiac muscles, and critically, the brain, apparently require some unidentified component of colostrum to achieve a maximum rate of protein synthesis and growth. The composition of colostrum is quite different from that of mature milk (Table 1.1). The most important components of colostrum can basically be broken down into two major categories: A) immune factors and B) growth factors. However, several other factors found in sow milk may also contribute to the passive/active protection of suckling neonates.

A) Immune factors

Immune factors can be further divided into components providing 1) passive and 2) active protection.

1) Passive protection

Immunoglobulins protect the neonate from infection until their own immune system is developed. Colostral / milk antibodies are directed against gastrointestinal or respiratory tract pathogens with which the mother has come into contact (Hosseini *et al.*, 2001). They are rapidly taken up by non-specific pinocytosis into the enterocytes of the small intestine of the newborn piglets (Porter, 1969). The critical event in controlling the transfer of intact immunoglobulins into the circulation of the piglet is cessation of transfer across the basolateral membrane of the enterocytes (Rooke and Bland, 2002). This 'gut closure' takes place as early as 24h of age in suckling pigs and it is stimulated by intake and absorption of nutrients rather than any specific component of colostrum (Mehrazar *et al.*, 1993).

Table 1.1. The main components of sow's colostrum and milk

<i>Nutrient</i>	<i>Colostrum¹</i>	<i>Milk²</i>	<i>Reference³</i>
Total Proteins (g/100g)	15.14	5.47	
Casein (g/100g)	1.48	2.74	
Whey (g/100g)	14.75	2.22	
Serum albumin (mg/ml)	15.79	4.61	
IgG (mg/ml)	95.6	0.9	
IgA (mg/ml)	21.2	5.3	
IgM (mg/ml)	9.1	1.4	
Amino Acids (g/100g amino acids)			
Lzine	6.5	7.5	
Methionine	1.8	1.7	
Cysteine	2.2	1.5	
Histidine	2.3	2.4	
Phenylalanine	4.5	3.9	
Tyrosine	4.4	4.2	
Threonine	5.5	3.9	
Isoleucine	3.0	3.8	
Leucine	9.8	8.8	
Valine	5.5	4.7	
Tryptophan	1.6	1.4	
Arginine	5.4	5.2	
Proline	9.2	11.3	
Glycine	3.3	2.8	
Glutamic acid	17.4	21.6	
Aspartic acid	7.8	7.9	
Serine	6.3	5.2	
Alanine	4.4	3.2	
Lactose (g/100g)	3.4	5.3	
Fat (g/100g)	5.9	7.6	
Ash (g/100g)	0.7	0.9	
Cholesterol (g/L)	-	0.34	e
Fatty acids (%)			e
Medium chain	-	0.65	
Long chain saturated	29	34	
Monounsaturated	45	45	
Polyunsaturated	24	20	
Vitamins (g/100ml)			
Vitamin A	169	96	
Vitamin D	1.58	0.95	
Vitamin E	390	266	
Vitamin C	7.2	8.42	
Vitamin B ₁₂	-	0.15	
Vitamin K ₃	9.68	9.37	
Biotin	-	1.4	

Table 1.1. (cont.) The main components of sow's colostrum and milk

<i>Nutrient</i>	<i>Colostrum¹</i>	<i>Milk²</i>	<i>Reference³</i>
Minerals (mg/100 g)			
Calcium	68.6	162.8	
Phosphorus	101.7	118.3	
Potassium	11.0	58.7	
Sodium	68.5	39.3	
Magnesium	7.9	9.0	
Iron	0.2	0.2	
Zinc	1.6	0.7	
Sulphur	-	3.6	
Copper	0.4	0.2	
Selenium (mg/L)	0.10-0.15	0.02-0.03	e
Chromium (mg/L)	0.60	0.46	e
Aluminium (mg/L)	3.5	1.8-3.7	e
Nickel (mg/L)	0.42	0.31	e
Chloride (mg/L)	1200	1000	e
Molybdenum (mg/L)	0.04	0.02-0.01	e
Ammonia (mmol/L)	2.01	1.25	e
Urea (mmol/L)	6.04	5.18	e
Lactoferrin (µg/ml)	~1600	~400	f
Bombensin (pg/ml)	1995	-	e
Neurotensins (pg/ml)	265	-	e
Polyamines (µmol/L)			h
Spermidine	~5	2.05	
Spermine	21.6	5.16	
Cells (%)			b
Neutrophils	62.7	27.2	
Macrophages	24.5	19.4	
Lymphocytes	8.8	6.0	
Eosinophils	1.5	1.7	
Epithelial cells	2.5	45.7	
Growth factors			
EGF (ng/ml)	~1500	150-250	c
IGF-I (ng/ml)	136	11	d
IGF-II (ng/ml)	291	17	d
TGF α - β (ng/ml)	9-12	-	c
Leptin (ng/ml)	53.7 \pm 15.2	25.5 \pm 2.5	i
Insulin (ng/ml)	10.8-18.2	1.2-5.5	c
Lysozyme (µg/ml)	9.2 \pm 3.9	5.2 \pm 2.0	g

Table 1.1. (cont.) The main components of sow's colostrum and milk

<i>Nutrient</i>	<i>Colostrum¹</i>	<i>Milk²</i>	<i>Reference³</i>
Gross energy (kcal/L)	1517	1114	e

¹ taken immediately postpartum; ² classified as a milk samples collected between 14 and 21 days postpartum; ³ from Darragh and Moughan, 1998 in Verstegen, Moughan and Schrama eds., *The lactating sow*, Wageningen Pers, The Netherlands, adapted partially from b) (Lee *et al.*, 1983); c) (Xu *et al.*, 2000); d) (Donovan *et al.*, 1994); e) (Burrin, 2001); f) (Yang *et al.*, 2000); g) (Schulze and Müller, 1980); h) (Motyl *et al.*, 1995); i) (Wolinski *et al.*, 2003).

Functionally, this process ensures that if the neonatal piglet has an adequate colostrum intake, a sufficient amount of IgG is absorbed in a minimum time thus reducing the chances of invasion by potentially pathogenic macromolecules prior to gut closure (Rooke and Bland, 2002). However, the exact mechanism by which the amount of nutrients absorbed induces closure is not clear and warrants further investigation.

Interestingly, although immunoglobulins are very important in the neonatal defense against infectious agents, they alone cannot kill anything. They protect the neonates by three basic mechanisms:

- 1) by coating the infectious agents in order to prevent them from attaching to or penetrating host cells
- 2) by agglutinating infectious agents to reduce their infectivity
- 3) by neutralizing toxins by direct binding to them

A very important function of immunoglobulins is that they mark (opsonize) infectious agents for destruction by complement system, phagocytic cells, and/or cytotoxic cells

(Roth, 1999). In doing so, the immunoglobulins can form a bridge between the phagocyte (e.g. macrophage) and the invader, bringing it close, and preparing it for destruction (phagocytosis) (Figure 1.2). In the piglet, immunoglobulin-producing cells first appear in the gut at the end of the first week of life and reach mature profile after a month (Bourne, 1976).

The three major immunoglobulin classes IgG, IgA and IgM have been isolated and characterized in the porcine mammal secretions (Porter, 1969; Curtis and Bourne, 1971) (Table 1.2).

Figure 1.2. Simplified drawing depicting the immunoglobulin “bridge” between bacteria and phagocytic cells. When immunoglobulins bind to bacterial surface (opsonize) they do so with their Fab regions, leaving their Fc regions available for binding Fc receptors on the surface of phagocytic cells (Sompayrac, 1999)

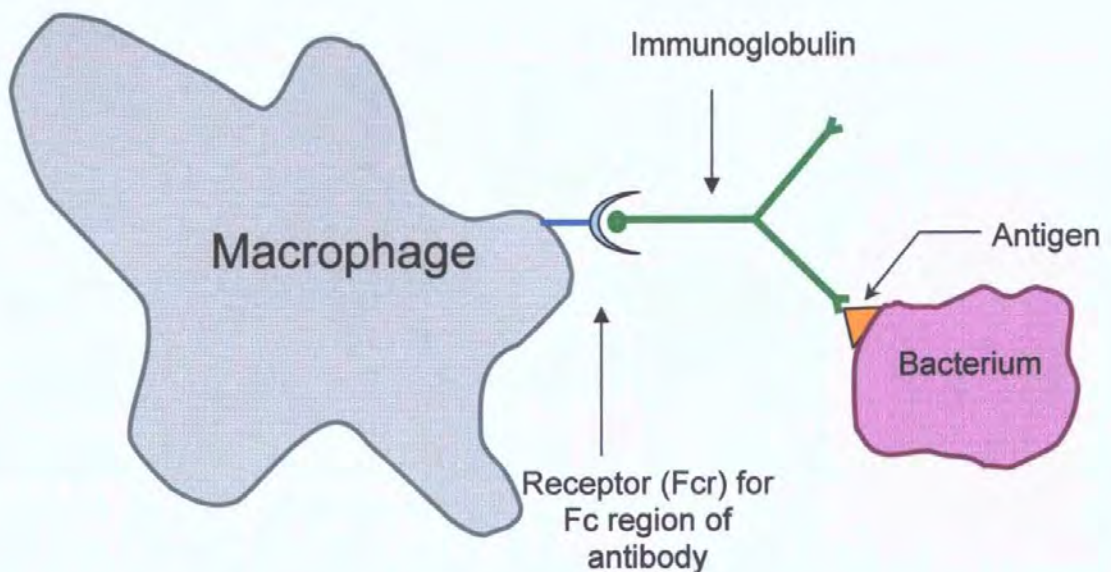


Table 1.2. Properties and biological activities of sow milk immunoglobulins.

Property / Activity	IgG		IgA	IgM	Ref.
	<i>IgG₁</i>	<i>IgG₂</i>			
Sedimentation coefficient $S_{20,w}$	6.7S	18S	10.6-11.2 S	17.8S	a
Concentration in colostrum (%)	60		6	5-10	c
Concentration in milk (%)	29		70	1	c
Factors influencing C					
Half lives after absorption (days)	6.5 - 22.5		2-3	3.5-6.5	b

a) (Porter, 1975), b) (Curtis and Bourne, 1973); c) (Butler, 1973)

In swine, all colostrum IgG was found to be derived from serum and although 60% of colostral IgA is produced locally, this represents only a small fraction of total colostral immunoglobulins. Therefore, sow colostrum can therefore be regarded as a serum transudate and not a true secretion as far as immunoglobulins are concerned, which only emphasizes its suitability in performing immune function (Bourne, 1976). There are several mechanisms that serve to protect immunoglobulins in the neonate's digestive systems in order to be absorbed as intact molecules. Colostrum is a good buffering compound and protects immunoglobulins from denaturation by extremes of pH. Furthermore, antibodies are protected not only by the presence of sow colostrum antitrypsin factor (Watson, 1980), but also by the presence of high amounts of proteinase inhibitors in the digestive system of the neonates (Ohlsson *et al.*, 1986) as well as by the function of chymosin. Chymosin represents the pig's most important protease in the immediate postnatal period as it acts specifically against the κ - casein fraction of milk

protein and clots the milk without further proteolytic breakdown of the peptide bonds (Yen, 2001a).

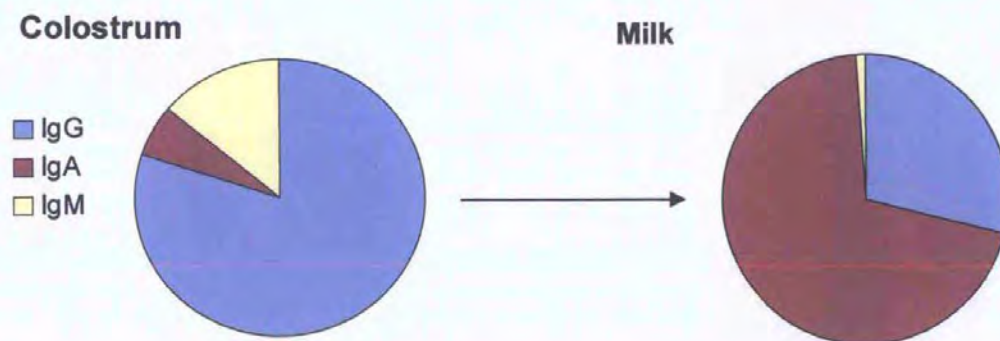
IgG represents quantitatively the most important immunoglobulin class in the pig, accounting for more than 60% of the immunoglobulin in sow colostrum (Roth, 1999). It also represents the first immunoglobulin synthesized by piglets and its major role is to provide the piglet with systemic immunity (Brandenburg and Wilson, 1973). However, with the onset of lactation, a sharp decrease in the IgG concentration and biological activity occurs over the first 2-3 days of suckling. The period of lactation during which colostral IgG persists at optimum level is very similar to the natural intestinal absorptive phase of the neonate which has been established to be 24-36 hours (Lecce and Morgan, 1962). It has been shown that concentration of IgG in piglet plasma depends on the amount of colostrum ingested, IgG concentration in colostrum and the timing of gut closure (Rooke and Bland, 2002). These findings only emphasize the importance of colostrum IgG concentrations as piglets born late in the birth order to a sow producing colostrum with low IgG concentrations would have reduced plasma IgG concentrations and therefore face a higher risk of potential disease. The importance of an adequate IgG intake from colostrum in the first day of life was also reinforced by recent evidence which suggests that the synthesis of IgG by the piglet is positively related to the amount of maternal IgG absorbed before the gut closure (Rooke and Bland, 2002). The concentration of colostral IgG is not constant but depends on many factors. Inoue *et al.*, (1980) analysed 13 possible factors on 157 samples of sow colostrum and it was revealed that the district, number of parturition, kind of feed, type of farming, and number of sows raised on a farm were the most influential. The mean IgG concentration was 53.03 mg ml⁻¹ with the maximum and minimum values 101.39 mg ml⁻¹ and 11.74 mg ml⁻¹, respectively. There are two main subclasses of porcine IgG (IgG₁ and IgG₂) with IgG₁ predominating in serum and

colostrum. The IgG₃ and IgG₄ subclasses were also found but in lesser concentrations (Roth, 1999).

IgM is quantitatively the least abundant of the 3 immunoglobulin classes, accounting for only 5-10% of the total immunoglobulin in colostrum (Roth, 1999). The molecule of IgM is a pentamer held together by disulfide bonds. This class of immunoglobulin carries the most potent antibacterial activity, and provides protection against pyrogenic and lethal effects of endotoxin from Gram-negative bacteria (Porter, 1969). Administration of *E. coli* antigens by oral route generates IgM and IgA antibody producing lymphocytes in the lamina of the gut (Allen and Porter, 1973).

IgA is the predominant isotype of milk. This means that a marked change occurs in the immunoglobulin profile of mammary secretions during the early part of lactation from an IgG to IgA predominance (Figure 1.3) (Bourne, 1976).

Figure 1.3. Immunoglobulin profile of sow colostrum and milk (adapted from Butler, 1973).



This difference in immunoglobulin composition of colostrum versus milk is hormonally regulated (Wagstrom *et al.*, 2000) and reflects the changing needs of the piglet, as absorption of whole proteins gives way to the maintenance of localized immune protection within the gut (Darragh and Moughan, 1998). In general, the IgG tends to protect against systemic infections while the prolonged intake of milk IgA protects against enteric disease (Tizard, 2001). Flow cytometric analysis of faecal bacteria showed that *in vivo* many bacteria (24-74%) are coated with IgA and less with IgG and IgM (van der Waaij *et al.*, 1996). The major action of IgA in the neonatal intestinal tract is to block the adhesion of enteropathogenic bacteria, viruses and bacterial enterotoxins to epithelial cells by which subsequent downstream effects of proinflammatory signals within epithelia are abrogated (Delneri *et al.*, 1997; de Araujo and Giugliano, 2000; Fernandes *et al.*, 2001; Hosseini *et al.*, 2001). IgA-depleted colostrum lost its inhibitory effect on enteropathogenic *E. coli* (EPEC) as well as enteroaggregative *E. coli* (EAEC) adhesion to Hep-2 cells (Carbonare *et al.*, 1997; Fernandes *et al.*, 2001). In addition, colostral IgA showed an ability to produce a physical-chemical change in the bacterial surface of *E. coli* (Hill and Porter, 1974). The ability of colostral sIgA and IgG to bind *E. coli* was studied by *in vitro* assay, as well as by transmission electron microscopy and immunofluorescence (Albanese *et al.*, 1994). Although both immunoglobulins bound *E. coli*, only sIgA completely prevented bacterial passage across the intact intestinal epithelial membrane. Immunofluorescence revealed aggregates of sIgA and *E. coli* on the mucosal, but not on the submucosal surface of the ileal membrane. Therefore, although IgA does not represent the main immunoglobulin in colostrum it seems to have an important function in the protection of neonatal pigs.

Sows' mammary glands are able to produce up to 30 g of IgA daily (Butler, 1998). This secretory IgA differs from serum IgA in possessing a complexed non-globulin component

called the 'secretory component' and therefore it appears in a higher molecular size range than serum IgG. The secretory component represents a part of polymeric IgA receptor (pIgR) exposed on the basolateral surface of the mammary gland epithelial cells (Kumura *et al.*, 2000). After dimeric IgA binds to the pIgR, the receptor-IgA complex is transported across the epithelial barrier to the lumen by receptor-mediated endocytosis into coated pits (Goldsby *et al.*, 2000). The pIgR is then cleaved enzymatically from the membrane and becomes the secretory component, which is bound to dimeric IgA. Its function is to mask sites susceptible to protease cleavage and this makes secretory IgA highly resistant to the action of proteolytic enzymes (Macpherson *et al.*, 2001) a factor which must assist in the retention of its antibody function in the gastrointestinal tract. An additional factor facilitating the action of milk IgA as an antibody in the intestine of the pig is the rate at which it passes from the stomach after ingestion (Porter *et al.*, 1970). The mean IgA concentration is 12.26 mg ml⁻¹ with maximum and minimum values 28.14 mg ml⁻¹ and 5.63 mg ml⁻¹, respectively (Inoue, 1981). Results of analyses of 12 possible factors influencing the IgA concentration in colostrum of farm raised sows revealed, that from the items analysed, breed and number of parturition were the most influential (Inoue, 1981). The IgA concentration was low until the 3rd parturition, and showed a tendency to be high between the 4th and 9th parturitions, but it became low again after the 10th parturition. In terms of breed, the IgA concentrations were low in colostrum of Landrace, Large White and other crossbreeds, but it was high in colostrum of Hampshire, Landrace x Large White and Landrace x Hampshire.

Porcine IgE, with similar physicochemical properties to the IgE of other species, has recently been purified and characterized (Roe *et al.*, 1993). The highest degree of similarity was found with sheep IgE.

Lactoferrin (Lf) is a Fe-binding glycoprotein that contains two high affinity iron binding sites per molecule. It is synthesized *in situ* in the mammary gland, intestinal tract, salivary glands and in neutrophils (Masson *et al.*, 1966). Lf exists *in vivo* in three major forms, apo-Lf, diferric Lf (both Fe^{3+} sites occupied), and ferric Lf (one Fe^{3+} site occupied) and in several isoforms (Lonnerdal and Iyer, 1995). The Fe-Lf complex is remarkably stable at a pH as low as 2 and resistant to proteolysis (Chierici, 2001). Many biological functions have been ascribed to Lf. They include bacteriostasis (Guttenberg *et al.*, 1990), transcriptional regulation (Penco *et al.*, 1995), facilitation of intestinal iron uptake and excretion (Ismail and Brock, 1993), antiviral activity (van der Strate *et al.*, 2001), anabolic function in neonates (Burrin *et al.*, 1996), growth promotion of intestinal epithelial cells (Hagiwara *et al.*, 1995; Oguchi *et al.*, 1995) as well as B- and T-lymphocytic cell lines (Hashizume *et al.*, 1983) and modulation of disease defense systems that relate to inflammation of the mucosal surface (Nakao *et al.*, 1997; Conneely, 2001; Ward *et al.*, 2002). Finally, Lf has been found to complex with lysozyme (Ellison and Giehl, 1991) and IgA (Stephens *et al.*, 1980; Vaerman, 1984), resulting in bactericidal effects at concentrations at which Lf and lysozyme alone are usually only bacteriostatic (Bernt and Walker, 2001). This brief summary of Lf functions indicates that milk Lf, apart from providing passive protection against bacteria and viruses, may also play an important role in the development of the newborn's own immune system. The exact mechanisms by which Lf exerts its antimicrobial, antiviral and mitogenic activity *in vivo* are complex and in many cases still poorly understood. In general, two basic biochemical properties of Lf contribute to its involvement in the host defense namely, the extremely powerful iron-chelating capability and the strong interaction with other molecules and surfaces (van Hooijdonk *et al.*, 2000). In addition to iron deprivation, Lf is also able to stimulate other delayed non-specific responses to improve protection against infection. These responses

are associated with the indirect effect of Lf on the production of plasma cytokines, compounds produced by immune cells during infection and inflammation to coordinate the defense against pathogens (van Hooijdonk *et al.*, 2000).

Substantial amounts of Lf are found in sow's milk averaging $1600 \mu\text{g ml}^{-1}$ near the time of farrowing, declining slowly during the first week of lactation to about $1200 \mu\text{g ml}^{-1}$ (Yang *et al.*, 2000). The concentration of Lf varies considerably among sows, but not with teat position (anterior to posterior) (Elliot *et al.*, 1984). Specific Lf receptors have been identified in the small intestine brush border membrane of humans (Kawakami and Lonnerdal, 1991), mice (Hu *et al.*, 1988), rhesus monkeys (Davidson and Lonnerdal, 1988) and piglets (Gislason *et al.*, 1993; Gislason *et al.*, 1995). Porcine transferrin, human, and bovine lactoferrin did not bind to the porcine lactoferrin receptor, which suggest that lactoferrin binding is highly specific (Gislason *et al.*, 1995). Lf-binding occurs throughout the intestine independent of the age of the piglet. Receptor number ($15 \times 10^{14} / \text{mg protein}$) and affinity ($K(d) = 3 \times 10^{-7} \text{ M}$) were relatively constant from birth until weaning (Gislason *et al.*, 1993). Performance of biological functions of Lf in the small intestine requires at least some resistance to degradation. In the study of Drescher *et al.*, (1999) digestibility of porcine Lf in the distal small intestine was significantly lower in suckling piglets compared with adult pigs. In contrast to that of formula-fed infants, the gut flora of breast-fed neonates is usually much richer in bifidobacteria and lactobacilli. This is usually associated with increased resistance against colonization by pathogens (van Hooijdonk *et al.*, 2000). The low Lf digestibility at the suckling stage of the piglet's life may be important to the biological function of Lf in the neonatal intestine and therefore it is likely that Lf, in conjunction with other factors in milk, contributes to this favourable microbial ecosystem in the gut.

Lysozyme (1,4- β -N-acetylmuramidase) is a small basic protein (enzyme) with antibacterial activity. It exerts its antimicrobial action by hydrolyzing glycosidic bonds of the peptidoglycan layer in the cell wall of Gram-positive bacteria. Peptidoglycan is a structure present only in microorganisms, which explains why lysozyme is non-toxic for animal cells. Because of a different cell wall structure, Gram-negative bacteria are able to resist the bactericidal effect of lysozyme. However, it has been shown that in the presence of Lf, the antibacterial action of lysozyme against Gram-negative bacteria can be enhanced (Yamauchi *et al.*, 1993). Ellison and Giehl (1991) demonstrated that while each protein alone is bacteriostatic, together they can be bactericidal for strains of *V. cholerae*, *S. typhimurium*, and *E. coli*. Interaction of Lf with the outer membrane of Gram-negative bacteria alters its permeability, with the subsequent release of lipopolysaccharide (LPS) molecules and an increase in bacterial susceptibility to antibiotics and lysozyme (Ellison and Giehl, 1987; Ellison *et al.*, 1988; Ellison *et al.*, 1990; Yamauchi *et al.*, 1993). As lactoferrin and lysozyme are present together in sow colostrum, it is probable that their synergistic action contributes to host defence.

The concentration of lysozyme in sow milk was estimated by Schulze and Müller (1980). The lysozyme level, during the first two days postpartum, was found to be between 6.8 and 11.0 $\mu\text{g ml}^{-1}$. It reached its highest point on the second day after parturition, followed by a rapid decline to a much lower value ($5.2 \pm 2.0 \mu\text{g ml}^{-1}$) which then remained constant up to the 30th day of lactation. Day of lactation and parity both appear to have a marked influence on lysozyme concentration. Such high lysozyme levels in sow colostrum over the first two days of age suggest that this protein might affect the bacterial population of the gastro-intestinal tract of suckling piglets. However, there is very little *in vivo* evidence for an involvement of milk lysozyme in the defence against infection in the newborn.

Besides its antimicrobial activity lysozyme has many other functions including inactivation of certain viruses (Gluck, 1989), enhancing phagocytic activity of polymorphonuclear leukocytes and macrophages (Biggar and Sturgess, 1977), stimulation of monocytes and lymphocyte proliferation (Lemarbre *et al.*, 1981; Rinehart *et al.*, 1982), antitumor activity (Lemarbre *et al.*, 1981; Warren *et al.*, 1981), and induction of fusion of phospholipid vesicles (Posse *et al.*, 1990; Vechetti *et al.*, 1997).

Phagocytic cells. Porcine mammary secretions contain a significant cellular component including phagocytes (neutrophils and macrophages), epithelial cells, eosinophils and lymphocytes (Lee *et al.*, 1983; Magnusson *et al.*, 1991) (Table 1.1). Estimates of the number of specific cells in porcine mammary secretions vary widely among researchers and between individual animals but, in general, the neutrophils predominate in colostrum and involution secretion, whereas in milk the epithelial cells predominate. Macrophages and lymphocytes are present throughout lactation (Lee *et al.*, 1983). According to Evans (1982) sow colostrum contained a mean cell yield of 1×10^7 cells ml^{-1} , but during the first week post-partum the yield decreased approximately 10-fold. Like other immune factors, cell types and numbers are also affected by stage of lactation as well as other individual conditions, but the piglet ingests an average of 500-700 million maternal cells daily (Le Jan, 1996). While polymorphonuclear neutrophils (PMN) decreased from about 56% of the total leucocytes at day 1 (colostrum) to 12-14% at day 14 and day 21, macrophages increased from 35% at day 1 to 77-80% at day 14 and 21 (Hurley and Grieve, 1988).

The first role of the mammary cells is to protect the gland itself against infection (Fetherston *et al.*, 2001). Phagocytes play an essential part in nonspecific immunity by ingesting and killing invading microorganisms and by releasing many soluble factors that

contribute to host defense and to inflammation (Quinn, 1990). In pigs, neutrophils represent the predominant lacteal phagocytic cells (Lee *et al.*, 1983). Like the macrophages, they exhibit a great capacity to engulf lipid, casein micelles and cellular debris. However, there is evidence to suggest that milk is unable to provide optimal conditions for phagocytic activity. Apparently, milk neutrophils neither respond as quickly to chemotactic agents nor move as rapidly as the same type of cell from the blood (Evans *et al.*, 1982; Norcross, 1982; Targowski and Niemialtowski, 1986; Dulin *et al.*, 1988). Numerous factors were suggested to be responsible for limited phagocytic activity of milk, including ingestion of casein, fat and cellular debris, the availability of energy, pO_2 level, low concentration of opsonizing milk immunoglobulins and complement as well as the presence of a blocking factor for phagocyte Fc receptors (Fetherston *et al.*, 2001). Only recently, a study by Osterlundh *et al.*, (2001) demonstrated differences between sow colostrum and milk. The phagocytic capacity of polymorphonuclear (PMN) leukocytes in sow colostrum was significantly less than that of cells in milk, which may predispose sows to coliform mastitis during the early postparturient period.

The second main role of the phagocytes and other cellular components from mammary secretions is to interact with the development of local immunity in the newborn, and to modulate active immunization of the neonatal intestine during this critical period when the development of adapted responses to antigens (protection/tolerance) is of crucial importance for the future of the young (Le Jan, 1996). During 24 to 48h after suckling, maternal pig colostral leukocytes were observed to intercellularly migrate between duodenal and jejunal epithelial cells to lamina propria and submucosal spaces where they conferred an immunomodulating effect on neonatal pigs (Tuboly *et al.*, 1988; Williams, 1993). Interestingly, Tuboly *et al.*, (1988) also demonstrated that only colostral

lymphocytes from the piglet's mother are allowed to cross the epithelium. Cells from unrelated sows and peripheral blood leukocytes from the piglet's mother were unable to cross the intestinal epithelium.

Both milk macrophages and neutrophils are able to take up lactoferrin and immunoglobulins from the surrounding fluids. Their attachment to the mucosa of the intestinal tract, followed by the release of ingested antibody, represents an important adjunct in support of the neonate's intestinal defence (Riedel-Caspari, 2001). It has also been shown that colostral and milk leucocytes secrete interferon, interleukin-6 (IL-6) (Taylor *et al.*, 1997) and prostaglandins (Blau *et al.*, 1983; Ledeist *et al.*, 1986), factors which are capable of modulating lymphoid cell proliferation in the suckling newborn. Finally, Honorio-Franca *et al.*, (1997) demonstrated that colostral mononuclear phagocytes are able to kill enteropathogenic *Escherichia coli*. However, bacterial opsonization with IgA was a prerequisite.

2) Active protection

Lymphocytes represent 10-25% of cells isolated from sow colostrum, while milk contains only few lymphocytes (0.5-2%) (Chabaudie *et al.*, 1993; Lejan, 1994). By using flow cytometry it has been shown that 70-90% of colostral lymphocytes were T lymphocytes, with T8 lymphocytes predominating over T4 (Lejan, 1994). B lymphocytes are less numerous than T lymphocytes in porcine mammary secretions, making up only about 30% of the lymphocyte population (Wagstrom *et al.*, 2000). As has already been mentioned, ingested milk lymphocytes can survive within the intestine and cross the epithelium in the duodenum and jejunum. By 24 hours post-feeding maternal lymphocytes are present in liver, lung, lymph nodes, spleen and gastrointestinal tissues of the suckling piglet

(Williams, 1993). The functional significance of the lymphocytes found in colostrum and milk has been difficult to determine. Nevertheless, it is clear that lymphocytes are functional and hence there is a probability that they play a role in the development of the immunity in the newborn (Tizard, 2001).

B) Growth factors

In addition to immunoglobulins and cell components, colostrum contains numerous hormones and growth factors (Wagstrom *et al.*, 2000). Growth factors are multifunctional, locally-acting small proteins (polypeptides) that play key roles in (Kendrew and Lawrence, 1994):

- cell growth, division and differentiation
- coordinating immune, nervous and hormonal systems
- enhancing cell communication
- competing with viruses for expression of the same genetic sites

Newborn piglets can be considered as a community of different types of cells whose individual proliferation, differentiation and physiological function must, in some way, be coordinated for the overall function of both individual tissues and the whole organism. This is achieved by specific intercellular signals (endocrine or paracrine) which control cell multiplication, differentiation and behavior. Growth factors, as paracrine signals, share a number of common biological properties. They often exert their biological actions at very low (typically $10^{-9} - 10^{-11}$ M) concentrations as their action is mediated by their association with specific, high affinity receptors expressed by the target cell type

(Kendrew and Lawrence, 1994). In addition, growth factors exhibit cell-type specificity, which means that the same growth factor can have very different biological effects depending on the type of cell with which it interacts. Growth factors present in porcine milk include epidermal growth factor (EGF) (Xu *et al.*, 2000), insulin, insulin-like growth factor I and II (IGF-I, IGF-II) (Donovan *et al.*, 1994), and transforming growth factor- β (TGF- β) (Xu *et al.*, 1999) (Table 1.1). There is increasing evidence showing that milk-borne factors can survive in the GI tract of the suckling animal (Shen and Xu, 1998; Shen and Xu, 2000a; Shen and Xu, 2000b), and that exogenous growth factors administered orally stimulate GI maturation in newborns (Jaeger *et al.*, 1990; Houle *et al.*, 1995; Houle *et al.*, 1997; Monaco *et al.*, 2000). However, the biological significance of milk growth factors remains to be fully elucidated. Understanding their role in postnatal GI adaptation as well as their mechanism of action may help us to prevent and treat many GI disorders in neonatal animals.

Epidermal growth factor (EGF) – is a 6 kDa peptide composed of 53 amino acids and it represents one of the first growth factors to be discovered (Kendrew and Lawrence, 1994). It is cleaved from a much larger transmembrane precursor protein (Odle *et al.*, 1996). EGF is mitogenic for a wide variety of cell types, particularly those of epithelial origin. To induce a mitogenic effect, EGF binds to a 170 kDa specific membrane-bound receptor consisting of an extracellular mitogen binding site and cytoplasmic domain with tyrosine-kinase activity (Xu *et al.*, 2000). Binding results in a second-messenger cascade that culminates in mitosis and/or differentiation of target cells (Odle *et al.*, 1996). Milk EGF could be derived from either maternal circulation or from the synthesis within the mammary gland itself. In sows, as well as in most other species, concentrations of EGF are highest in colostrum and decline rapidly thereafter (Table 1.1). Receptors for EGF have

been identified immunohistochemically throughout the luminal mucosa of 1 to 28-d old piglets, from the esophagus to ileum (Jaeger and Lamar, 1992), and found predominately in the basal regions of the villi and in the crypts (Kelly *et al.*, 1992b). Based on many studies, both in animals and humans, there is no doubt that EGF can have some very potent and multiple effects on the gastrointestinal tract. These may be related to the control of growth and development, to the regular control of cell renewal, cytoprotection as well as to the induction of gene expression such as mucosal enzymes (Goodlad and Wright, 1996). The brush-border surface area of rabbit's jejunum was significantly increased in EGF-treated tissue after 30 (42%) and 120 min (60%) (Hardin *et al.*, 1993). A study by Opletamadsen *et al.*, (1991) suggested that EGF modulates development of transport function during the postnatal period both by stimulating mucosal growth and by inducing specific transport processes. The effect of oral EGF on recovery of damaged intestine was studied in rats (Petschow *et al.*, 1993). The results showed that oral EGF was capable of modulating mucosal protein levels and stimulating enterocyte hydrolase expression during repair of the intestinal mucosa. Furthermore, Jaeger *et al.*, (1990) demonstrated that after 3 days of treatment, orally administrated EGF increased jejunal lactase and sucrase specific activities in newly weaned piglets. The benefit of high physiological levels of EGF has also been shown in a study with neonatal piglets infected with porcine group A rotavirus (Zijlstra *et al.*, 1994). Finally, a very recent study by Dvorak *et al.*, (2002) demonstrated the potential of EGF to reduce the incidence and severity of necrotizing enterocolitis in a rat model. A review of the intestinal effect of EGF in the young pig is provided by Odle *et al.*, (1996) and Xu *et al.*, (2000). Despite the solid basis of experimental studies on EGF, its precise role in the control of gastrointestinal functions is still not fully resolved.

The **Insulin-like growth factors (IGF-I, IGF-II)** are single chain polypeptides with a

molecular mass of 7.5 kDa that retain 70% amino acid homology with each other and 50% homology with proinsulin (Louveau *et al.*, 2000). They exert both metabolic and mitogenic effects *in vitro* (Reiss *et al.*, 1998; Chen and Donovan, 2000; Sirotkin *et al.*, 2000) and *in vivo* (Xu *et al.*, 1994; Houle *et al.*, 1995; Xu *et al.*, 1996; Monaco and Donovan, 1997). Porcine milk contains both IGF-I and -II, with IGF-II predominating (Donovan *et al.*, 1994). They interact with two types of receptors, type I and type II, that differ in their primary as well as secondary structure, ligand binding specificity and signaling mechanism (Rechler and Nissley, 1985). While the type I receptor generally binds IGF-I more tightly than IGF-II and also interacts weakly with insulin, the type II receptor prefers IGF-II over IGF-I and does not recognize insulin (Nissley *et al.*, 1985). Both types of IGF receptors have been identified throughout the gut of many species, including pigs (Xu *et al.*, 1994).

The IGFs are almost always tightly associated with a family of structurally-related binding proteins (IGFBPs) (Louveau *et al.*, 2000). To date, five genetically distinct IGFBPs (with molecular weight of 43, 39, 34, 28, and 24 kDa) have been identified in pig serum and six IGFBP of similar molecular weight, as serum IGFBP, have been reported in porcine milk (Coleman *et al.*, 1991; Donovan *et al.*, 1994). Despite the presence of these IGFBPs in milk, there is little evidence to indicate whether these binding proteins either serve to protect IGF-I from intestinal proteolytic activity or facilitate its binding to the enterocyte IGF- receptors.

To determine biological function of milk-borne IGFs, many studies have focused on the growth of the gastrointestinal tract, because this is the first tissue exposed to ingested growth factors. Xu *et al.*, (1994) used a cell labeling technique to show that both IGF-I and IGF-II stimulated cell proliferation in the small intestinal crypts of newborn piglets,

although he pointed out that the substantial gastrointestinal tissue growth during the neonatal period is unlikely to be due to these growth factors alone. Further experimental evidence summarized in the review of Xu *et al.*, (2000) confirms the regulatory role of these milk-borne growth factors in stimulating GI tissue growth and function maturation, and in enhancing repair of damaged GI mucosa in the suckling young. However, potential therapeutic applications of milk-borne growth factors in agricultural animals warrant further investigation.

Transforming growth factor- β (TGF- β) is a dimeric protein with a molecular weight of 25 kDa (Xu *et al.*, 2000), which is also a prototype for a large family of over 40 different proteins belonging to the TGF β superfamily (Flanders and Roberts, 2000). In mammals there are three isoforms of TGF- β (β_1 , β_2 and β_3), each one encoded by a unique gene on different chromosomes and expressing different biological action within the body (Lawrence, 1996). While TGF- β_1 is the predominant isoform in most cells and tissues, it is TGF- β_2 which is predominantly found in porcine and bovine colostrum (Xu *et al.*, 1999). Most cell types synthesize TGF- β as a large latent complex (L-TGF- β) that must be converted to an active form before TGF- β can interact with its cell surface receptors (Khalil, 1999). This activation of latent TGF- β , which may involve plasmin, thrombospondin and possibly acidic microenvironments, appears to be a crucial regulatory step in controlling their effects (Lawrence, 1996; Nunes *et al.*, 1996). The TGF- β and their receptors are very ubiquitously expressed, suggesting that the cellular targets are not restricted to any lineages or cell types and that the regulation of TGF- β activity is likely to be complex and multifactorial (Khalil, 1999). Indeed, the TGF- β represents the most pleiotropic and multifunctional proteins from the large and still growing TGF β family of growth factors. It affects processes ranging from regulation of cellular differentiation and

growth to inflammation, tissue repairing, immunoregulation and bone formation and contributes to the pathogenesis of many diseases (Roberts, 1998; Barcellos-Hoff and Ewan, 2000).

Xu *et al.*, (1999) reported that the concentration of TGF- β in porcine colostrum ranged between 126 and 260 ng ml⁻¹ at the time of parturition and decreased rapidly as lactation proceeded. Most of the TGF- β in porcine colostrum is in a latent form, which can be activated at pH 3.5 or less, similar to the pH of the neonatal stomach. According to data from animals and *in vitro* studies TGF- β is the most potent known growth inhibitor for normal and transformed epithelial cells, endothelial cells, fibroblasts, neuronal cells, lymphoid cells and other hematopoietic cell types, hepatocytes, and keratinocytes (Flanders and Roberts, 2000). The extent of growth inhibition induced by TGF- β depends on the cell type, on the concentration of TGF- β , and on the presence of other factors. Isoform TGF- β_1 was found to control the life and death decisions of T lymphocytes (Chen *et al.*, 2001). Both thymic and peripheral T cell apoptosis was increased in mice lacking TGF- β_1 . Kalliomaki *et al.*, (1999) also demonstrated that TGF- β in human colostrum has a crucial effect on 2 essential parts of the mucosal immune system: promotion of IgA production in synergy with IL-2 and IL-5, and oral tolerance induction. A comprehensive review of the structure, function and mechanism of action of TGF- β *in vitro* and *in vivo* is provided by Flanders and Roberts (2000).

Other protective substances of sow milk

In addition to the milk-borne immune factors, with well-established immunological functions, several other protein and non-protein components have been found in sow milk

that may also contribute to the passive/active protection of neonates through suckling. Several cytokines including interleukin-1 β (IL-1 β) (Hawkes *et al.*, 1999), IL-6 (Rudloff *et al.*, 1993), IL-8 (Hashira *et al.*, 2002), IL-10 (Garofalo *et al.*, 1995), IL-12 (Bryan *et al.*, 1999), IL-18 (Takahata *et al.*, 2001) tumor necrosis factor- α (TNF- α) (Rudloff *et al.*, 1992), macrophage colony stimulating factor (M-CSF) (Hara *et al.*, 1995), and interferon- γ (IFN- γ) (Goldman *et al.*, 1997) have been detected in human colostrum. Cytokines are important signaling molecules in the immune system, as they regulate numerous physiological responses including development of cellular and humoral immune responses, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation and the induction of wound healing (Durum, 2000). However, limited data are available on the type, concentration and function of cytokines in porcine colostrum (Rooke and Bland, 2002).

In addition to cytokines, milk polyamines (spermine, spermidine) are another key factor essentially involved in various processes of cell growth and differentiation and thus can contribute to the neonatal defense system (Loser, 2000). The presence of these nitrogen compounds in porcine colostrum and milk has been demonstrated (Kelly *et al.*, 1991; Motyl *et al.*, 1995). The average concentration of spermidine throughout the lactation in sows ranged from 7.14 $\mu\text{mol L}^{-1}$ in the second week to 2.05 $\mu\text{mol L}^{-1}$ in the fifth lactation week (Motyl *et al.*, 1995). Interestingly, a high positive exponential correlation existed between number of piglets and spermidine concentration in sow's milk. The concentration of spermine was higher than spermidine throughout the entire lactation and ranged from 21.6 $\mu\text{mol L}^{-1}$ at the beginning of lactation to 5.16 $\mu\text{mol L}^{-1}$ in the fifth week of lactation (Motyl *et al.*, 1995). In this case, the relationship between number of piglets and spermine concentration was lower and not significant. This highest secretion of both polyamines in

the mammary gland at the beginning of lactation corresponds with the highest demands of a newborn piglet for polyamines, especially in the development of the gastrointestinal tract. Very recently it has been also demonstrated that sows colostrum/milk contain remarkable amounts of bioactive leptin (Table 1.1), a protein responsible for controlling adipose tissue mass, food intake and body weight in animals (Zabielski *et al.*, 2003). However, in addition to these functions, milk-borne leptin play an important maturation role in the development of the neonatal GI-tract (Zabielski *et al.*, 2003).

Finally, milk lipids can serve not only as nutrients but also as a nonspecific protective factor against viruses, bacteria and protozoa (Isaacs, 2001). The lipids develop antimicrobial activity in the gastrointestinal tract of suckling neonates as a result of lipolytic activity which converts milk triglycerides to antimicrobial fatty acids and monoglycerides.

1.1.3 Development and microbial ecology of the neonatal gastrointestinal (GI)-tract

The GI-tract is the first neonatal body system with which ingested colostrum comes into contact and through which nutrients, electrolytes and fluids enter the body. It also provides a protective barrier against infectious agents and represents the largest endocrine organ in the body (Yen, 2001b). Immediately after birth, the rapid somatic growth is accompanied by marked anatomical, physiological and chemical composition changes of individual body tissues. GI-tract ontogenesis involves extensive epithelial cell proliferation and cytodifferentiation including changes in the expression of enzymes, receptors and transport systems (Kelly *et al.*, 1992a). The development of the GI-tract in the pig commences early

in foetal life. Primitive villi are present at day 35 of gestation and their proper development is achieved by day 110 (Kelly and King, 2001a). At birth, the GI-tract of pig must be able to cope with the shift from parenteral nutrition to enteral nutrition. To meet this challenge, a rapid rate of postnatal intestinal development is of great importance. A series of studies in foetal pigs have shown that both hormonal and luminal factors influence this rapid phase of GI-tract development. Among the potential hormonal regulators of development, cortisol plays a pivotal role (Sangild *et al.*, 2000). The components of the digestive system which are mainly responsible for the digestion and absorption of food are the stomach, small intestine, pancreas and liver (Kidder and Manners, 1978). The ability of the pig to carry out digestive and absorptive functions will depend on the physical capacity of the gut, the nature and amount of the secretions it can provide (e.g. acid, enzymes, bicarbonate and bile), the development of mechanisms to control these secretions, and the digestive and absorptive capacity of the mucosal surface of the small intestine (Cranwell, 1995). Following weaning, pigs require relatively larger digestive systems than suckling pigs if they are to digest and absorb the inherently less digestible post-weaning diets satisfactory and maintain an acceptable growth rate (Cranwell, 1995). The period of time it takes the piglet digestive system to mature is thus one of the limitations affecting post-weaning performance. Therefore, it is important to know the factors that influence the GI-tract development during the neonatal period.

Maturity of the digestive system can be defined as 'the ability, which is possessed by the adult pig, to digest a wide range of different foodstuffs' (Kidder, 1982). In the pig, some key GI-tract maturational events do not take place until immediately before and after birth (Sangild, 2001). The small intestine grows more rapidly than the body as a whole during the last 3-4 weeks of gestation, as well as within the first weeks postnatally. In 24 hours

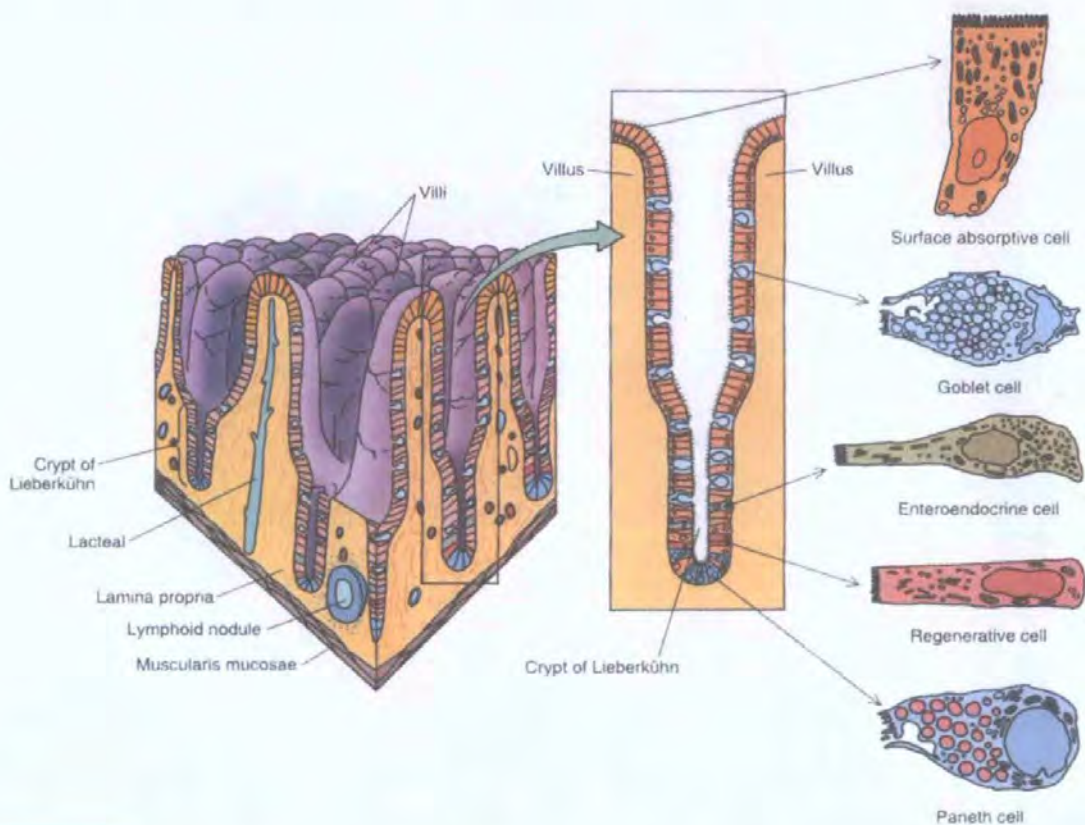
after birth the pig intestine has a 50% higher relative weight than at birth (Sangild, 2001). As has been discussed previously there are many bioactive agents present in colostrum (lactoferrin, IGF-I, IGF-II, EGF, polyamines, some aminoacids, prolactin, TGF) which promote this rapid maturation of the intestinal epithelium. The anatomy, histology, ontogenesis and maturation of the neonatal stomach, pancreas and liver have been reviewed by several authors (Kelly and King, 2001a; Yen, 2001a; Yen, 2001b). This review will focus on the neonatal intestine and the importance of its rapid maturation.

1.1.3.1. Structure and function of neonatal intestine

The anatomy of the small intestine is quite similar in most mammals (Figure 1.4). It is a part of the GI-tract where essential events of digestion and absorption take place. The small intestine of the neonatal piglet is 2-4 m long and consists of duodenum, jejunum, and ileum; although the differentiation of jejunum and ileum is not clear at this stage of life (Yen, 2001a). The wall of the small intestine is made up of 3 concentric layers; mucosa, submucosa, and the muscle layer covered by serosa (Liebler-Tenorio *et al.*, 1999). Typical features of the small-intestinal mucosa are tall villi, which increase the absorptive and digestive capacity of the small intestine. A rapid increase in the length of the villi begins in the first days of life. The villi are covered by a sheet of absorptive epithelial cells, punctuated at intervals by goblet cells, which are not directly involved in epithelial transport and secrete viscous mucous into the intestinal lumen. This mucous coat is composed of a solution of glycoproteins (mucin) which differs between animal species as well as developmental stages and even within localized regions of the intestinal tract (Sanderson and Walker, 1999). Mucin molecules are classified into neutral and acidic subtypes and apparently it is the acidic subtype of mucin that protects the host against

bacterial translocation (Deplancke and Gaskins, 2001). Mucus has sufficient adhesive and elastic strength to be retained on the epithelial surface despite the force of peristalsis. Mucus acts as the outermost sensory “organ” of the mucosal immune system due to it possessing the function of a selectively permeable barrier (Cone, 1999).

Figure 1.4. Structure of the small intestine (Gartner and Hiatt, 2001).



It must permit rapid entry and exit of nutrients, gases and wastes, but at the same time it has to select and regulate what the cellular immune system will encounter. Due to mucus' short life time, goblet cells produce it continuously. For example, humans secrete approximately 10 liters of mucus per day (Cone, 1999). By continuous secretion, the mucus layer adjacent to the epithelial cells is rapidly replaced and thus pathogens must advance through the blanket of a mucus gel that is moving outwards, if they are to reach the epithelial surface and cause pathological change. The thickness of the mucus layer

within the intestine varies greatly depending on digestive activity. However, no mucus is secreted in the region surrounding M cells which make these cells relatively unprotected and vulnerable to bacterial attack. In one way, the absence of the mucus is actually beneficial for their proper function. M cells are key sites of antigen sampling for the mucosal associated lymphoid system (MALT) and therefore they represent essential components of the mucosal immunity (Sansonetti and Phalipon, 1999). But on the other hand, they have recently been recognized as a major site of adherence and ports of entry for enteric pathogens (Jepson, 1998). More recently, it has been recognized, that in addition to mucus, goblet cells secrete also trefoil peptides, a family of abundant proteins that share a distinctive and highly conserved structural motif of a 'three-leafed clover' shape (Thim, 1997). This unique structure, in which six cysteine residues form three disulphide bonds, is likely responsible for the marked resistance to protease digestion that permits these peptides to remain structurally and functionally intact despite secretion onto the mucosal surface where they are exposed to a variety of proteases. The trefoil peptides are normally expressed in a site specific pattern within the gastrointestinal epithelium and it is now well established that they have cytoprotective functions as well as playing an important role in mucosal healing processes (Modlin and Poulson, 1997; Longman *et al.*, 1999; Dignass, 2001).

Apart from absorptive epithelial cells and goblet cells there are also enteroendocrine cells present within the intestinal epithelium, mainly based within the crypts and scattered on villi. Their function is to produce the hormones (e.g. enteroglucagon) that are involved in the regulatory process of the cellular renewal system (Falk *et al.*, 1998). The epithelial cells are attached to the basement membrane which separates them from the connective tissue of the lamina propria. The lamina propria forms the core of a villus and contains

lymphoid nodules (Peyer's patches), free lymphocytes, blood vessels and neurons (Yen, 2001a). At the level of the bases of the villi, the lining epithelium dips into the substance of the lamina propria to form the so-called crypts of Lieberkühn. Within these crypts, cell division takes place, so that villous cells are produced, which migrate from the crypts to the tip of the villi and are extruded into the lumen of the gut, where they disintegrate and are digested. During migration, the crypt cells differentiate into absorptive cells and goblet cells (Lipkin, 1985). This process of enterocyte renewal occurs continuously and, in the case of pigs, the epithelium is replaced by new epithelium approximately every 3-4 days (Yen, 2001a). The most distinctive feature of the absorptive cell is a brush border (microvilli) which faces the lumen and which increases the apical surface of enterocytes by about 14-40 fold (Yen, 2001a). Just beneath the brush border, along the sides of the cells, special functional structures, the so-called "tight junctions", are present which hold the cells together in a more or less continuous sheet. Tight junctions used to be thought of as passive structures of the epithelial barrier. However, studies over the past few years have documented that they are actually highly regulated gates that open and close in response to events in the lumen, signals from the lamina propria, and even messages from the epithelium itself (Perdue, 1999; Nusrat *et al.*, 2000). In the brush border membranes, enzymes (peptidases, carbohydrases) are found which participate in digestive and absorptive processes of nutrients from the lumen. It also provides adhesion sites for lectins, which are bacterial surface receptors (Liebler-Tenorio *et al.*, 1999). For example, enterotoxigenic *Escherichia coli* K88, a common cause of diarrhoea in newborn and weaned piglets, binds to the receptors present in both the intestinal brush border membrane and intestinal mucus (Blomberg *et al.*, 1993c; Erickson *et al.*, 1994; Billey *et al.*, 1998; Francis *et al.*, 1999). This attachment allows bacteria to avoid elimination by intestinal peristalsis. It has been demonstrated that activity of some K88 receptors (e.g. IMTGP-1

and IMTGP-2) is dependent on the maturational state of the intestine (Blomberg *et al.*, 1993a; Francis *et al.*, 1998). While brush borders from immature intestinal cells possess the highest concentrations of these receptors, a progressive decrease in receptors is observed as the enterocytes mature. This only emphasizes the importance of making sure that the newborn piglet ingests colostrum as soon as it is born, in order to obtain the highest concentrations of all factors responsible for intestinal growth and maturation.

The large intestine (3.5-6 m) of the pig consists of a short caecum and long colon. Its main function is the absorption of fluids and electrolytes and digestion by microbial fermentation (Liebler-Tenorio *et al.*, 1999). The mucosal membrane of the large intestine has no villi and its lining comprises short columnar epithelial cells with characteristic striated borders containing microvilli. Unlike the small intestine, microvilli in the large intestine do not contain digestive enzymes (Yen, 2001a).

1.1.3.2. Bacterial colonization of the neonatal intestine

The microflora in the GI-tract of pigs is a complex ecosystem, consisting of several hundred microbial species (Conway, 1997). The GI-tract of newborn pig, which is sterile at birth, rapidly acquires its characteristic microflora by contact with its mother and the environment into which it is born. By 24 hours, *Escherichia coli*, streptococci, and clostridia have been found in high numbers (over 10^8 g^{-1}) in the faeces of piglets. After a further 24 hours, these bacteria were accompanied by similarly high numbers of lactobacilli (Smith and Crab, 1961). Three major groups of *Lactobacillus* (*L.*) spp. have been isolated from the GI-tract of pigs (Du Toit *et al.*, 2001):

1) obligately homofermentative lactobacilli

L. acidophilus, *L. delbrueckii*, *L. salivarius*, *L. amylovorus*, *L. crispatus*

2) facultative heterofermentative lactobacilli

L. plantarum, *L. intestinalis*, *L. reuteri*

3) group, biochemically related to *L. plantarum*

L. paraplantarum

Lactobacillus species and biovars as well as other bacterial groups change with neonatal growth (Naito *et al.*, 1995; Katouli *et al.*, 1997). While *L. reuteri* colonised the piglet's gut on the first day of birth, in one-week's time it was *L. acidophilus* that became the predominant strain (Naito *et al.*, 1995). In general, the first bacteria, which become established in the piglet gut, are derived from the aerobic and anaerobic flora of the dam's birth canal. Comparison of biochemical fingerprints of faecal flora on the 3rd day of suckling indicated a high similarity between the flora of piglets in each litter and their dams (Katouli *et al.*, 1997). However, despite the close contact of piglet with its mother, the similarity was lost after the first week of lactation, which suggests that, although the vaginal and faecal bacteria are ingested during farrowing, they do not normally seem to colonize the piglet's intestinal tract. This indicates that the newborn pig must possess a very efficient microbial selection system, to be able to modify the various bacterial ecosystems it encounters and finally produce relatively constant internal gut conditions (Ewing and Cole, 1994). Thus, although the neonatal pig is commonly exposed to a wide range of different bacteria, not all are able to establish themselves in the neonatal intestine. Ingestion of new types of microorganisms occurs continually, to a greater or lesser degree, depending on the extent of faecal contamination. Sansom and Gleed (1981) demonstrated that suckling piglets consume a significant quantity of sow's faeces and bedding material

(18-25 g day⁻¹). In addition, the surface of sow's teats is usually heavily contaminated with microbes from faeces and the environment. The composition of the 'normal' microflora in a specific region of the GI-tract is difficult to define even within the given individual, not only because of the problem of distinguishing resident from transient species, but also because of the difficulty in culturing most components *ex vivo* (Falk *et al.*, 1998). However, thanks to molecular biology, visualization of bacteria that are difficult to cultivate or to detect by other methods has become a reality. During the past few years, molecular approaches, largely based on PCR technologies, have been developed for microbial strain classification and analysis of complex microbial ecosystems (Vaughan *et al.*, 2000). These techniques work on the basis of DNA sequence diversity. Thus, further advances in our understanding of developmental microbial ecology in the neonatal gastrointestinal tract are dependent on the application of these modern molecular techniques. Recently, Pryde *et al.*, (1999) used nucleotide sequencing strategies that target bacterial ribosomal DNA in order to compare the information gained from cultural and molecular analyses. The 16S rDNA sequence analysis of the pig colon and caecum confirmed the concern of many microbiologists, that despite applying strictly anaerobic conditions, the culture approach does not reflect much of the diversity present in the colonic wall sample and is likely to overestimate the contributions of certain bacterial groups to the flora. In addition, molecular analyses of human faecal microflora revealed the differences between the microflora of the colonic wall and that of colonic lumen, which strongly indicates that the faecal microflora, even if it reflects the microflora of colonic lumen accurately, may not reflect that of the colonic wall (Wilson and Blichington, 1996). This fact was also confirmed very recently in the study of Zoetendal *et al.*, (2002). The predominant mucosa-associated bacterial community was host specific and uniformly distributed along the colon but significantly different from the faecal community. As will

be described later, the bacterial communities associated with the intestinal wall and intestinal mucosa are potentially of great importance as this is the main site of interaction with the host and host immune system. This finding could have a significant implication in the research area of probiotics, as it is probably worthwhile to reconsider whether existing laboratory bacterial isolates are the most appropriate to use in attempts to adjust the intestinal microflora.

Table 1.3. Factors affecting the microflora of the GI-tract (Holzapfel *et al.*, 1998).

1. *Host mediated factors*

pH, secretions such as immunoglobulins, bile salts, enzymes
Motility, e.g. speed, peristalsis
Physiology, e.g. compartmentalization
Exfoliated cells, mucins, tissue exudates

2. *Microbial factors*

Adhesion
Motility
Nutritional flexibility
Spores, capsules, enzymes, antimicrobial components

3. *Microbial interactions*

Synergy:
Metabolic cooperation
Growth factors and vitamin excretion
Changes to Eh, pH, O₂ tension
Antagonism/Stimulation:
Short-chain fatty acids, amines
Changes to Eh, pH, O₂ tension
Antimicrobial components, siderophores
Nutritional requirements etc.

4. *Diet*

Composition, non-digestable fibres, drugs, etc.

The latest study on diversity of the intestinal bacterial community in pigs only confirmed that our understanding of complex pig microbial ecosystem is still far from complete. Comparative 16S rDNA sequence analysis on the intestinal samples collected from 24 pigs representing a variety of diets, ages, and herd health status revealed that the majority of bacterial species colonizing the pig GI-tract represent yet-uncharacterized bacterial genera or species (Leser *et al.*, 2002).

Table 1.4. Comparison of selected properties of germfree (absence of microflora) and conventional (presence of microflora) animals (Tannock, 2001).

Biochemical, physiological, and immunologic host characteristics	Conventional	Germfree
Bile acid metabolism	Deconjugation, dehydrogenation and dehydroxylation	Absence of deconjugation, dehydrogenation and dehydroxylation
Bilirubin metabolisms	Deconjugation and reduction	Little deconjugation; absence of reduction
Cholesterol	Reduction to coprostanol	Absence of coprostanol
β -Aspartylglycine	Absent	Present
Intestinal gases	Hydrogen, methane and CO ₂	Absence of Hydrogen, methane, less carbon dioxide
Short-chain fatty acids	Large amounts of several acids	Small amount of few acids
Tryptic activity	Little activity	High activity
Urease	Present	Absent
β -Glucuronidase (pH 6.5)	Present	Absent
Organ weights (heart, lung and liver)	High	Low
Cardiac output and oxygen utilization	High	Low
Mucin contents of intestinal mucus	High	Low
Extent of degradation of mucins	More	Less
Cecal size (rodents)	Small	Large
Enzyme activities associated with duodenal enterocytes	Low	High
Intestinal wall	Thick	Thin
Intestinal mucosal surface area	Great	Small
Rate of enterocyte replacement	Fast	Slow
Peristaltic movement of the contents through small bowel	Fast	Slow
Body temperature	High	Low
Serum cholesterol concentration	Low	High
Lymph nodes	Large	Small
γ -globulin fraction in blood	More	Less

There are numerous regulatory factors influencing bacterial colonization of the GI-tract, which are briefly summarized in Table 1.3. The indigenous microflora of the intestine has several functions by which it contributes to the host's overall health (Rolfe, 2000).

Comparisons of germfree animals with their conventional counterparts have shown that animals do not require microbial colonization for survival, but these two groups differ in many physiological and biochemical parameters (Table 1.4) and germfree animals are more susceptible to infection (Midtvedt, 1985; Tannock, 2001). As is summarized in Table 1.4, indigenous microflora stimulates a range of important functions, from postnatal intestinal maturation and motility to maintenance of the mucosal barrier and nutrient absorption (Falk *et al.*, 1998). Since the resident microflora makes it difficult for newly arrived bacteria to become established, it also provides a first line of defense against pathogenic bacteria, a function described as 'colonization resistance' (Adlerberth, 1999).

Many possible mechanisms have been suggested by which these beneficial bacteria might help promote health and combat the proliferation of pathogenic species of bacteria. However, at present, it is still unclear which of these mechanisms might be the most important or whether they might act in synergy. Adhesion of pathogenic bacteria to mucosal surfaces is considered to be the first step of intestinal infection (Finlay and Falkow, 1997). Therefore, the theory of 'competitive exclusion (CE)' has received considerable attention. This term has been used to describe the protective effect of the natural or indigenous flora of the intestine in limiting the colonization of some bacterial pathogens (Figure 1.5).

Figure 1.5. Competitive exclusion of pathogens due to the preferential attachment of indigenous bacterial flora (adapted from Ewing and Cole, 1994).

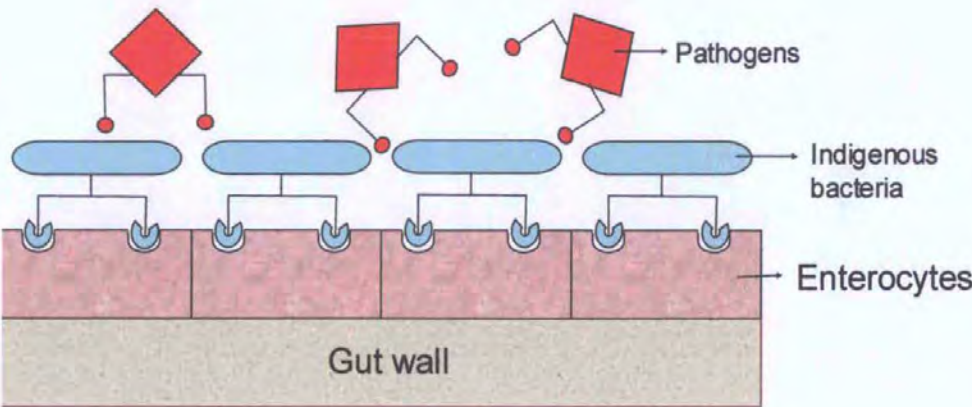
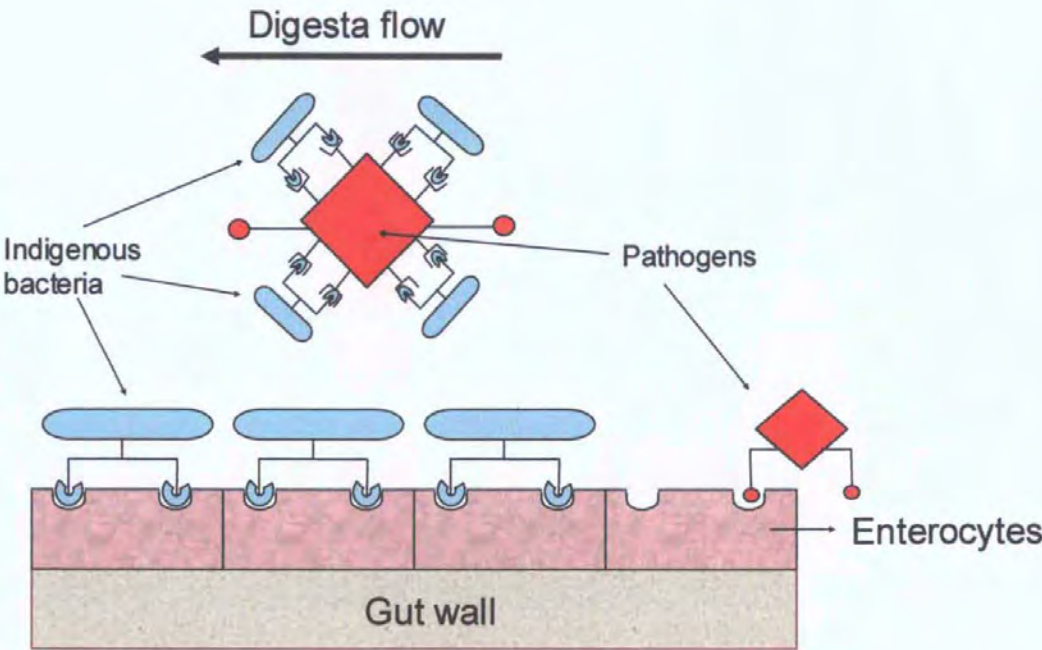


Figure 1.6. Accumulation of grouping of non-pathogens to pathogens in areas of quick digesta flow may allow their removal from the gut (adapted from Ewing and Cole, 1994).



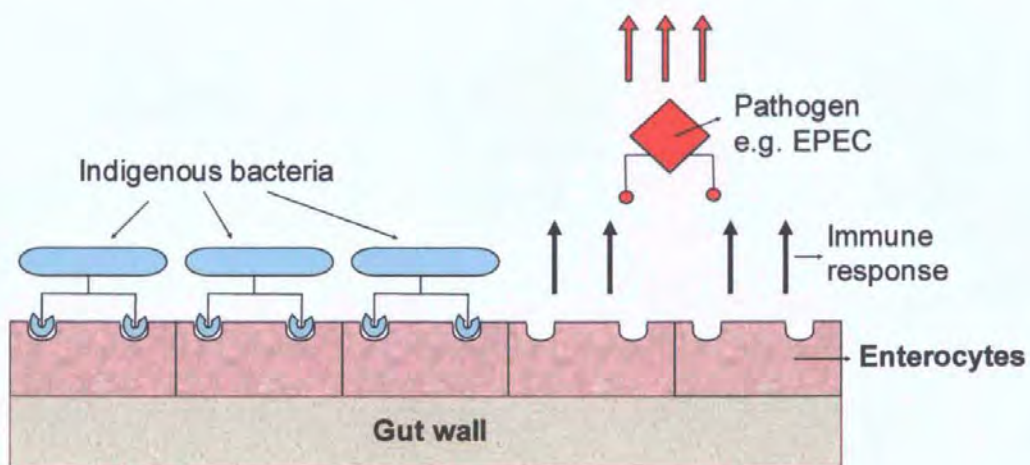
There is a considerable amount of literature on this subject however, only in the last few years has this theory been developed to the point that commercial preparations of CE products are available for use in poultry in order to protect them against pathogenic bacterial colonization particularly *Salmonella* and *E. coli* (Hofacre *et al.*, 2002; Nisbet, 2002; Wagner *et al.*, 2002).

The similar approach was also used in pigs and it has been shown that the mucosal competitive exclusion culture, isolated from the cecal microflora of a young, healthy pig, may represent a useful way to control *Salmonella* (Anderson *et al.*, 1999; Fedorka-Cray *et al.*, 1999; Harvey *et al.*, 2002) as well as pathogenic *E. coli* (Genovese *et al.*, 2000; Harvey *et al.*, 2002). In fact, using similar technology as in poultry, a CE commercial product that decreases shedding of salmonellas in neonate and weaned pigs, has been developed (Nisbet, 2002).

Other studies with CE product isolated from the pig cecum demonstrated their efficiency against *E. coli* strain F-18, and *E. coli* serotype O157:H7 (933). CE could also be as a result of aggregation of non-pathogens to pathogens, preventing binding to attachment sites and leading to their removal from the gut (Figure 1.6). Reid *et al.*, (1988) observed that type 1 fimbriated *E. coli* coaggregated with *L. casei* and suggested that there was a potential for lactobacilli to prevent pathogenic adhesion in the urinary tract by coaggregation. Coaggregation was also observed between intestinal lactobacilli of porcine origin and the pig pathogen *E. coli* K88, indicating that similar prevention could be obtained in the intestine (Hillman *et al.*, 1994). The exact mechanism by which CE products work is still under investigation. However, it has been suggested that in addition to physical obstruction of attachment sites for pathogens, increased production of volatile

fatty acids (VFA) and lactate as well as other antimicrobial substances (e.g. bacterial enzymes, bacteriophage, bacteriocins, hydrogen peroxide) together with competition for essential nutrients could also contribute to this protective effect of CE products. It has been shown that the adhesion potential of LAB to intestinal glycoproteins is strain-dependent and that different probiotics might express different effects on the subsequent adhesion of pathogens such as *E. coli* and *Salmonella* (Tuomola *et al.*, 1999). While adhesion of *S. typhimurium* was significantly inhibited by *L. johnsonii* and *L. casei* Shirota, increased pathogen adherence was associated with *Lactobacillus* GG and *L. rhamnosus* (human isolate). Interestingly, the same strains which were able to increase *Salmonella* adhesion were responsible for reduction of *E. coli* adhesion. These results suggest that the mechanism for probiotic action depends on both the probiotic strain and the type of pathogen.

Figure 1.7. Antigens from non-pathogens potentiate the host's immune response to pathogens. Pathogen bindings are inhibited (adapted from Ewing and Cole, 1994).



Finally, a great deal of work has been undertaken recently to demonstrate the important effect of commensal bacteria on the host mucosal immune system (Salminen *et al.*, 1998a;

Bonet *et al.*, 1999; Vitini *et al.*, 2000; Isolauri, 2001). A study by Michail (2002) showed that *L. plantarum* v299 can play an important role in reducing the secretory changes of Caco-2 cells in response to EPEC infection, possibly through inhibition of its binding. However, this effect of *L. plantarum* was preventive rather than therapeutic as the presence of the probiotic agent before the infection was a prerequisite (Figure 1.7). Therefore, it is clear that a disturbance of this ecological balance in the GI-tract may lead to a detrimental effect on the host. It has previously been mentioned that different forms of stress (disease, starvation, transport, trauma, parturition, etc.) can negatively affect the composition of GI-tract bacterial flora. The general trend is for lactobacilli to decrease and coliforms to increase (Arbuckle, 1968; Maclean and Thomas, 1974; Ewing and Cole, 1994).

Adequate knowledge of the types of microorganisms present, as well as the factors that influence the timing and process of colonisation, may provide opportunities to modulate the intestinal microbiota especially when modulation is necessary to enhance its beneficial function.

1.1.4 Gut microflora and the mucosal immune system

Apart from all its other functions, the GI-tract is also an immune organ, and the lymphoid tissue within it is collectively referred to as Gut-Associated Lymphoid Tissue or GALT (Goldsby *et al.*, 2000). In reality, 70-80% of the immune cells of the body are in the gut (Castro and Arntzen, 1993). As there is a considerable antigenic load on the intestine immediately after birth, it is obvious that the crucial role of the intestinal immune system is to discriminate between pathogenic and non-pathogenic, self and non-self, beneficial and harmful constituents (Simmons *et al.*, 2001). However, like the neonatal GI-tract, immune

system after birth is also functionally and structurally immature. Exposure to bacterial antigens is now recognized to be of great importance, both postnatally in order to prime the immune system in the correct way and throughout life, to maintain a functional immune system (Kelly and King, 2001b). Hence, knowledge of the gut bacteria that provide the correct signals to the gut immune system could have several practical and theoretical implications including vaccine design or alterations of enteric flora with probiotics.

Discrimination between dangerous and harmless antigens within the intestine requires continual sampling of the microenvironment by multiple potential pathways involving many cells of the innate and adaptive immune system (Shanahan, 2000). Immune cells in the GALT are distributed in three basic intestinal areas (Figure 1.8):

Lamina propria – the area below the epithelial monolayer with loose, barely organized clusters of lymphoid cells, including large numbers of B lymphocytes, plasma cells, activated T_H cells and macrophages. The plasma cells secrete mainly IgA, which is transported across the epithelial cells and released into the lumen. Thus, IgA antibodies can potentially encounter antigens in three anatomic compartments in relation to mucosal epithelium: 1) in the luminal secretions, 2) within the epithelial cells during transcytosis, and 3) in the lamina propria (Lamm, 1998).

Peyer's patches – well-organized nodules of lymphoid follicles, located in the submucosa layer of the small intestine. After antigen sampling within the follicles, if a positive response is induced, antigen-specific precursor T cells and IgA precursor B cells clonally expand during migration through the mesenteric lymph nodes, where further maturation and multiplication occurs. Finally,

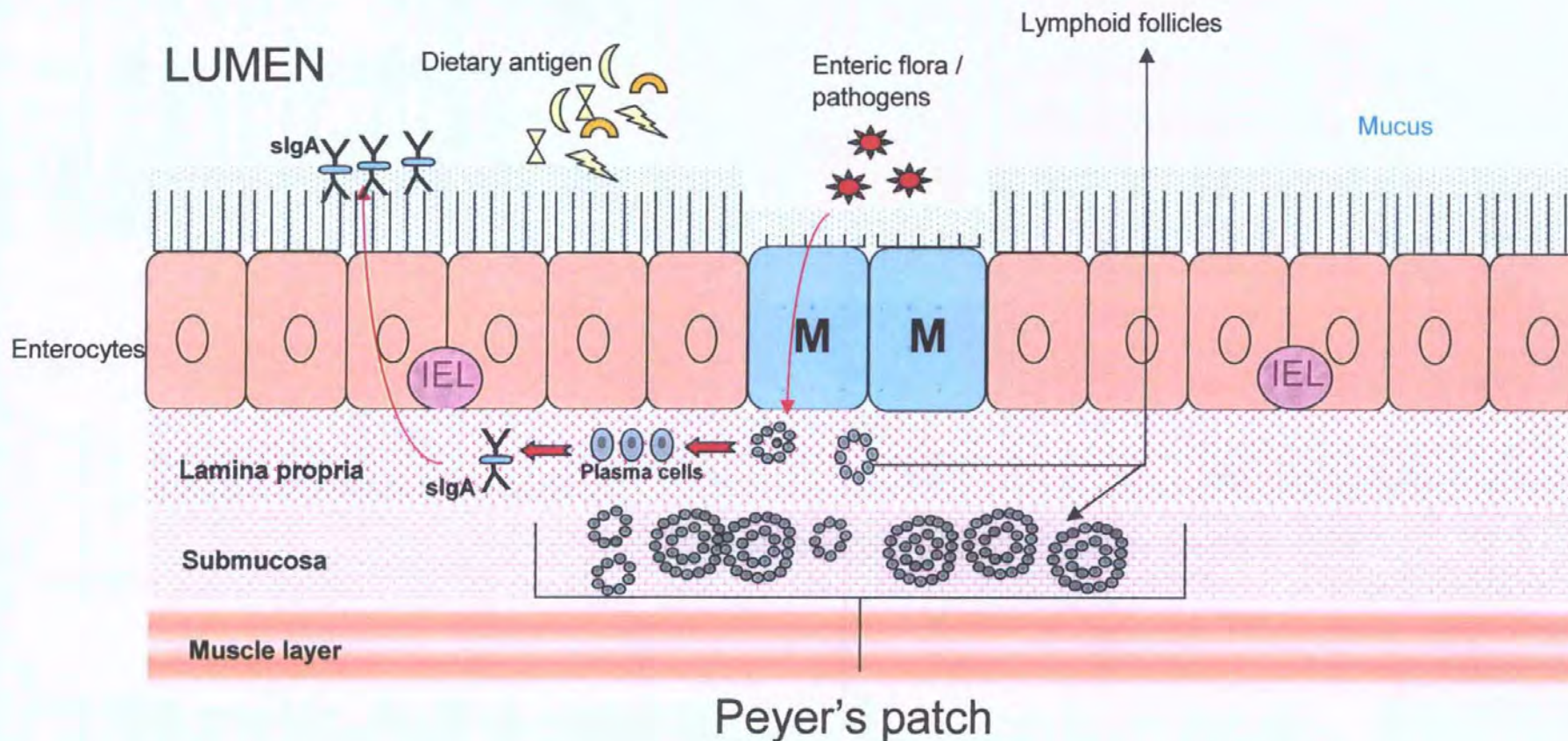
they migrate via the thoracic duct and circulation back to the lamina propria and epithelium and also populate distant mucosal tissues. Thus an immune response to enteric antigens may be reflected at a distant mucosal site such as the mammary gland. This explains the passive transfer of immunity to the same antigen by colostrum and milk to the suckling neonate (Shanahan, 2000).

Intraepithelium – lymphocytes located in the basolateral spaces between luminal epithelial cells, beneath the tight junctions (Figure 1.8). The majority of these lymphocytes are T cells with unusual $\gamma\delta$ T-cell receptors and limited diversity for antigen (Ag). They differ from $\alpha\beta$ -T cells in their mode of antigen recognition, as $\gamma\delta$ T-cell do not require antigen processing (Shanahan, 2000). They have an ideal location for meeting Ag and crosstalk with epithelial cells and other cell immune populations. Once activated by injured epithelium, they produce epithelium specific growth factors, chemokines, and other cytokines that promote healing, initiate an adaptive immune response, and recruit inflammatory cells (Shanahan, 2000).

Another important components of the GI immune system are epithelial cells together with tight junctions and M cells. **M-cells**, an epithelial cell phenotype, are found in the epithelium overlaying the Peyer's patches and represent key sites of antigen sampling for the GALT (Sansonetti and Phalipon, 1999). They are specialized to pick up foreign antigens from the lumen, and transport them to the underlying lamina propria that contains large amounts of immune cells (Figure 1.8) (Neutra, 1998). This is one manner in which the intestinal immunological system is activated. The apical membranes of M cells and enterocytes in the intestine show remarkable differences that reflect their differing

Figure 1.8. Diagram of the gut-associated immune system (GALT). Uptake of antigens occurs primarily by specialized epithelial M cells overlying lymphoid follicles. Antigens transported across the M-cells can activate B cells within lymphoid follicles in the lamina propria. The activated B cells differentiated into plasma cells, which leave the follicles and secrete the IgA class of antibodies, which are subsequently transported across the epithelial cells and released as a secretory IgA (adapted from Goldsby *et al.*, 2000).

IEL - intraepithelial lymphocytes, **sIgA** - secretory immunoglobulin A, **M** - membranous epithelial cells (M cells).



functions (Neutra *et al.*, 1999). The typical enterocyte has its absorptive area amplified by closely-packed microvilli, whose tips are coated with a thick layer of membrane anchored glycoproteins (glycocalyx) and adsorbed pancreatic enzymes. This structure of the brush border facilitates digestion but also is an effective diffusion barrier that minimises the uptake of antigens and pathogens by enterocytes. M cell apical surfaces lack this typical brush border arrangement. Their microvilli (often called “microfolds”) are variable, and the glycocalyx coat varies widely in thickness and composition (Neutra *et al.*, 1999). They can easily be recognized by their high endocytic activity and basolateral lymphocyte-containing pocket (Neutra *et al.*, 1996). All of these structural features make the apical surfaces of M cells vulnerable to pathogenic viruses and bacteria and indeed, the M cells are nowadays considered as a pathogen translocator toward immunocompetent area of the gut. So far, at least 14 different species of non-pathogenic and pathogenic bacteria, including enteropathogenic *E. coli* (von Moll and Cantey, 1997) and *Salmonella typhimurium* (Clark *et al.*, 1996; Jepson and Clark, 2001) have been shown to selectively adhere to the apical surface of M cells. However, a lot more remains to be understood about the molecular mechanisms of pathogen – M cells interactions. The fact is that once bacteria have been transported through the M cells, they face innate immune defences, particularly the macrophages, and the outcome of the invasive process will rely heavily on the way bacteria escape killing by these immune cells (Sansonetti and Phalipon, 1999).

Intestinal Epithelial Cells (IEC, enterocytes)

Although the main role of IECs is absorption of nutrients in the form of small molecules (e.g. amino acids, sugars), enterocytes are also able to sample intact protein antigens (Perdue, 1999). Intracellular processing of these antigenic proteins within enterocytes

appears to be important for oral tolerance (Mayer, 1998). This form of immunological tolerance, which is by definition '*a state of unresponsiveness that exists for non-pathogenic antigens present within the gut lumen*', is not programmed into the germline but is acquired during postnatal maturation (Strobel, 1995). In reality, this state of unresponsiveness is not a passive lack of response but rather involves the active dampening of the immune responses (Mayer, 1998). However, the mechanisms that account for oral tolerance, especially with respect to the role of epithelium, are still not completely elucidated. IECs represent the cornerstone of intrinsic mechanisms protecting the underlying elements of the intestinal mucosa from luminal agents (Podolsky, 1999). They have been grouped into a category of non-professional antigen-presenting cells (APCs), which means that they do not constitutively express class II MHC molecules and do not activate T cells in a conventional manner (Mayer, 1998). However, several unique features of the epithelial cells have been described (recently e.g. complex of gp180 and CD1d molecules), making their potential role as APCs a critical part of mucosal homeostasis (Mayer, 1998).

Moreover, it is well established that IECs produce a range of cytokines and chemokines by which they communicates with the immune compartments of the mucosa (Fiocchi, 1997; Haller *et al.*, 2000b). The expression of these mediator molecules is controlled by specific transcription factors, which are activated after interaction of microorganisms with IECs. Nuclear Factor- κ B (NF- κ B) is considered as a central regulator of cellular responses to pathogens, non-pathogens and stress (Makarov, 2000; Mahida and Johal, 2001). However, despite rapid accumulation of our knowledge on epithelial cells we are still just at the beginning of understanding the critical role of epithelial cells in regulating the response to ingested antigens.

Recent years have seen increasing interest in the field of gut microflora and its effects on the host immune system and it is widely accepted that there is a life-long dialog between bacteria and GALT, which commences immediately following colonisation at birth. For obvious reasons, considerable efforts have been devoted to the understanding of infectious diseases, including the biology of pathogens, host resistance and therapy in animals including humans. Unfortunately, bacteria that do not cause disease have long been ignored. As a result little is known about these intimate residents. Indeed, the vast majority have not even been named. It should be stressed that studying the cross-talk between indigenous microorganisms and their mammalian hosts represents an experimental challenge because these interactions are typically subtle and the microbial communities that associate with mammalian hosts are very complex and dynamic (Falk *et al.*, 1998). Therefore, exactly how these so-called 'good' bacteria affect the immune system of the host organism is not fully understood. However, knowledge about the cells and molecules important in regulating mucosal immune response is expanding rapidly (Hessle *et al.*, 1999; Haller *et al.*, 2000b; Iijima *et al.*, 2001; Christensen *et al.*, 2002). Studies with germ-free animals showed that both the innate and the acquired immune system are affected by the lack of resident bacteria (McCracken and Lorenz, 2001). The one way by which commensal bacteria can directly influence immune function is by modulating intestinal cytokine profiles (Christensen *et al.*, 2002). Bacterial effects on cytokine production and their regulation of the immune system have been studied intensively in the last ten years in cell lines and primary cells of both rodents and humans (Miettinen *et al.*, 1996; Tejada-Simon *et al.*, 1999a; Miettinen *et al.*, 2000; Iijima *et al.*, 2001; von der Weid *et al.*, 2001; Christensen *et al.*, 2002). Special attention has been focused on the lactic acid bacteria (LAB) and the bifidobacteria populations, both those that are naturally present within this complex ecosystem and those that are ingested as probiotics in functional foods. Immuno-

histochemical analyses of cytokine profiles in the study of Maassen (1998) revealed that differential immuno-modulation can be obtained depending on the *Lactobacillus* strain applied. Lactobacilli were able to enhance or inhibit the development of disease in mice, which only emphasized the importance of proper strain selection in order to influence the immune system in the right direction. Two important immunoregulatory cytokines produced by cells of the innate defence system in response to bacteria are interleukin-12 (IL-12) and (IL-10), which have largely opposite effects on the immune system. They represent the key messenger molecules which bridge the gap between innate and acquired immunity. IL-12 activates proliferation, enhances cytotoxicity and interferon- γ (IFN- γ) secretion by T cells and NK cells, whereas IL-10 inhibits these functions and instead stimulates B cell maturation (Esche *et al.*, 2000). IL-12 also enhances resistance to a variety of infectious diseases and exhibits potent antitumor immunity (Esche *et al.*, 2000). Interferon- γ induced by IL-12 has a multitude of cellular biological functions e.g. it promotes phagocytosis, inhibits viral replications, upregulates microbial killing, upregulates endothelial cells and induces antibody class-switching by B-cells to complement fixing antibody isotypes such as the IgG2a (Billiau and Vandenbroeck, 2000). It has been demonstrated that non-pathogenic Gram-positive bacteria are strong IL-12 inducers with substantial differences among the strains whereas Gram-negative bacteria preferentially stimulated secretion of IL-10 (Hessle *et al.*, 1999; Kato *et al.*, 1999; Haller *et al.*, 2000a; Hessle *et al.*, 2000; Christensen *et al.*, 2002). It has been shown that both dendritic cells (Christensen *et al.*, 2002), which plays a key immunoregulatory role in the Th1, Th2, and Th3 cell balance as well as NK cells (Haller *et al.*, 2000a), which provide a first line of defence against intestinal pathogens and viral infections, could represent the major targets for modulation by gut microbes, including probiotics. The specific cell wall components or cell layers (e.g. lipopolysacharides, peptidoglycans and lipoteichoic acids)

may act as adjuvants and increase immune responses (Heumann *et al.*, 1994; Huang *et al.*, 1999; Tejada-Simon *et al.*, 1999a). Gram-positive and Gram-negative bacteria have also been shown to induce different patterns of costimulatory molecules on antigen-presenting cells, which could contribute to diverging T cell responses to these bacteria depending on the structure of their cell wall (Keller *et al.*, 1995). The presence of specific receptors for these cell-wall components of LAB on lymphocytes and macrophages has also been reported (Dziarski, 1991). Therefore, one of the reasons why lactobacilli hardly cause disease might be their potent ability to induce the production of IL-12 and thereby activate macrophages resulting in a more effective clearance of the bacteria, and thus decreasing their infectivity (Hessle *et al.*, 1999).

The role of diet in health and wellbeing has changed as the science of nutrition has evolved. Research interest is currently directed towards improvement of defined physiological functions beyond the nutritional impact of food, including the potential to reduce the risk of disease. This is also a focus on probiotic research (Isolauri *et al.*, 2002). The word probiotic is derived from the Greek meaning 'for life' (Shortt, 1999). There have been many definitions of probiotics proposed during the years but none of them has received universal acceptance. However, the most widely used is that of (Fuller, 1989) where probiotic is defined as

'a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance.'

Recently Salminen (1999) proposed the following definition, which included current application and scientific data on proven effects of probiotics:

'Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host.'

While Fuller's definition stresses the need for probiotic to be viable and is limited to the intestinal tract, the second definition implies that probiotics do not necessarily need to be viable and that not only whole microbial cells, but also parts of cells have been observed to improve host health. Exploiting probiotic bacteria provides an opportunity for food scientists to develop food products tailored to prevent specific diseases and promote overall human/animal gastrointestinal health. Indeed, the market for functional foods, such as probiotics, is undoubtedly the fastest growing area of new food product development (O'Brien *et al.*, 1999). Current safety criteria for successful probiotics have been defined in several reviews (Salminen *et al.*, 1998b; O'Brien *et al.*, 1999). These criteria represent the outcome of a multidisciplinary approach to probiotic safety evaluation, involving the contributions of pathologists, geneticists, toxicologists, immunologists, gastroenterologists, and microbiologists. Probiotic bacteria are generally, though not exclusively lactic acid bacteria (LAB) (Table 1.5). Members of the genera *Lactococcus* and *Lactobacillus* are most commonly given **Generally-Recognised-As-Safe** (GRAS) status whilst members of the genera *Streptococcus* and *Enterococcus* and some other genera of LAB contain some opportunistic pathogens (Salminen *et al.*, 1998b). LAB used for feeding animals are subjected to EC regulation for feed additive (SCANN, 2000). In terms of sows and pigs, there are currently only 12 probiotic additives permitted within the European Union (Table 1.6). A review of the literature shows that there are a considerable number of studies, which report, in some shape or form, the use of various *Lactobacillus* strains as probiotic agents. In addition to their nutritional and antimicrobial effects, many of them express

immunomodulatory activity. De Roos (2000) reviewed 33 human probiotic intervention studies of which 26 dealt with the prevention or treatment of diarrhoeal disease and 7 with the stimulation of the immune system.

Table 1.5. Microorganisms used in probiotic products around the world (Shortt, 1999).

Lactobacilli	Bifidobacteria	Other LAB	Non-LAB
<i>L. acidophilus</i>	<i>B. animalis</i>	<i>E. faecium</i>	<i>B. cereus</i>
<i>L. casei</i>	<i>B. breve</i>		<i>E. coli</i>
<i>L. jonsoni</i>	<i>B. infantis</i>		<i>S. boulardii</i>
<i>L. reuteri</i>	<i>B. longum</i>		<i>Cl. butiricum</i>
<i>L. rhamnosus</i>	<i>B. adolescentis</i>		
<i>L. salivarius</i>	<i>B. lactis</i>		
<i>L. plantarum</i>	<i>B. bifidum</i>		
<i>L. crispatus</i>			

The most widely studied probiotic bacteria were *Lactobacillus* GG, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Enterococcus faecium*. The data presented in Table 1.7 shows that probiotic bacteria are able to interact with the immune system at many levels, including cytokine production, mononuclear cell proliferation, macrophage phagocytosis and killing, modulation of autoimmunity, and immunity to bacterial pathogens. Perdigon *et al.*, (2000) suggested that the different effects of LAB on mucosal immunostimulation are related to the different pathways of gut internalisation used to make contact with the immune cells associated with the intestinal lamina propria. However, it is worth noting that some strains do not have this immunostimulation influence in healthy individuals (Spanhaak *et al.*, 1998).

Table 1.6. Microorganisms approved as probiotics in the EU (Council Directive 70/524/ EEC) for piglets and sows (Official Journal of the European Communities, 2002).

EU No.	Strain	Culture collection number	Tradename	Target animal categories	Producer	Activity
6	<i>Saccharomyces cerevisiae</i>	CNCM I-1079	Levucell SB20®	Gestating/lactating sows Postweaned piglets	Lallemand, France	Reduction of pathogens Stimulation of the immune system Reinforcement of the intestinal microflora equilibrium
9	<i>Pediococcus acidilactici</i>	MA 185M	Bactocell ®	Postweaned piglets Fattening pigs	Lallemand, France	Production of lactic acid (L+) Regulation of microbial ecosystem of liquid feed, intestine and faeces Reduction of pathogens Robustness and stability in feed processing systems
E170 0	<i>Bacillus licheniformis</i> <i>Bacillus subtilis</i>	DSM 5749 DSM 5750	Bioplus 2B ®	Nursery piglets Sows and pigs for fattening	Chr. Hansen, Denmark	Assists digestion May improve feed intake, average daily gain and feed conversion Reduces odour in manure
10	<i>Enterococcus faecium</i>	NCIMB10415	Cylactin LBC®	Piglets and pigs for fattening, sows	Cerbios Pharma, Switzerland	Improves feed intake, feed efficiency and live weight gain, stabilizes the health
11	<i>Enterococcus faecium</i>	DSM 5464	Microferm ®	piglets	Medipharm/Ceva Sweden	Maintaining normal digestive function Suppresses pathogenic bacteria such as <i>Salmonella</i> and <i>E.coli</i>
13	<i>Enterococcus faecium</i>	DSM10663/ NCIMB 10415	Oralin ®	Pigs for fattening	Chevita GmbH	Production of lactic acid Competitive exclusion of pathogens Stabilisation of physiological gut flora Improvement of weight gain and feed conversion

Table 1.6 (cont). Microorganisms approved as probiotics in the EU (Council Directive 70/524/ EEC) for piglets and sows (Official Journal of the European Communities, 2002).

EU No.	Strain	Culture collection number	Tradename	Target animal categories	Producer	Activity
18	<i>Enterococcus faecium</i>	CECT 4515	Fecinor plus®	Piglets and pigs for fattening	Norel SA, Spain	Improvement of daily weight gain Prevent from weaning stress Prevents from E.coli invasion Reduces incidence of sticky droppings
15	<i>Enterococcus faecium</i>	NCIMB 11181	Lactiferm ®	Weaned / starter pigs	Medipharm AB Sweden	Antimicrobial activity against <i>E.coli</i> , <i>Salmonella</i> and <i>Shigella</i>
16	<i>Enterococcus faecium</i> <i>Lactobacillus rhamnosus</i>	DSM 7134 DSM 7133	Bonvital ®	Pigs for fattening	Schaumann, Austria	Stabilizes the intestinal flora Increases appetite and performance Suppresses pathogenic bacteria
12	<i>Lactobacillus farciminis</i>	CNCM MA67/4R	Biacton ®	piglets	Bioarmor, France	Increased digestive safety Increased growth
3	<i>Saccharomyces cerevisiae</i>	NCYC Sc 47	Biosaf SC47®	Piglets and sows	Lesaffre, France	Better use of foodstuff Improved performance (increased growth and milk yield) Reduction of diarrhoea and mortality
14	<i>Sacharomyces cerevisiae</i>	BCCM/MUC L 39885	Biosprint ®	Piglets and pigs for fattening	Prosol SpA, Italy	Increased growth rates and milk yield Improved milk quality Less digestive upsets

Table 1.7. *In vivo* studies involving dietary supplementation with LAB that have shown enhanced immune response.

Reference:	Probiotic treatment	Consequence of probiotic feeding and immune correlate
(Kato <i>et al.</i> , 1983)	<i>L. casei</i>	Macrophage activation
(Kato <i>et al.</i> , 1984)	<i>L. casei</i>	Augmentation of mouse natural killer (NK) cell activity
(Perdigon <i>et al.</i> , 1990b)	<i>L. casei</i> and <i>L. acidophilus</i>	Increased survival, decreased pathogen translocation to the spleen and liver, increased serum and gut mucosal anti- <i>Salmonella</i> antibody titres
(Perdigon <i>et al.</i> , 1991)	<i>L. casei</i>	Increased IgA secretion in the intestinal lumen
(Davidkova <i>et al.</i> , 1992)	<i>L. bulgaricus</i>	Activation of phagocytic/secretory functions of monocytes
(Mao <i>et al.</i> , 1996)	<i>L. reuteri</i> , <i>L. plantarum</i>	Increased ileal and colonic secretory IgA concentrations and elevated CD4 and CD8 numbers
(Maassen <i>et al.</i> , 1998)	<i>L. reuteri</i> , <i>L. brevis</i> , <i>L. gasseri</i> , <i>L. casei</i> , <i>L. murines</i> , <i>L. plantarum</i> (x2), <i>L. fermentum</i>	Induction of cytokine profile: study demonstrating that individual <i>Lactobacillus</i> strain can induce differential cytokine profiles in the murine gut following oral administration
(Herias <i>et al.</i> , 1999)	<i>L. plantarum</i> 299v	Increased serum IgA levels; increased density of CD25(+) cells in the lamina propria
(Kato <i>et al.</i> , 1999)	<i>L. casei</i> strain <i>Shirota</i>	Induction of the production of interleukin-12 and interferon-gamma by mouse splenocytes
(Kirjavainen <i>et al.</i> , 1999b)	<i>L. acidophilus</i>	Enhanced basal proliferation and response of B-cells of mice

Table 1.7 (cont.) *In vivo* studies involving dietary supplementation with LAB that have shown enhanced immune response.

Reference:	Probiotic treatment	Consequence of probiotic feeding and immune correlate
(Perdigon <i>et al.</i> , 1999)	<i>L. casei</i>	Stimulation of IgA and IgM synthesis in malnourished mice, increasing the number of T-Lymphocytes and activity of intestinal macrophage
(Vitini <i>et al.</i> , 2000)	<i>L. casei</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. plantarum</i> , <i>Lactococcus lactis</i> , <i>Streptococcus thermophilus</i>	Increased number of IgA producing cells associated to the lamina propria of small intestine
(Madsen <i>et al.</i> , 2001)	VSL#3 (<i>Bifidobacterium longum</i> , <i>B. infantis</i> , <i>B. breve</i> , <i>L. acidophilus</i> , <i>L. casei</i> , <i>L. delbrueckii</i> subsp. <i>L. bulgaricus</i> , <i>L. plantarum</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>)	Treatment of IL-10 gene deficient mice with the probiotic compound VSL#3 resulted in normalisation of colonic physiologic function and barrier integrity in conjunction with a reduction in mucosal secretion of TNF α and interferon γ and an improvement in histologic disease.
(Gill and Rutherford, 2001)	<i>L. rhamnosus</i> HN001	Enhanced phagocytic activity of blood and peritoneal leucocytes
(Matar <i>et al.</i> , 2001)	<i>L. helveticus</i>	Increased number of sIgA at both the intestinal and bronchial levels, indicating that a cellular migration had occurred.
(Shu and Gill, 2001)	<i>Bifidobacterium lactis</i>	Increased phagocytic capacity of blood neutrophils and peritoneal macrophages
(Hori <i>et al.</i> , 2002)	<i>L. casei</i> strain Shirota	Increased natural killer activity of splenocytes and lung cells; induction of gamma interferon (IFN- γ) and tumour necrosis factor alpha (TNF- α),
(Dalloul <i>et al.</i> , 2003)	<i>Lactobacillus</i> -based commercial probiotic (Primalac)	Altered intraepithelial lymphocytes (IEL) population, increased numbers of IEL expressing the surface markers CM, CD4, CD8, and $\alpha\beta$ TCR

In the years between 1980 and 1990, a number of animal experiments were published that demonstrated the immunomodulatory effect of lactobacilli *in vivo*. It has been shown that probiotics may reinforce different lines of gut defense such as immune exclusion, immune elimination and immune regulation. Lessard and Brisson (1987) fed piglets with rehydrated skim milk powder fermented with a mixture of lactobacilli and reported slightly increased serum IgG levels. Perdigon *et al.*, (1986a) reported enhanced macrophage and lymphocyte activity in mice after oral administration of a mixed culture of *L. casei* and *L. acidophilus*.

Further studies involving oral challenge with enteropathogens have investigated the protective effects of probiotic feeding against *Salmonella* or *Escherichia coli*. Mice fed *L. casei* prior to *Salmonella typhimurium* or *E. coli* challenge exhibited increased protection against either pathogen. This effect was concurrent with increased synthesis of pathogen-specific sIgA antibodies in intestinal fluid of these animals (Perdigon *et al.*, 1991). Protective effects against diarrhoea have been demonstrated in a large animal neonatal model by feeding immune-modulating bifidobacteria. Shu and Gill (2001) showed that pre-feeding piglets with *Bifidobacterium lactis* (strain HN019), prior to weaning, in conditions that would pre-dispose the piglets to environmental pathogen exposure, could effectively reduce the cumulative morbidity index in these animals and, as a consequence, the probiotic-fed animals maintained a greater rate of food intake and exhibited a higher feed conversion efficiency compared to a non probiotic-fed group of animals. This study also provided further evidence that pertinent cellular and humoral immune parameters could be increased by probiotic feeding. Both serum and GI-tract pathogen specific antibody titres, as well as blood-derived neutrophil phagocytic capacity and T cell proliferative responsiveness to concanavalin A mitogen, were significantly elevated in probiotic-fed animals. Finally, a very recent study by Dalloul *et al.*, (2003) provides further clear

evidence of the immunomodulating potential of lactobacilli. This study investigated the effect of feeding a *Lactobacillus*-based probiotic to chicken on the intestinal intraepithelial lymphocyte (IEL) subpopulations and subsequent protection against coccidiosis caused by *Eimeria acervulina*. Differences in IEL subpopulations were assessed by flow cytometry at 21 d postprobiotic treatment. Probiotic-fed chickens had more IEL expressing the surface markers CM, CD4, CD8, and $\alpha\beta$ TCR as well as producing significantly less oocysts than control birds.

Collectively, these studies demonstrated that significant progress has been made in our understanding of the impact of indigenous bacteria on the GI-tract, especially its immune part, and their likely significance to the host's health and wellbeing. There is strong evidence that certain strains of LAB have a capacity to stimulate both non-specific and specific immune function, which highlights the opportunities for health food industries to develop novel immunity-enhancing food products. It has been shown that the efficacy of immunomodulating LAB could be enhanced by using live cultures rather than killed bacteria (Vesely *et al.*, 1985; De Simone *et al.*, 1986; Portier *et al.*, 1993; Kaila *et al.*, 1995; Miettinen *et al.*, 1996) and by delivering them in fermented rather than non-fermented products (Perdigon *et al.*, 1986b; Saucier *et al.*, 1992). However, it is clear that the research in this field is far from complete. It has been shown that a large variation exists in the ability of different LAB strains to modulate the immune system and that this effect is dose-dependant. There is still lack of clear answers on questions such as: what is the efficacy of LAB in relation to the host age, physiological status and dietary intake. Another very important issue is to define the effective dose for each strain as well as elucidate their exact mechanism of action. Finally, as the majority of the immunostimulatory experiments have been carried out with pure strains and *in vitro*

conditions, the next area in which knowledge has to be improved is the understanding of how the introduced strains will interact within the microbial biofilm of the host GI-tract. Therefore, studies investigating the influence of probiotics on the immune function in swine have yet to be reported. Other beneficial effects of feeding probiotics to pigs are listed in Table 1.8:

Table 1.8. Other beneficial effects of feeding probiotics to pigs.

Reference	Probiotic treatment	Probiotic effect
(Abe <i>et al.</i> , 1995)	Lactobacilli, Bifidobacteria	Increased weight gain and reduced neonatal mortality of the neonatal piglets
(Nemcova <i>et al.</i> , 1998) (Bomba <i>et al.</i> , 1998)	<i>Lactobacillus casei</i>	Probiotic-fed gnotobiotic piglets consumed more milk and gained more weight than germ-free piglets
(Kumprecht and Zobac, 1998)	<i>Enterococcus faecium</i>	Increased weight gain and feed efficiency of the piglets
(Kyriakis <i>et al.</i> , 1999) (Alexopoulos <i>et al.</i> , 2001)	<i>Bacillus licheniformis</i> <i>Bacillus cereus</i>	Improved weight gain and feed conversion and reduced diarrhea and mortality in piglets.

1.1.5 Fermented liquid feed (FLF) and gut health

Probiotic bacteria are delivered to humans mainly in fermented foods for several reasons: 1) fermented foods, and dairy products in particular, already have a record as being healthy; 2) consumers are familiar with the fact that fermented foods contain living microorganisms (bacteria); and 3) probiotics used as starter organisms combine the positive images of both fermentation and probiotic cultures (Heller, 2001). Fermentation of the feed before its presentation to the pigs represents a new development in liquid feeding.

This approach is creating great interest in Europe as producers strive to find alternatives to antibiotic growth promoters, which are being removed from the market. The three primary effects of antibiotic growth promoters are: 1) increased growth; 2) improved feed efficiency, and 3) a lower incidence of certain diseases. To be effective, alternatives to antibiotics should generate similar benefits. The modes of action of growth-promoting antibiotics and their alternatives can differ considerably. Sub-therapeutic growth promoters work in part by decreasing the microbial load in the gut, resulting in a reduction in energy and protein required to maintain and nourish the intestinal tissues (Ferket, 2003). Thus any reduction in gut tissue mass can have a significant impact on the amount of energy available for growth and caloric conversion efficiency. The reduced microbial load in the gut by sub-therapeutic levels of antibiotics also reduces immunological stress, resulting in more nutrients partitioning toward growth and production rather than toward mechanisms of disease resistance. In contrast, most alternative compounds do not reduce overall microbial loads in the gut and thus will not promote growth by a mechanism similar to antibiotics. Instead, they alter the gut microflora profile by limiting the colonisation of unfavourable bacteria while promoting the fermentation of more favourable species. Consequently, the alternatives to antibiotics promote gut health by several possible mechanisms including altering gut pH, maintaining protective gut mucins, selection for beneficial intestinal organisms or against pathogens, enhancing fermentation acids, enhancing nutrient uptake, and increasing the humoral immune response (Ferket, 2003).

Fermented liquid feeding represents a very promising feeding practice which may compensate to some extent for the reduction or elimination of antibiotic growth promoters in feeds. Moreover, its liquid form is very suitable for the inclusion of additional alternative products currently available on the market (e.g. enzymes, metal chelates,

prebiotics, probiotics, organic acids, herbs and immunostimulators). The subject of fermented liquid feed for pigs has been thoroughly reviewed in the literature (Jensen and Mikkelsen, 1998; Brooks, 1999; Brooks *et al.*, 1999; Scholten *et al.*, 1999; Brooks *et al.*, 2001) and therefore this review will highlight only the main points.

Fermented liquid feed is characterised as a diet which is high in lactic acid bacteria ($>9 \log \text{CFU ml}^{-1}$) and yeast ($\sim 6\text{--}7 \log \text{CFU ml}^{-1}$), high concentration of lactic acid (64.0–105.3 g kg^{-1}), and has a low pH ($\text{pH} < 4.0$) (Geary *et al.*, 1996; Mikkelsen and Jensen, 1997). Liquid feeding of pigs is not new as the idea of providing newly weaned pigs with frequent small meals mixed with water and fed as warm as sow's milk has been recommended from at least the early 19th century (Henderson, 1814; Youatt, 1847). The advantage of this type of feeding during post-weaning period is that the piglet only needs to obtain its food and water requirements from a single source rather than learn to satisfy their drives for hunger and thirst separately (Brooks *et al.*, 2001). In addition, liquid feed encourages feed intake and reduces post-weaning stress because it has a dry matter concentration more like that of sow's milk (Partridge and Gill, 1993). It has been also shown that providing the diet in a liquid form appears to improve its digestibility (Barber *et al.*, 1991; Brooks *et al.*, 1999). Finally there is growing evidence that liquid feeding reduces gastrointestinal disorders and diarrhoea (Tielen *et al.*, 1997; van der Wolf *et al.*, 1999). In the study of 320 farms in The Netherlands, the incidence of *Salmonella* positive herds was 10 times lower on farms with liquid feeding, than on farms utilizing dry feed systems which was attributed to the antagonistic activity of the organic acids present in the diet, due to the indigenous LAB fermenting the water-soluble carbohydrates present (Tielen *et al.*, 1997; van der Wolf *et al.*, 1999). Nowadays, it is also becoming more common to feed lactating sows on liquid diets, as this can increase their feed intake. However, the major problem with feeding a

liquid diet is that wet feed does not stay fresh in the trough for very long and spoilage bacteria together with molds can also accumulate without regular cleaning. Therefore, it would be advantageous if a sterilant material could be found that could be added to liquid diets which would both be economically viable and maintain the palatability of the feed. Study in our lab showed that chlorine dioxide, a strong oxidizing and sanitizing agent with broad antimicrobial spectrum, could represent very promising sterilant reagent for liquid feed (Demeckova *et al.*, 2001). No coliforms, yeasts or LAB were detected after 24 hours in the liquid feed treated with 300 ppm chlorine dioxide or higher.

Fermentation of a liquid feed could represent another solution how to maintain the liquid feed in a hygienic and palatable state. The production and use of FLF is simple in concept, relying on a natural process that has been used for centuries to preserve human food and in the last century for ensiling herbage for ruminants. In early studies at Plymouth, the fermentation of liquid feed relied upon naturally occurring LAB (Russell *et al.*, 1996). The LAB, epiphytic to cereal component of the diet, will proliferate in a liquid feed system and reduce the pH of the diet (Smith, 1976). The naturally fermented diets in Russel study were well accepted by weaners and there was no evidence of post-weaning scours. However, despite these satisfactory results, the uncontrolled nature of this system gave cause for concern as not all pig producers will be able to exercise the same levels of control on farm that could be achieved in experimental facilities. The high unpredictability of natural fermentation of liquid feed was recently demonstrated in our laboratory (Beal *et al.*, unpublished data). A lactic acid concentration of 70 mMol was found to be bacteriostatic, but higher levels (> 100 mMol) are needed in order to be bactericidal. Unfortunately, natural fermentation cannot be relied upon to produce these concentrations of acid. For example, in samples of wheat from across the UK fermented for 24 hours at 30°C the lactic

acid level varied from 0 to 50 (8.7 ± 12.2) mMol. After 72 hours the range was from 0.14 to 135 (48 ± 38) mMol lactic acid. Only circa 10% of natural fermentations achieved the threshold level of 100 mMol lactic acid needed to eliminate *Salmonella* (Beal *pers. commun.*). Therefore, a more recent approach has relied upon controlled fermentation with LAB inoculants that produce lactic acid rapidly, and to a high concentration. Despite number of LAB species are capable of producing more than 100 mMol lactic acid in 24 hours (Beal, unpublished data), currently only one of these (*Pediococcus acidilactici* MA18/5M; Bactocell, Lallemand) could be added to diets, as it is only one microorganism that is registered as a feed additive. Inoculant for feed fermentation must possess the following basic criteria (adapted from Bückenhuskes (1993)):

- Non-pathogenic and safe status
- Promotion of homolactic fermentation
- Ability to grow and reduce pH rapidly
- Ability to utilize a broad range of carbohydrates
- Propagation must be feasible from an economical point of view
- Resistance to in-feed antimicrobials
- Starter organism that dominate the indigenous microflora of feed

Apart from these criteria it would be beneficial if strain used for feed inoculation would express some probiotic properties as well (e.g. immunomodulatory, adhesive, bacteriocin production). This way the inoculant could play double role: controlling the fermentation and expressing potential health benefit for the consumer. However, both probiotic and fermentative properties do not have to be necessarily expressed by single strain. The same effect could be achieved by right combination ('cocktail') of LAB strains with different

properties. In fact, it has been shown that the combination of probiotic strains with synergistically acting components seems to be the best way of enhancing the efficacy of probiotic preparations from practical point of view (Salminen *et al.*, 1998a).

Continuous fermentation can be maintained by retaining about half of the feed each day and adding half fresh feed and water to the next mixture. The importance of temperature in controlling fermentation and lowering pH of the feed was demonstrated in the study of Jensen and Mikkelsen (1998) as well as Beal *et al.*, (2002). The death rate of *Salmonella typhimurium* in fermented feed was much faster at 30°C than at 20°C. The temperature of water added to the system represents another important issue which needs to be taken in account in order to achieve desirable fermentation. Water added directly from tap might cold-shock the system leading to following adverse effects: 1) it will negatively affect the growth of LAB; 2) it will allow the yeasts to become dominant; and 3) it will induce the production of protective 'cold shock' proteins in enteropathogens which will allow them to persist in the feed for much longer. If fermentation of liquid feed is controlled properly, the resultant feed is well accepted by pigs and has a beneficial effect on stomach pH, gut architecture and microbiology. Review of the FLF research showed numbers of proven benefits of FLF for pigs:

- improved feed intake post-weaning (Jensen and Mikkelsen, 1998; Moran, 2001)
- improved post-weaning growth performance (Urlings *et al.*, 1993; Geary and Brooks, 1998; Jensen and Mikkelsen, 1998; Scholten *et al.*, 1998)
- improved bacterial ecology of the gastrointestinal tract (Mikkelsen and Jensen, 1997; Mikkelsen and Jensen, 1998; Hansen *et al.*, 2000; Moran, 2001; van Winsen *et al.*, 2001b; van Winsen *et al.*, 2002)

- it reduces stomach pH (Mikkelsen and Jensen, 1998; van Winsen *et al.*, 2001b)
- it supplies lactic acid bacteria (Urlings *et al.*, 1993; van Winsen *et al.*, 2001b)

The challenge now is to refine equipment and develop management practices that will enable the potential of this feeding method to be realized on commercial pig units.

1.3 Rationale of the study

The neonatal pig is born with no intestinal microflora. It gains its microflora during passage through the vagina and from the environment into which it is born. The most significant factor affecting the microflora of the environment into which it is born is the sow. Therefore, management interventions that influence the microbiology of the sow's faeces are likely also to influence the neonate. This study will examine the hypothesis that feeding the sow a diet fermented with lactic acid bacteria can have significant benefits for her offspring. Antagonism against intestinal pathogens, the barrier effect of the microflora, and modulation of the host's immune function are among some beneficial effects that some lactic acid bacteria provide to the host. The gastrointestinal tract is probably the most exposed body site with respect to pathogenic microorganisms. The gut associated lymphatic tissues (GALT) play a major role in both local and systemic immunological responses. Recent studies strongly support the hypothesis that orally administered lactic acid bacteria stimulate the immune system, both at the local level and the systemic level. This immunomodulatory effect has significant beneficial consequences, such as preventive and therapeutic effects on intestinal infections.

Therefore the aim of the present study was to examine the hypothesis that feeding the sow a diet fermented with *Lactobacillus* can have significant benefits for her offspring.

The specific objectives were:

- to investigate whether feeding sows FLF modifies the faecal microflora at farrowing when a coliform 'bloom' usually occurs
- whether any changes in the faecal microflora of farrowing sows affects bacterial colonization of the gut of the newborn piglet
- whether feeding FLF has a beneficial effect on the quality of colostrum produced by the sows
- to select a new *Lactobacillus* inoculum of porcine origin that would have a potential double role: i) control fermentation and ii) probiotic role
- to determine the *in vivo* effect of the selected inoculum on microbial and immunological parameters of farrowing sows

Chapter 2

The effect of fermented liquid feed, fermented with *Lactobacillus plantarum*, on faecal microbiology and colostrum quality of sows.

All protocols for the animal experiments were approved by the University Animal Ethics Committee.

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2.1. Introduction

Under normal circumstances, the newborn pig, which is sterile at birth, rapidly acquires its characteristic microflora by contact with its mother and the environment into which it is born. The most significant factor affecting the microflora of the piglet's environment is the sow. It has been shown that suckling piglets ingest a large quantity of sow's faeces and bedding material (Sansom and Gleed, 1981). In addition, the sow's teats could be heavily contaminated with microbes from faeces and the environment. Therefore, management interventions and nutritional regimes that influence the microbiology of the sow's faeces in a beneficial way are likely also to influence the neonate. The relative proportion of different organisms in the sow's faeces and the extent to which the piglet comes into contact with the faeces may affect colonisation of the gut. Colonisation of the pig gut occurs very rapidly after birth. By 24 hours, *Escherichia coli*, streptococci, and clostridia were found in high numbers (over 10^8 g^{-1}) in the faeces of piglets. After a further 24 hours, these bacteria were accompanied by similarly high numbers of lactobacilli (Smith and Crab, 1961). The problem is that this rapid bacterial colonisation can not be efficiently controlled by the piglet's immune system, as the immune system at this stage is very naive. In addition, the gastrointestinal tract is also in the process of developing, which means that pathogenic bacteria have easy access to the neonate body systems. This is the reason why the neonates have an increased susceptibility to many pathogens that results in high levels of morbidity and mortality on many pig units. Strict hygiene can reduce the bacterial challenge in the neonatal pig's environment. However, the sow remains a source of potentially pathogenic bacteria. Therefore, if the microflora that the sow introduces into the environment can be modified in a beneficial way this may also have a beneficial effect on colonisation of the piglet's gut.

The most well studied external factor in influencing the establishment of the intestinal microbiota is the diet. Work at the University of Plymouth and at Foulum in Denmark has shown that feed may be fermented successfully with lactic acid bacteria (LAB) and that this process reduces the number of salmonellas and coliforms in the feed (Brooks *et al.*, 1996; Geary *et al.*, 1996; Moran, 2001). Feeding such a diet to pigs does not appear to produce any significant effect on the number of LAB throughout the gut but it does dramatically reduce the number of coliforms in the lower small intestine, caecum and colon (Jensen and Mikkelsen, 1998; Hansen *et al.*, 2000; Moran, 2001). In addition, the presence of high numbers of LAB in the FLF can also contribute to the pig's health status. Recent studies strongly support the hypothesis that orally administered LAB stimulate the immune system both, at the local and systemic level (Perdigon *et al.*, 1990a; Maassen *et al.*, 1998; Erickson and Hubbard, 2000). This stimulation of the immune system can be reflected in higher levels of colostral immune factors. Therefore, we can postulate a beneficial sequence of events. Namely, that if lactating sows were fed FLF this could modify their gut microflora. This in turn would affect the composition of the microflora in their dung and possibly the production of immune factors in their milk. A changed ratio of LAB to coliforms in the sows' faeces might affect microbial colonisation of the piglet's gut and additionally, by ingesting hyperimmune colostrum, effective neonatal defence would be provided from the time of the first infective challenge. This combination of effective immunity and reduced level of environmental contamination with faecal pathogens is the basis of maintenance of good health for both sow and piglet.

Therefore, the aim of this study were to investigate

- whether feeding sows FLF modifies the faecal microflora at farrowing when a coliform bloom usually occurs
- whether any changes in faecal microflora affect bacterial colonization of the gut of the newborn piglet
- whether feeding FLF has a beneficial effect on the quality of colostrum produced by the sows

A preliminary experiment was performed in order to examine the potential of chlorine dioxide (ClO_2) (Sanitech; Alltech, Inc., Kentucky, USA) to suppress natural bacterial contamination of pig feed and hence allow the starter culture to become the predominant strain in the feed. Sanitization of feed before inoculation will not only prevent uncontrolled fermentation, but allow us to associate possible immunomodulatory effects of FLF with the dominant strain in the diet - *L. plantarum*. ClO_2 has recently received attention due to its potential advantages over chlorine based sanitizers (Berg *et al.*, 1986).

2.2. Material and Methods

2.2.1. Preliminary sanitization experiment with Chlorine dioxide (ClO_2)

An experiment was conducted using a two factor factorial design in which factor 1 was the concentration of chlorine dioxide (ClO_2) added at preparation to liquid diets (0, 100, 200, 300, 400, 500 ppm) and factor 2 was the time at which coliforms, yeasts and lactic acid bacteria (LAB) were enumerated (0, 3, 6, and 24 hours). Active chlorine dioxide was prepared by mixing 6.27 ml of ClO_2 concentrate and 2.5 g of citric acid (Sigma-Aldrich, UK) in a 1litre jar. The reaction was allowed to proceed for five minutes until the pH had dropped to approximately 2.6 and the activated solution turned yellow-green. The solution was then added to 500 ml of tap water and poured over the pig feed. A liquid feed was prepared from a first stage weaner pig diet (220 g protein, 79 g oil, 25 g fibre, 65 g ash and 150 mg Copper per kilogram) in a water to feed ratio of 2.5:1. Liquid feed was steeped at 30°C for 24 hours. Coliforms were enumerated on Violet Red Bile agar (VRBA) (Oxoid, England) using a serial dilution and double-layered pour plate technique. Plates were incubated aerobically at 37°C for 24 hours. Yeasts were counted on using Rose-Bengal Chloramphenicol agar (Oxoid, England), by aerobic incubation at 20°C for 3 days. LAB numbers were assessed using de Man-Rogosa-Sharpe agar (Oxoid, England) with the plates being incubated anaerobically at 37°C for 48 hours. The results were log transformed and analysed by general linear model – analysis of variance and comparison of means was performed by Tukey's post-hoc test.

The feeding experiment was conducted according to a randomised block design, with six replicates. A replicate consisted of 3 animals, each fed one of three dietary treatments from 14 days prefarrowing to 7 days post farrowing.

2.2.2. *Experimental animals*

Eighteen multiparous (ranges 2nd to 9th parity) sows (Large White x Landrace) and their piglets were used in the experiment. They were selected on the basis of expected farrowing date. Sows on different replicates were marked for identification with different coloured stock marker sprays. All piglets in the litter of each sow were tagged and weighed at birth, one week of age and 3 weeks of age. Neither sows nor piglets received any medication before or during the experiment.

2.2.3. *Dietary treatments*

Sows were randomly allocated to one of three dietary treatments, while the piglets only received maternal milk. Feed was given twice daily. The dietary treatments were:

- Dry pelleted feed (DPF)
- Non-fermented liquid feed (NFLF)
- Fermented liquid feed (FLF)

Preparation of feed

DPF - was supplied by BOCM Pauls Ltd, (diet specification in Table 2.1). The sows were given 3.5 kg of DPF day⁻¹.

NFLF - 3.5 kg of DPF was mixed with 7 kg of water, containing 300 ppm chlorine dioxide (ClO_2) (Sanitech 2%; Alltech Inc., Kentucky). Active chlorine dioxide was prepared by mixing 125.4 ml of ClO_2 concentrate and 12.5 g of citric acid in a 25-litre fermentation bin. The reaction was allowed to proceed for five minutes until the pH had dropped to approximately 2.6 and the activated solution turned yellow-green. 7 kg of water (30°C) was added to the fermentation bin and 3.5 kg of the feed added slowly and constantly mixed to ensure an homogenous product. The feed was then left to steep for 24 h at 30°C .

Table 2.1. Declared composition of the experimental diet fed as either a liquid meal or in a dry pelleted form.

Ingredient	Inclusion g kg^{-1} Dry matter
Oil	65
Protein	175
Fibre	50
Ash	65
Lysine	10
Moisture	138
Copper	25 mg kg^{-1}
Sodium selenite-selenium	0.40 mg kg^{-1}
Vitamin A –retinol	10 000 iu
Vitamin D3 –cholecalciferol	2 000 iu
Vitamin E. α -tocopherol	100 iu
Digestible energy (DE)	14 MJ Kg^{-1}

FLF - 3.5 kg of DPF was mixed with 7 kg of water, containing 300 ppm chlorine dioxide (Sanitech; Alltech Inc., Kentucky) and left to steep for 24h at 30°C . *Lactobacillus plantarum*^{rif} (rifampicin resistant), spontaneous mutant, was used as a starter culture. Antibiotic resistant isolates of *L. plantarum* strain (PC-81-11-02, Alltech, Kentucky) were

obtained by serial culturing on MRS medium (Oxoid Ltd., Basingstoke, England) containing increasing concentration of rifampicin (Sigma-Aldrich Ltd. Dorset, England) to a final concentration of $100 \mu\text{g ml}^{-1}$. Rifampicin ([3-(4-methylpiperazinyl-iminomethyl) rifamycin SV]) was prepared as a stock solution (10 mg ml^{-1}) in dimethyl sulfoxide (DMSO) and used at a final concentration of $100 \mu\text{g ml}^{-1}$. Stock *Lactobacillus* cultures were maintained at -80°C in 1 ml aliquots of MRS broth with 20% v/v glycerol. The strain was always sub-cultured twice in 10ml MRS broth (Oxoid Ltd., Basingstoke, England) at 30°C prior to being used to inoculate feed. After 24-hour sanitation with chlorine dioxide the feed was inoculated with liquid *Lactobacillus* starter culture to give a final concentration of between 6 and 7 \log_{10} CFU ml^{-1} liquid feed. The inoculated feed was fermented for 96 hours at 30°C .

2.2.4. Monitoring of feed

The dry matter of fermented and non-fermented feed was determined daily by oven drying at 103°C for 3-4 days (Method: ISO 6469/NEN 3332). The results were expressed as percentage dry matter.

Samples (~20 mls) of liquid feed were also removed daily from each batch for pH measurement, for microbial and chemical analyses. pH of liquid feed was measured using an electronic pH meter (W.G. Pye & Co. Ltd., Cambridge, UK).

Microbial analyses of the feed samples were conducted after sanitation of NFLF and FLF with ClO_2 and at the end of fermentation process. All selective media used were obtained from Oxoid, Basingstoke, UK. Representative samples were serially diluted 10-fold in

Maximum Recovery Diluent (MRD) (1 ml sample in 9 ml MRD). Relevant dilutions were plated out on selective media and plates were incubated at the recommended temperature. Coliforms were enumerated on VRBA agar using double-layered pour-plate technique and incubated aerobically for 24 hours at 37°C.

Lactic acid bacteria were enumerated on Rogosa agar and incubated anaerobically for 72 hours at 30°C.

Lactobacillus plantarum starter culture was selectively enumerated on Rogosa agar containing 100 µg ml⁻¹ rifampicin and incubated anaerobically for 72 hours at 30°C.

Yeasts were enumerated on Rose Bengal Chloramphenicol agar (RBCA) and incubated aerobically for 72 hours at 30°C.

2.2.5. Collection of faecal samples and culture from samples.

Fresh faecal samples (approximately 100 g) were collected manually from the rectum of each sow 7, 5 and 2 days before anticipated farrowing date, at parturition (Day 0) and 2, 5, and 7 days after parturition. Dry matter concentration of each faecal sample was determined by oven drying at 103°C for 3 days. Viable plate counts were performed immediately upon receipt of the samples. Faeces were suspended in sterile MRD as 10-fold dilutions (wt/vol) from which further 10-fold dilutions (vol/vol) were made. To evaluate LAB populations, relevant dilutions were plated out on Rogosa agar by double-layered pour-plate technique and plates were incubated anaerobically at 37°C for 48 hours. To evaluate coliform populations, relevant dilutions were plated out on VRBA agar by double-layered pour-plate technique. Plates were incubated at 37°C for 24 hours. To screen for the presence of *L. plantarum*^{rif}, appropriate dilutions were plated on Rogosa agar containing rifampicin (100 µg ml⁻¹). Plates were incubated anaerobically for 72 hours at

37°C. Random colonies, selected daily from antibiotic plates, were checked by light microscopy for cell morphology and Gram reaction to confirm recovery of *L. plantarum*.

2.2.6. *Quantification of Short-Chain Fatty acids (SCFA) and lactic acid*

SCFA analyses of feed and faecal samples were conducted by the HPLC method. Faecal samples were homogenized and diluted 1:10 with sterilized distilled water. After the addition of sulphuric acid (at final concentration of 0.1 g l⁻¹) as the internal standard, a proportion of the processed sample was centrifuged (10000 g for 15 min) and filtered (filter pore size 0.45 µm). HPLC separations were performed using a P580 HPG pump and GINA 50 autosampler (Dionex, Cheshire, UK) with an Animex HPX-87H (30 cm x 6.8 mm) cation exchange column (Bio-rad, Herts, UK) and refractive index (RI) detector. The column temperature was maintained at 55°C and the flow rate at 0.7 ml min⁻¹. Determinations were based upon retention time in relation to authentic reference compounds. All calibrations were produced from peak height determinations utilising integration software (Chromeleon, Dionex-Softtron GmbH, Gemering, Germany).

2.2.7. *Collection and processing of colostral samples*

Colostrum samples (10-15 mls) were collected on the day of parturition by manual milking. These samples were centrifuged at 17 000 g for 15 min to remove fat. The aqueous phase of whey samples was separated and stored at -20°C until used.

2.2.8. Mitogenic activity on intestinal epithelial cells (IEC-6)

Cell culture. The rat intestinal epithelial crypt cell line IEC-6 was obtained from the European Collection of Animal Cell Cultures (EAACC, Salisbury, UK). Cells (passage 36) were cultured at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine and 5% foetal calf serum (FCS) (Sigma-Aldrich, UK).

Mitogenic assay. IEC-6 cells were cultured in 24-well-plates (Corning Costar, High Wycombe, UK) at a density of 1×10^4 cells / well. Cells were allowed to adhere for 18 hours and were then washed twice in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, UK). Twenty-eight hours prior to colostrum stimulation the complete medium was replaced with DMEM without serum. The cells were then treated for 24 hours with 100 µl colostrum. Cell culture with added foetal calf serum (100 µl) was used as a positive control, and cell culture with no added stimulants represented the negative control. After 18 hours of culture, cells were labeled for the final 6 hours with ³H-thymidine (1 µCi/well). Plates were then washed with HBSS, fixed with methanol for 5 min at 4°C, then with 10% Trichloroacetic acid (TCA) for 30 min and solubilized with 0.4 N NaOH for 1 hour at 50°C. IEC-6 cells were transferred to scintillation vials and radioactivity counted by scintillation spectrometer (Packard BioScience).

2.2.9. Mitogenic activity on blood lymphocytes

Lymphocyte cells were isolated from pig blood by Histopaque®-1077 procedure (Sigma-Aldrich Ltd. Dorset, England, Procedure No.1077). The blood lymphocytes were diluted to

a density of 5×10^6 cells / ml in the culture medium, and 100 μ l volumes were dispensed into the wells of microtitre plates. Lymphocyte transformation was tested by the addition of 1 μ g / well and 10 μ g / well of Phytohemagglutinin (PHA) (non-specific stimulation of lymphocyte replication; positive control) in 200 μ l of culture medium. Some of the lymphocytes did not receive any stimulants except for 100 μ l of culture medium (unstimulated lymphocytes; negative control). After a 48 hours incubation period in a humidified, CO₂ atmosphere (5%) at 37°C, 0.5 μ Ci of tritiated thymidine was added to each well. The culture was incubated for further 16h. The cultured cells were then harvested and the radioactivity (counts per minute (CPM)) associated with the harvested cells was measured in a scintillation counter after the addition of liquid scintillation fluid.

2.2.10. Immunoglobulin analysis of colostrum samples

The concentrations of IgG and IgA were determined by the sandwich ELISA method using commercial ELISA Quantitation Kits (Bethyl laboratories, Inc., USA) and following manufacturer's instructions (Appendix 1).

2.2.11. Total protein contents of colostrum samples

Protein content in defatted colostrum samples was determined by Lowry protein assay (Lowry *et al.*, 1951), using bovine serum albumin (BSA) (Sigma Aldrich, UK) as the standard. The following reagents were used in this procedure:

Lowry Reagent A:

40 g Sodium carbonate (anhydrous), 8 g Sodium hydroxide, 0.4 g Sodium potassium tartrate dissolved in 1L distilled water

Lowry Reagent B:

1.28 g Copper (II) sulphate-5-hydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) dissolved in 100 ml distilled water

Folin-Ciocalteu's Phenol Reagent was prepared by its dilution with distilled water (1 part of the Folin-Ciocalteu reagent (Sigma-Aldrich, Dorset UK) and 14 parts distilled water).

Bovine Serum Albumin (BSA) standard

200 mg of BSA were dissolved in 100 ml 0.1 M sodium hydroxide.

Sodium hydroxide (NaOH)

4 g NaOH pellets dissolved in 1L distilled water.

BSA standard was diluted with sodium hydroxide to reach the final concentrations 0, 10, 20, 30, 40, 60, 80, and 100 $\mu\text{g BSA ml}^{-1}$. 50 μl of each standard dilution were then pipetted in duplicate into microtiter plate wells (Imunolon-1, Dynatech Laboratory Inc., Virginia, USA) (A1, A2- H1, H2). Colostral samples were also serially diluted and 50 μl of each sample dilution was pipetted in duplicate into plate wells. Then 100 μl of the Copper reagent was added to each well. The Copper reagent was prepared by mixing 50 volumes of Reagent A with 1 volume of reagent B. After the samples had been left for 10-15 min at room temperature 100 μl of Folin-Ciocalteu reagent was added to each well and left to stand at room temperature for a further 15-30 min. Finally, the absorbance was measured with a well scanner reader (Dynatech, MR5000) at 630 nm.

2.2.12. Statistical analyses

All the original dilution and colony count data were recorded and calculated. The bacterial count per gram of faeces was further log transformed, tabulated, and statistically analysed

by ANOVA. Arcsine transformation was used for all data presented in percentages. Mitogenic experiments were carried out in triplicate determinations and repeated at least twice. Significant differences between treatment means were compared by Tukey's HSD test (Zar, 1999). The statistical analyses were undertaken using Minitab v. 10.2 (Minitab Inc., Pennsylvania, USA, 1994).

2.2. Results

Preliminary sanitization experiment with Chlorine dioxide (ClO₂)

The inactivation of coliforms, LAB and Yeasts in liquid feed with different concentrations of chlorine dioxide at Time 0 and 24 hours is shown in Table 2.2. No coliforms, LAB or Yeasts were detected ($< 3.0 \log_{10} \text{ CFU ml}^{-1}$) in the liquid feed containing 300 ppm ClO₂ after 24 hours steeping. This is a dramatic improvement compared to the control where approx. $8.0 \log_{10} \text{ CFU ml}^{-1}$ coliforms and LAB were present after 24 hours steeping. The interaction between chlorine dioxide concentration and length of steeping time was highly significant ($P < 0.001$).

Table 2.2. Effect of chlorine dioxide concentration and time on the sanitation of liquid feed.

		Chlorine dioxide concentration (ppm)				<i>P</i>		
	Time (h)	0	100	200	300	s.e.d.	Time	ClO ₂
Coliforms	0	3.2 ^a	3.2 ^a	2.7 ^a	<2.0 ^b	0.12	< 0.001	< 0.001
	24	8.2 ^a	6.3 ^b	4.0 ^c	<2.0 ^d			
LAB	0	4.1 ^a	<3.0 ^b	<3.0 ^b	<3.0 ^b	0.06	< 0.001	< 0.001
	24	8.4 ^a	5.5 ^b	3.8 ^c	<3.0 ^d			
Yeasts	0	4.9 ^a	4.7 ^a	4.6 ^{ab}	4.0 ^b	0.05	< 0.001	< 0.001
	24	6.5 ^a	3.9 ^b	3.1 ^c	<3.0 ^d			

^{a,b,c,d} Within rows, means with a common superscript are not statistically different ($P > 0.05$)

Microbiology and analyses of the feed

While sanitation of liquid feed with chlorine dioxide eliminated coliforms successfully, it did not always eliminate LAB and yeasts. It depended on how strictly the aseptic techniques, usually during mixing of feed, were maintained. But most often, there were approximately 10^3 to 10^4 CFU ml⁻¹ LAB and yeasts still remaining after 24-hour steeping. The pH of the liquid feed after the sanitation process was around 5.2. After 72-hour fermentation at 30°C, the pH of the feed dropped to 3.9 ± 0.2 and remained around this value for the rest of the experiment. Microbial analyses of fermented feed showed the presence of high numbers of LAB (around 10^9 CFU ml⁻¹) (Figure 2.1) while coliform level was below the detectable limit in each tested feed sample. The survival of *L. plantarum*^{rif} in relation to total LAB counts is shown in Figure 2.1. These results confirmed *L. plantarum*^{rif} as the predominant species (89% - 94%) in all replicates. It was found that it was necessary to prepare the starter culture from stock *Lactobacillus* culture because with further subculturing the resistance to rifampicin decreased rapidly. This was reflected in higher variations of *L. plantarum*^{rif} counts in the first two replicates (Figure 2.2). Preparing the starter culture from stock *Lactobacillus* culture successfully overcame this problem in replicates III - VI (Figure 2.3).

Figure 2.1. Survival of *L. plantarum* starter culture in relation to total LAB. Data are expressed as a mean \pm SD.

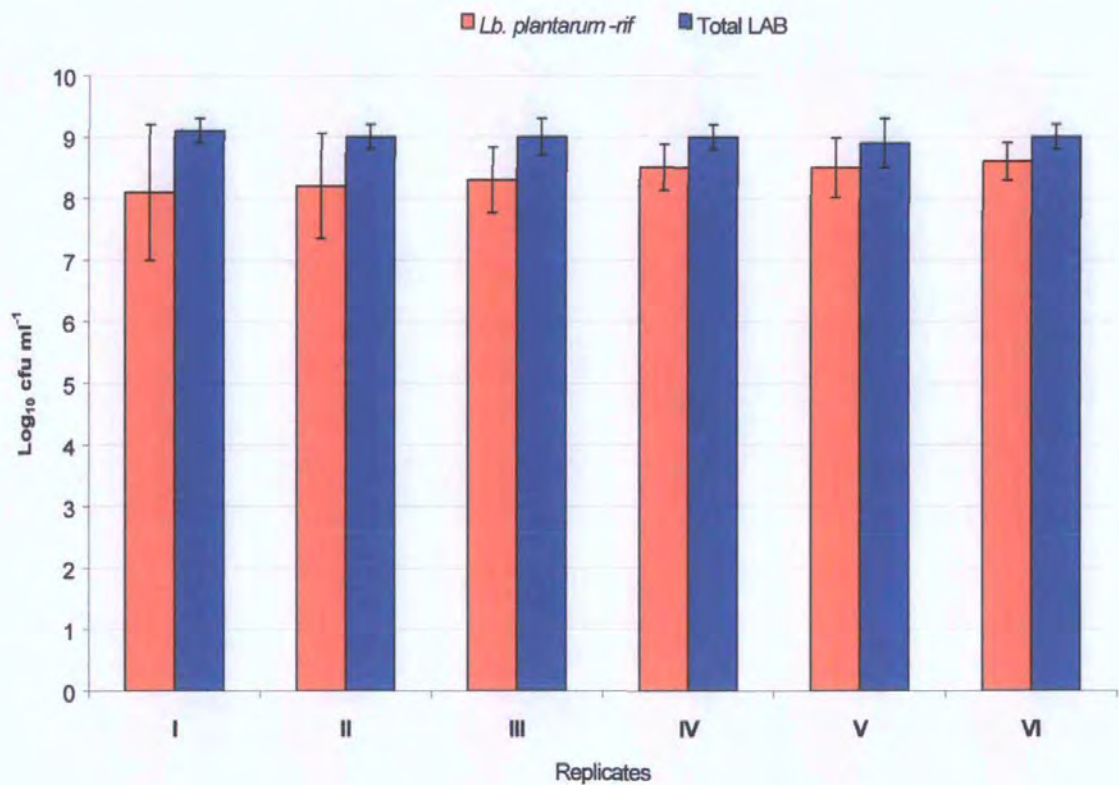


Figure 2.2. *L. plantarum*^{rif} in FLF in the I and II replicate.

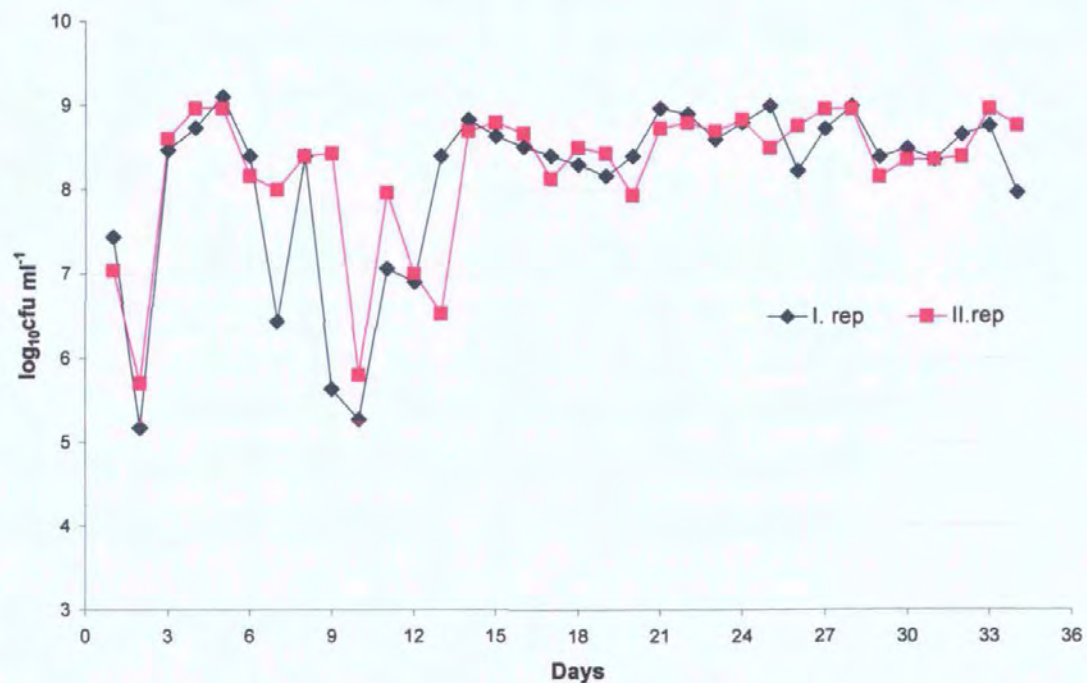
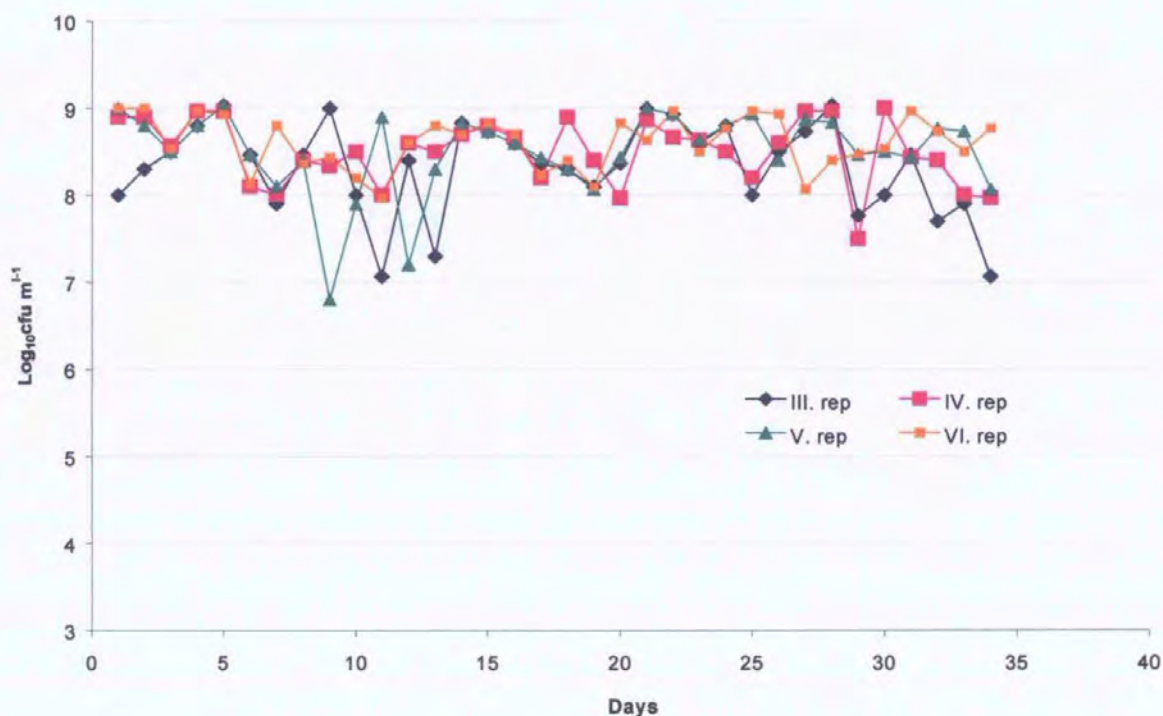
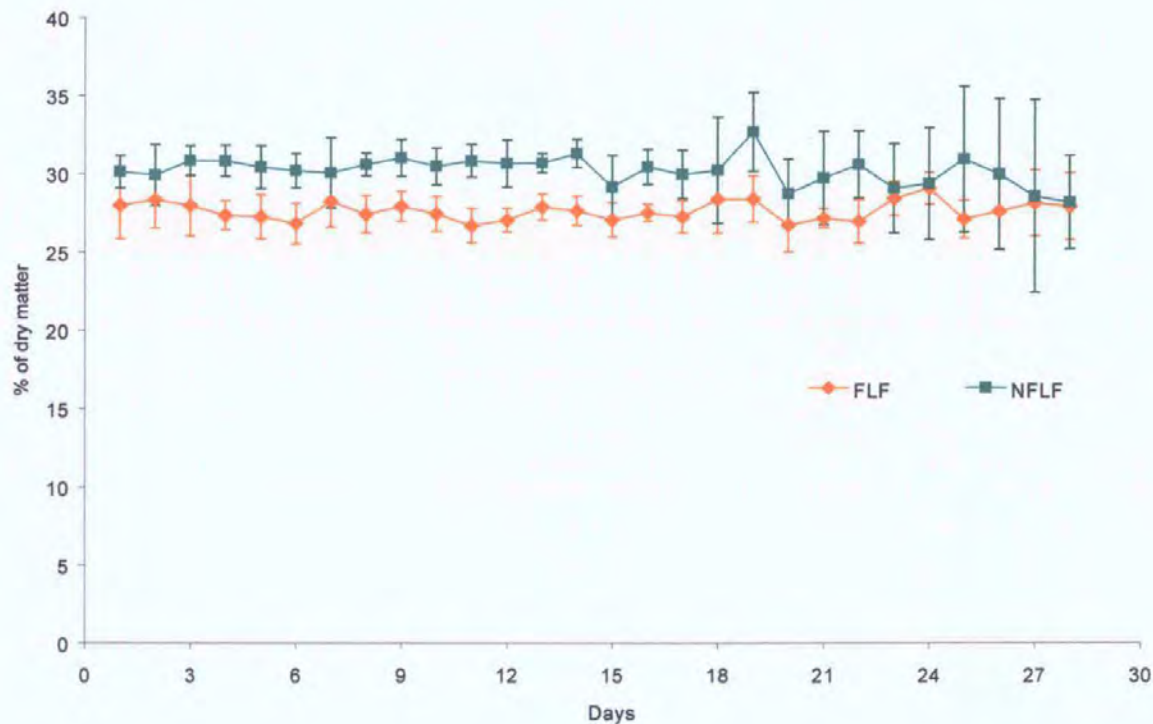


Figure 2.3. *L. plantarum*^{rif} in FLF in the III, IV, V, and VI replicate.

The dry matter (DM) concentrations of the FLF and NFLF were stable over the 28-day period with no significant differences between replicates (Figure 2.4). The average DM concentration for FLF was 27.8 ± 2.2 %, which was significantly lower ($P < 0.001$) than the average DM concentration of NFLF (29.6 ± 3.1 %). That means that approximately 6.1% of the DM was converted to volatile components (e.g. SCFA) and possibly some level of CO₂, or ethanol due to not complete elimination of yeasts and/or heterofermentative LAB by sanitation with ClO₂ under farm condition.

Figure 2.4. Dry matter (%) of FLF and NFLF during 4 weeks. Data are expressed as a mean \pm SD.

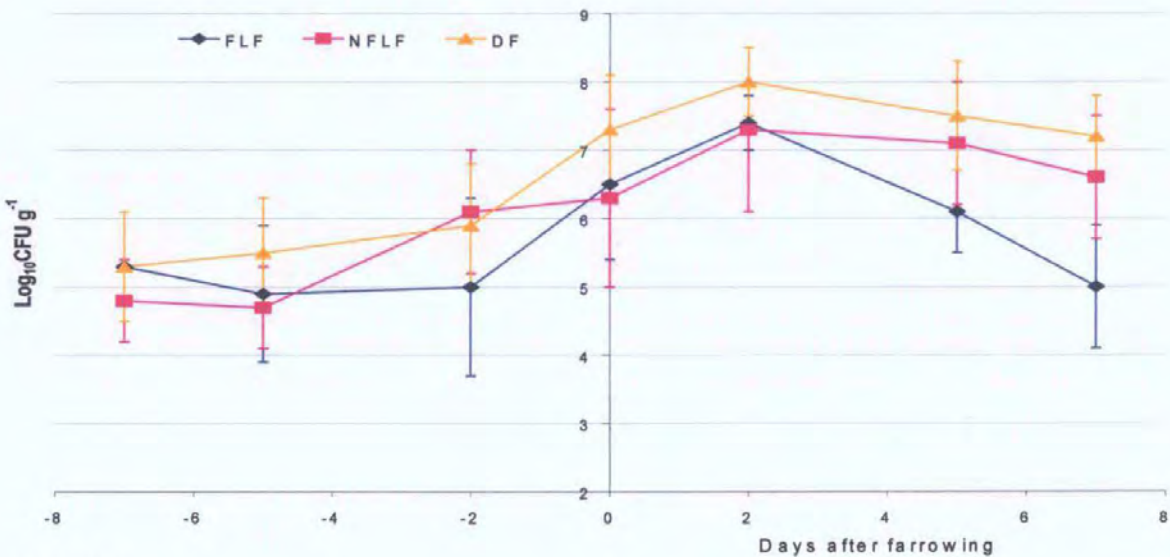


Microbiology of the sows' and piglets' faeces

Numbers of faecal LAB and coliforms in the faeces of sows fed FLF, NFLF, and DF are shown in Figure 2.5. While the LAB population was not significantly affected by dietary treatments, the most significant differences in coliform population were observed in the sows' faecal samples taken 7 days after parturition.

Figure 2.5. Faecal counts of coliforms (A) and LAB (B) in the sows fed different diet (FLF, NFLF and DF) for the period of 1 week before farrowing till 1 week after parturition.

A) Coliforms



B) LAB

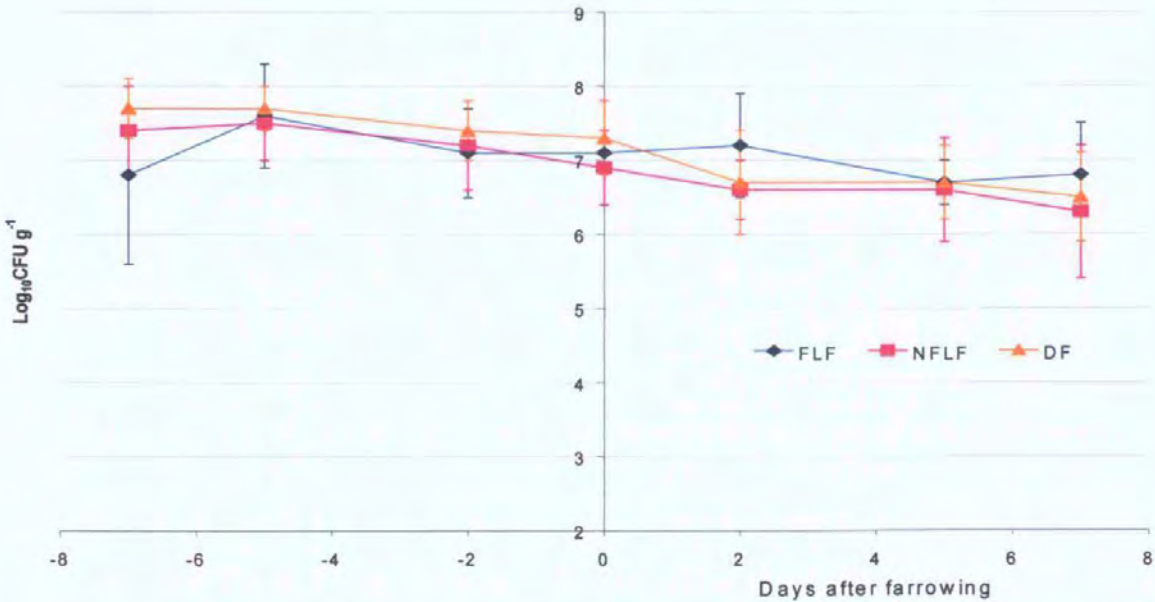
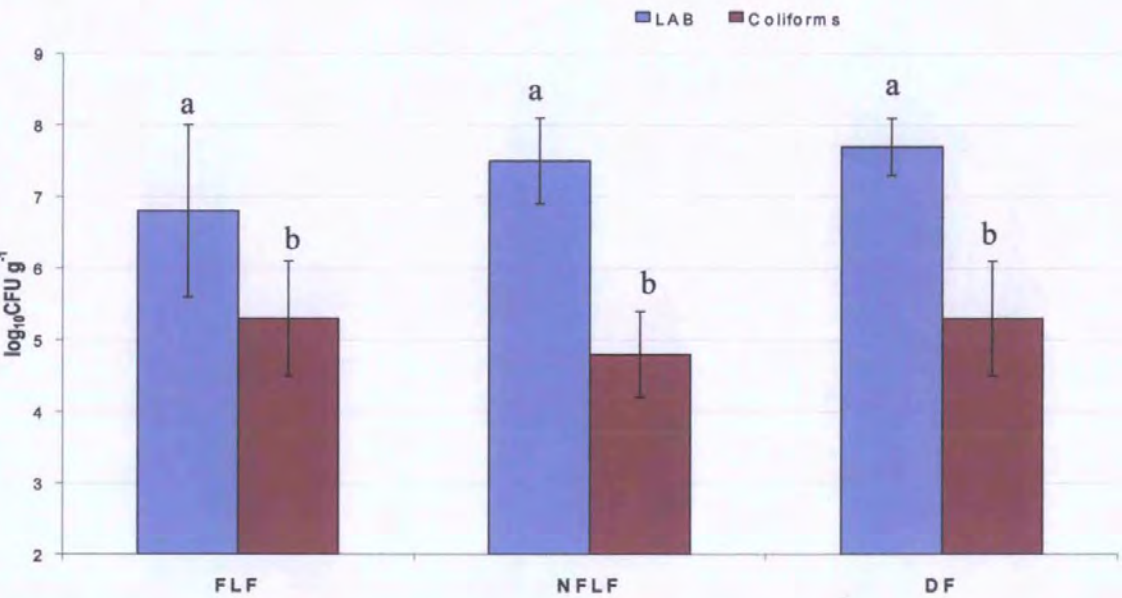
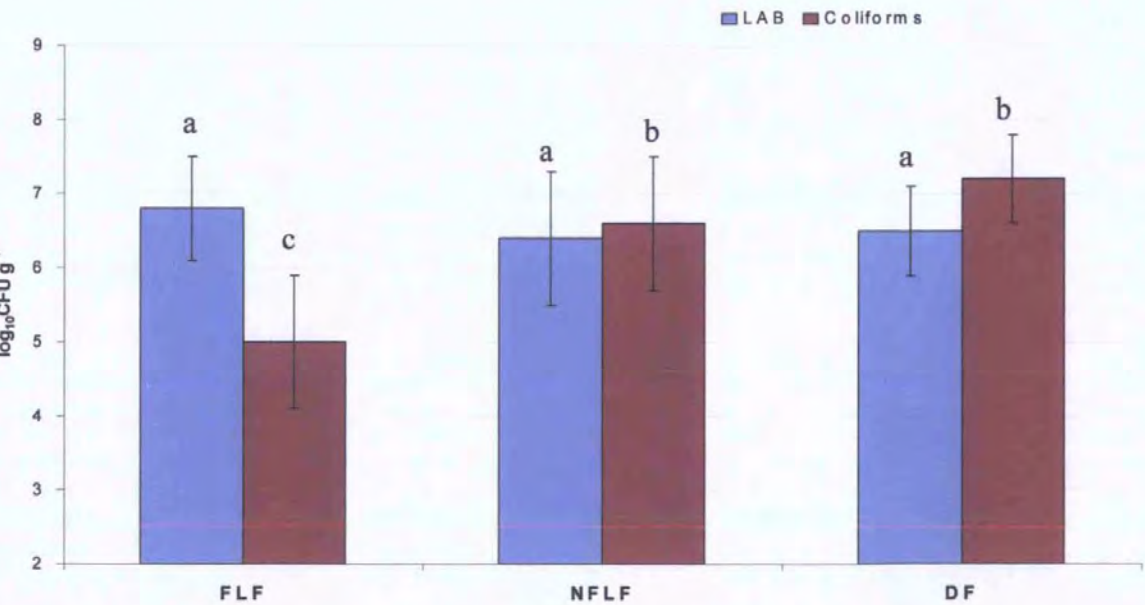


Figure 2.6. LAB and coliforms numbers in the sow's faeces 7 days before (A) and 7 days after (B) parturition.

A) 7 days before



B) 7 days after



a,b,c,d Means with the same superscript are not significantly different. Data are expressed as mean ± SD.

Faeces excreted from FLF fed sows had significantly ($P < 0.001$) lower numbers of coliforms compared with NFLF and DF fed sows (Figure 2.6). Although none of the dietary treatments were able to prevent a high coliform bloom (10^7 - 10^8 CFU g^{-1}) around farrowing time (Figure 2.5A), FLF reduced this rapid coliform increase more rapidly than the other two treatments. By the 5th day after farrowing there was already a significant ($P < 0.05$) decrease in coliform excretion by FLF-fed sows compare to DF-fed sows (Figure 2.5A). Two days later the size of the coliform population in FLF faeces dropped to approximately 10^5 CFU g^{-1} , which was significantly lower ($P < 0.001$) than the coliform numbers in the faeces of both NFLF and DF-fed sows (Figure 2.6). The large increase of coliforms around farrowing was usually accompanied by slight decrease of LAB population (Figure 2.5B).

Consequently, piglets from FLF-fed mothers excreted faeces that were significantly higher ($P < 0.01$) in LAB and significantly lower ($P < 0.001$) in coliforms than faeces from the piglets of DF-fed dams (Table 2.3). No significant differences were observed between FLF and NFLF piglets. These results were reflected in more favourable faecal LAB: Coliform ratio in the piglets belonged to the mothers fed on liquid diets (Figure 2.7).

Table 2.3. Lactic acid bacteria (LAB) and coliforms (\log_{10} CFU g^{-1}) in the faeces of 7 day old piglets born to sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF).

	FLF	NFLF	DF	SE _D	P
LAB	7.70 ^a	7.65 ^a	7.30 ^b	0.145	**
Coliforms	7.52 ^d	7.80 ^{cd}	8.07 ^c	0.139	***

^{a,b,c,d} within rows, means with different superscript are significantly different; ** $P < 0.01$; *** $P < 0.001$

Figure 2.7. LAB: Coliform ratio (log transformed numbers) in the faeces of 7 days old piglets as determined by dietary treatment.

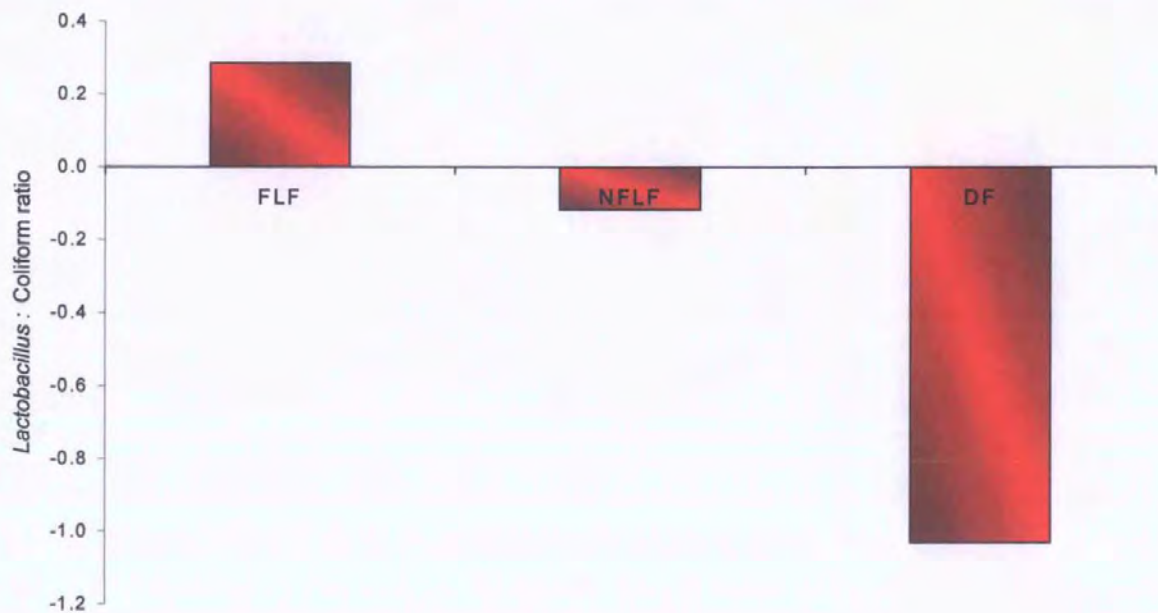
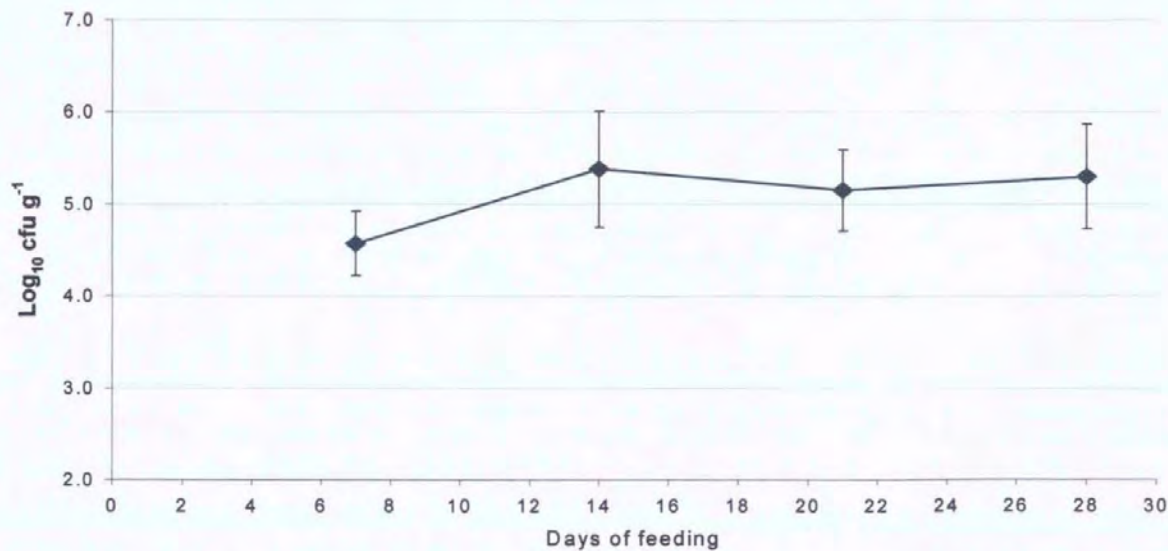


Figure 2.8. *L. plantarum*^{rif} in the sows' faeces after 7, 14, 21, and 28 days of feeding.



Data are expressed as a mean ± SD.

Antibiotic plate counts from sows' and piglets' faecal samples confirmed that the antibiotic-resistant *L. plantarum* strain, used to ferment the feed, survived passage through the intestinal tract (Figure 2.8). No rifampicin-resistant colonies were detected in any group of sows prior to introduction of liquid feed fermented with rifampicin resistant *L. plantarum*. After 7 days of feeding, the *Lactobacillus* strain maintained a population of about 3.7×10^4 CFU g⁻¹ of faeces. During a further 7 days the population of the introduced strain increased significantly ($P < 0.05$) up to 2.5×10^5 CFU g⁻¹ and fluctuated about this value for the rest of the 2-week testing period. The presence of the test strain was confirmed also in piglets' faeces on the 7th day of suckling but the counts constituted only a very small fraction of the total LAB, usually below 1%.

Dry matter and SCFA concentration of the sows' faeces

The dry matter of the sows' faeces was not significantly affected by any dietary treatment during the period of 2 weeks before farrowing. Significant increase ($P < 0.01$) of the dry matter concentration was observed only in the faeces of DF fed sows at 1 week after farrowing (Table 2.4).

The mean values of SCFA concentrations in the sow faeces are presented in Table 2.5. Significant increases in the total faecal concentration of acetic acid ($P < 0.05$) and propionic acid ($P < 0.05$) were recorded in the FLF group after 3 weeks of intake compare to NFLF and DF fed sows. There was also a significant increase ($P < 0.05$) in butyric acid in FLF-fed sows after 3 weeks of feeding. No significant changes in the SCFA acid concentration were observed in the DF group of sows. In all experimental groups, acetate was the predominant SCFA, propionate was the second most predominant acid and butyrate was

usually in the third position in terms of concentration. Neither lactic nor valeric acids were analysed in any experimental group.

Table 2.4. Dry matter content (g kg^{-1}) of faeces of sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF).

Days	FLF	NFLF	DF	P
14 before farrowing	28.33 \pm 1.97	30.67 \pm 7.73	30.54 \pm 7.35	ns
7 before farrowing	29.55 \pm 5.64	25.28 \pm 3.08	28.35 \pm 4.51	ns
7 after farrowing	31.02 \pm 3.61 ^b	30.38 \pm 3.87 ^b	38.03 \pm 3.98 ^a	**

Data are expressed as a mean \pm SD; ^{a,b} within the rows, means with the same superscript are not significantly different.

Table 2.5. Short chain fatty acid (SCFA) concentration (mmol g^{-1}) of the sows' faeces at day one and after 3 weeks of feeding.

SCFA		FLF	NFLF	DF	P
Acetate	Day 1	77.2 ²	38.8 ²	54.8 ¹	ns
	Day 21	137.2 ^{a,1}	69.1 ^{b,1}	64.7 ^{b,1}	*
Propionate	Day 1	37.5 ²	22.9 ²	33.8 ¹	ns
	Day 21	64.6 ^{a,1}	35.2 ^{b,1}	46.1 ^{a,b,1}	*
Butyrate	Day 1	0.8 ^{b,2}	4.0 ^{a,b,1}	4.4 ^{a,1}	*
	Day 21	5.16 ¹	8.0 ¹	5.1 ¹	ns

ns = not significant * $P < 0.05$

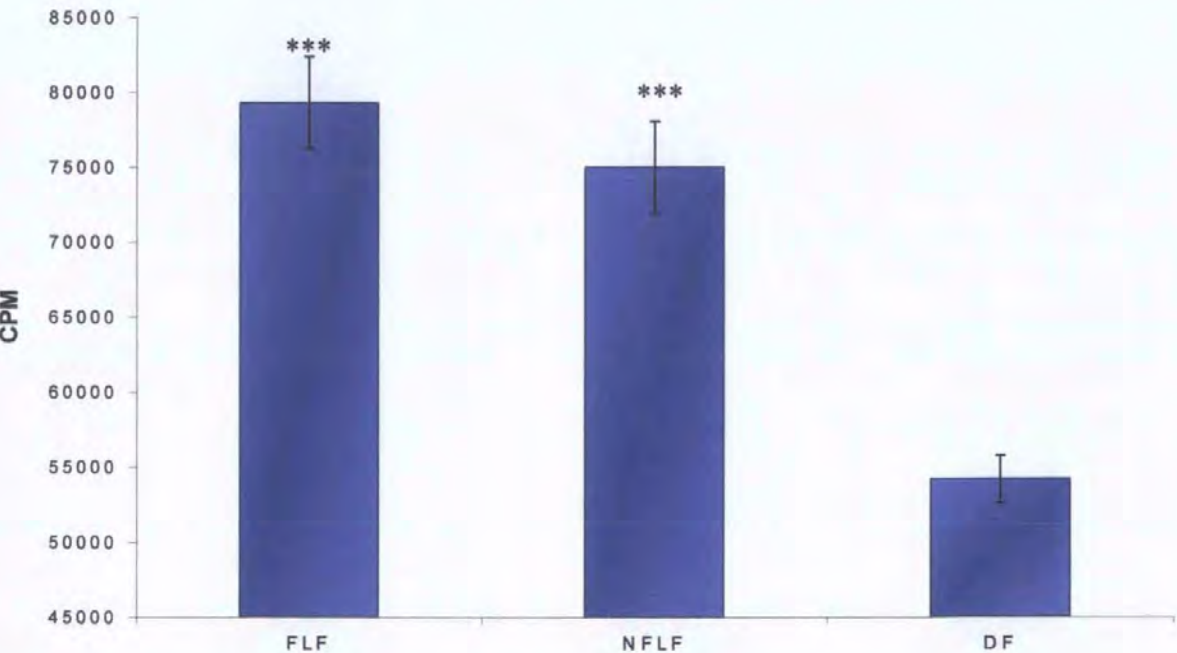
^{a,b} within rows, means with the same superscript are not significantly different

^{1,2} within columns, means with the same superscript are not significantly different

Mitogenic activity on IEC-6 and blood lymphocytes

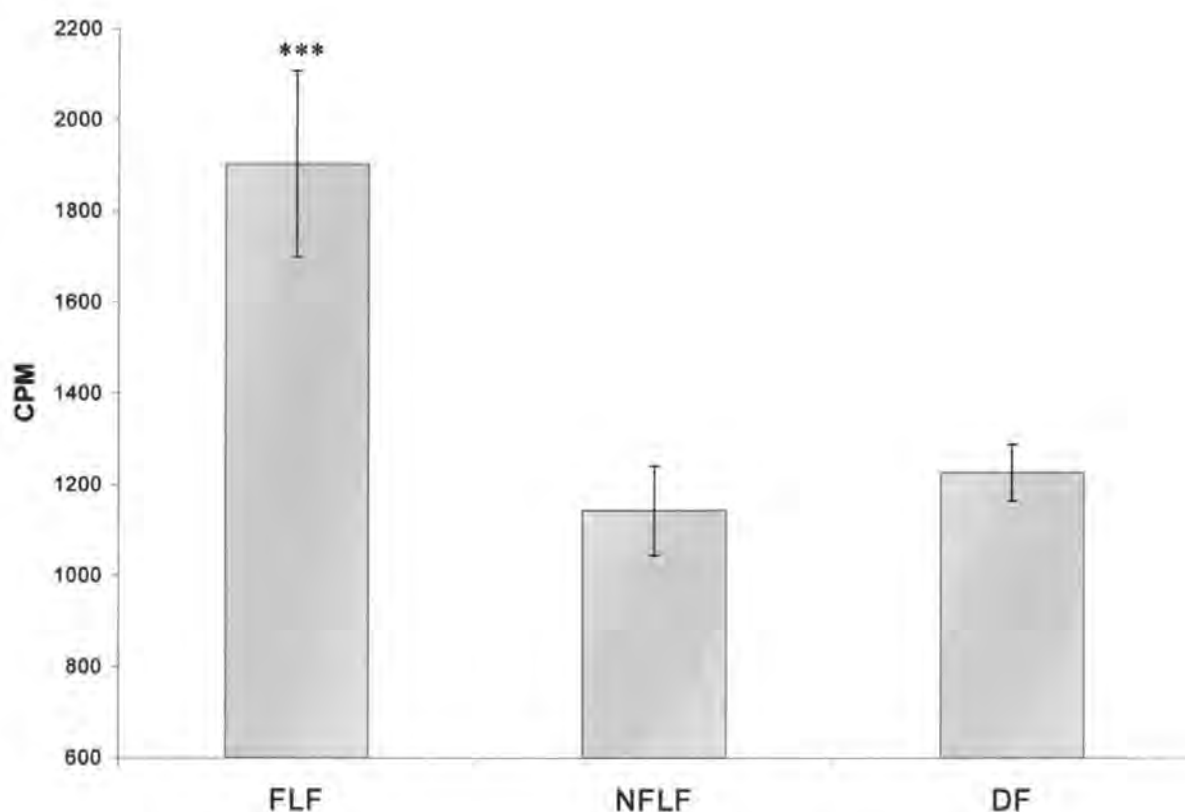
The mitogenic response (uptake of tritiated thimidine measured as counts per minute of harvested cells) of intestinal epithelial cells (IEC-6), exposed to the colostrum samples taken from the sows on different diets, is presented in Figure 2.9. Although all colostrum samples stimulated proliferation of IEC-6 compare to the control, colostrum from liquid feed fed sows had a significantly greater ($P<0.001$) mitogenic activity than colostrum from DF-fed sows. Lymphocyte mitogenic activity only confirmed significantly ($P<0.001$) higher stimulation potential of colostrum produced by FLF-fed sows (Figure 2.10). In this case, there were no significance differences between the NFLF and DF colostrum samples.

Figure 2.9. Mitogenic activity of sow colostrum on IEC-6.



Data are expressed as a mean counts per minute (CPM) \pm SEM; *** $p<0.001$

Figure 2.10. Mitogenic activity of sow colostrum on blood lymphocytes.



Data are expressed as a mean counts per minute (CPM) \pm SEM; *** $p < 0.001$

Protein and Immunoglobulin contents of colostrum samples

The mean concentrations of the total protein as well as the immunoglobulins A and G in the colostrum of sows fed different diet are given in Table 2.6. A large variation in the amounts of both immunoglobulins and proteins was observed between colostrum samples obtained from sows of different parity. There was a tendency for both IgA and IgG concentrations to gradually increase with increasing lactation number. Although such differences were not statistically significant because of the large SD (Table 2.6), the trend suggested that despite lower parity of sows, colostrum of sows fed FLF had a higher level

of both immunoglobulins and total proteins than colostrum of sows fed DF. It is also noteworthy to point out quite large differences in colostral Ig concentrations between NFLF and DF-fed sows, as the parity of these groups was almost the same, but the level of the immunoglobulins tended to be considerable higher in NFLF group.

Table 2.6. Concentration of proteins (mg ml^{-1}) and immunoglobulins A and G ($\mu\text{g mg}^{-1}$ of total protein) in colostrum of sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF), and dry feed (DF).

	Parity	Proteins	IgG	IgA
FLF	2,2,3,3,4,8	325.9 ± 42.6	169.3 ± 53.8	34.4 ± 11.8
NFLF	2,5,5,6,8,9	273.8 ± 74.7	271.9 ± 138.4	59.5 ± 47.6
DF	2,5,5,6,7,9	297.3 ± 88.8	156.4 ± 20.2	20.0 ± 6.8

Data are expressed as a mean \pm SD; to evaluate statistical differences between Ig(s) the sow parity was used as a covariate.

2.3. Discussion

Preliminary sanitization experiment with Chlorine dioxide (ClO₂)

Chlorine dioxide is a strong, oxidising and sanitising agent with a broad antimicrobial and antiviral spectrum, which has recently received attention due to its potential advantages over chlorine based sanitizers (Foschino *et al.*, 1998). It is a synthetic yellowish-green gas with chlorine odor. In contrast to chlorine, ClO₂ does not hydrolyze in water to any appreciable extent but remains in solution as a dissolved gas (Aieta and Berg, 1986). Its major advantages over chlorine based sanitizers include reduced reactivity with organic matter and greater activity at neutral pH (Fukayama *et al.*, 1986). In addition, ClO₂ also removes tastes and odors more efficiently than chlorine (Fiessinger *et al.*, 1981). The one disadvantage of ClO₂ is that it cannot be compressed or stored commercially as a gas because it is explosive under pressure and for that reason it is never shipped but must be created at the point of use. Solution of sodium chlorite represent most common precursor chemical for generation of ClO₂ and one way to activate the reaction is by acidification with organic or inorganic acid (Fukayama *et al.*, 1986). It has been shown that microbial susceptibility to ClO₂ differs with bacterial strain and environmental conditions of applications (Berg *et al.*, 1982; Foegeding *et al.*, 1986; Berg *et al.*, 1988; Wei *et al.*, 1995; Winniczuk and Parish, 1997; Foschino *et al.*, 1998; Han *et al.*, 2000; Wisniewsky *et al.*, 2000; Han *et al.*, 2001; Du *et al.*, 2002). In the current study, the inclusion of 300 ppm ClO₂ into liquid feed immediately reduced the coliforms to below detectable levels and produced a feed that remained free of coliforms for at least 24 hours. In addition, the same concentration (300 ppm) of ClO₂ eliminated naturally occurring LAB and yeasts. This is particularly beneficial in order to avoid problems with uncontrolled fermentations as yeasts

and heterofermentative LAB can either produce excessive alcohol or make the diet unpalatable. This can lead to behavioural problems such as aggression and the subsequent development of vices. In addition, (Moran, 2001) showed that sanitation of liquid feed with ClO_2 did not affect the palatability of the diet, or intake of feed by the weaned piglets.

Studies in rats revealed that ClO_2 is converted to chloride (Cl^-), chlorites (ClO_2^-) and chlorates (ClO_3^-), which are eliminated from the body more rapidly than chlorine, and they do not appear to increase trihalomethane concentrations (Abdel-Rahman *et al.*, 1982). Another rat study showed that neither clinical signs of toxicity nor adverse effects on any reproductive parameter examined were observed in the rats exposed to ClO_2 during gestation and lactation periods (Carlton *et al.*, 1991). Litter size, pup viability, and pup weight were unaltered by chlorine dioxide exposure.

In the US the acidified sodium chlorite has been approved for use on certain meats, seafood, poultry, and raw fruits and vegetables as either a spray or dip in the range of 500-1200 ppm (CFR, 2000). Applications of 500 ppm acidified ClO_2 significantly reduced population of *E. coli* O157:H7 on germinated alfalfa seeds (Taormina and Beuchat, 1999). However, there is still little information concerning the mode of action by which ClO_2 inactivates bacteria and viruses. A mechanism by which chlorine dioxide kills *Escherichia coli* cells was reported by Berg *et al.*, (1986). The loss of permeability control of the outer bacterial membrane was shown to be the primary lethal event at the physiological level.

Therefore, based on these results, 300 ppm ClO_2 was chosen to be the concentration used for inclusion into liquid feed preparations in the following feeding trial. This concentration

should be sufficient enough to allow the FLF inoculant (*L. plantarum*) to become the predominant strain in the feed system and thus ensure controlled fermentation.

Microbiology and analyses of the feed

By using chlorine dioxide in a concentration 300 ppm, a successful elimination of coliforms below detectable limit was obtained in the feed after 24 hours of steeping in each feed sample during the whole experimental period. This confirmed our previous study performed in laboratory conditions. However, under farm conditions it is impossible to maintain a sterile environment, which explains the low numbers of LAB and yeasts still remaining in the majority of feed samples tested. This could possibly have happened during opening the lids of fermentation batches in order to mix the feed and during mixing. In general, sanitation was quite successful and allowed the starter culture *L. plantarum*^{nif} to become a predominant LAB strain in each replicate.

The loss of dry matter (6%) in FLF in the current study was less than in the study of Moran (2001), (12%) but higher than in the studies of Geary (1997) and Jensen and Mikkelsen (1998) (3% DM). However, because the total organic acids and ethanol were not quantified in the FLF their loss during the drying process could not be estimated. Therefore, no corrections were made in the dry matter calculations for the volatile components of the FLF diet during this trial. According to CVB (1999), 8% of lactic acid, 50% of acetic acid and 100% of ethanol is lost due to volatilisation during the procedure to determine dry matter content.

Microbiology of the sows' and piglets' faeces

Gastrointestinal infections associated with *E. coli* represent a serious problem for neonatal pigs. These bacteria are present in the sow's intestine in large numbers but increase dramatically just prior to farrowing due to stress occasioned by movement and parturition (Arbuckle, 1968; Maclean and Thomas, 1974). Therefore, the newborn piglet is, at this time, exposed to a greater risk of infection. The stability of the GI microbiota or successional changes, in bacterial populations are influenced by numerous factors such as intestinal pH, microbial interactions, physiological factors, peristalsis, host secretions, immune responses, drug therapy, and bacterial mucosal receptors (Mackie *et al.*, 1999). Therefore, it is logical that the microorganisms entering newborns are more likely to colonise than are those entering healthy adults with stable and complex microbiota and fully functional immune and GI systems (Mackie *et al.*, 1999).

Diet represents one of the most studied external factors that influences the establishment or beneficial changes of the intestinal microbiota. Antimicrobial effects of FLF, in terms of elimination of coliforms bacteria from the gut, have been reported (Jensen and Mikkelsen, 1998; Hansen *et al.*, 2000; Moran, 2001). Despite this fact, the rapid coliform bloom around farrowing was not affected by feeding FLF in this experiment. However, according to results presented, FLF had the potential to eliminate high faecal coliform numbers at farrowing time more rapidly than NFLF or DF. Multiple factors may account for this beneficial effect of FLF, which may act independently or synergistically. The low pH of the diet, the high numbers of LAB and high concentration of lactic acid represent the most important characteristics of FLF in terms of its protective effect. The ability of LAB to inhibit the growth of various Gram-negative bacteria, especially pathogenic *E. coli*, is well

documented for both *in vitro* (Hillman *et al.*, 1995; Jin *et al.*, 2000) and *in vivo* conditions. The experiments *in vivo* were performed with different animal species such as pigs (Muralidhara *et al.*, 1977), mice (Perdigon *et al.*, 1990a; Perdigon *et al.*, 1990b), and poultry (Rada, 1995). Establishment of low pH due to production of lactic acid and short chain fatty acids (SCFA) plays very important role in the elimination of many pathogens, which are not able to tolerate low pH conditions (Muralidhara *et al.*, 1977; Jin *et al.*, 2000). FLF-fed sows were exposed to a large intake of lactic acid (288 mM) and acetic acid (34.4 mM). In addition, significant increases in the concentration of acetic, propionic, and butyric acids were obtained in the faeces of the FLF-fed sows, indicating increased fermentation in the colon. SCFA's represent the product of the fermentation of soluble carbohydrates and other nutrients by intestinal microbiota. The antimicrobial effect of lactic acid and SCFA is not determined by their quantity but depends on their degree of dissociation (Thompson and Hinton, 1996). Undissociated forms of organic acids are 10-600 times stronger in bactericidal effect than that of their dissociated forms (Helander *et al.*, 1997). The extent of dissociation is directly determined by pH of the environment. At pH's above 7 the SCFA are primarily in the dissociated form and unable to inhibit the growth of enteric bacteria. With lower pH the higher proportion of undissociated forms of the acids can penetrate the microbial cell and interfere with essential metabolic functions, resulting in cell death (Rolfe, 1997). A confirmation of this statement may be the work of Mathew (1993) who compared the numbers of total *E. coli* in the terminal ileum of two groups of pigs fed different diets. While the concentrations of SCFA were not different between the groups, lower ileal pH in pigs fed diet containing 1% galactan resulted in lower numbers of the total *E. coli*. Prohászka (1982) reported that pig's colon contains approximately 120 to 140 mmol kg⁻¹ of SCFA. In addition, they performed few studies on *Salmonella* and *E. coli* shedding by pigs fed dry feed (base on maize, barley, soybean

meal) and wet feed (maize silage diluted with water) (Prohászka and Baron, 1982; Prohászka and Lukács, 1984; Prohászka *et al.*, 1990). In these studies the pH-dependent antibacterial effect of colonic SCFA was demonstrated. The quantity and composition of SCFA were similar in both groups, but there were considerable differences in pH values of the large intestinal contents, which were reflected in excretion of lower numbers of *Salmonella* and *E.coli* by the sows fed wet diet. This effect was attributed to the different base values of the diets. The base value expresses *the bicarbonate load resulting from the consumption of 1 kg of feed* (Prohászka and Lukács, 1984). Bicarbonate is product of gastric parietal cells and its amount is equivalent to the quantity of HCl needed for gastric digestion. Diets of high base value produce a higher bicarbonate overload, which is subsequently compensated not only by renal function, but also by secretion of bicarbonate in the colon. The base value of dry feed was about three times higher than that of wet feed, indicating a higher bicarbonate overload and therefore higher pH (6.7-7.3) of the intestinal contents of pigs fed dry feed compare to pigs fed wet diet (pH=6). This could be one of the possible reasons for lower numbers coliforms in the faeces of pigs fed FLF in this experiment. A study by Canibe and Jensen (2000) showed significantly lower pH in the stomach of growing pigs fed FLF compare with pigs fed NFLF and DF. In contrast, Moran (2001) reported no significant effect of FLF on the pH of the pig lower GI tract. But taking in to account the different diet (weaning diet vs sow diet) and considerable lower amount of ingested lactic acid in Moran's experiment, further work is needed before possibility that FLF has no influence on the pH of the lower GI tract can be rejected.

It is important to realise that all SCFA's are rapidly absorbed from the hindgut, leaving only 5-10% in the faeces (Berggren *et al.*, 1993). This indicates that faecal SCFA's do not reflect the overall changes in SCFA production in the colon. However, for practical

reasons, faecal excretion of SCFAs is considered to reflect to some extent SCFA production in the proximal colon (Johansson *et al.*, 1998). Different acids, produced by intestinal bacteria, can be detected in the faeces, with acetic acid the predominant organic acid (Cummings *et al.*, 1979; McNeil, 1982; Johansson *et al.*, 1998). The predominance of acetic acid was confirmed also in this study with the highest concentration in the faeces of FLF-fed sows. This can represent another benefit in terms of coliform reduction, as acetic acid is even more inhibitory than lactic acid due to the higher concentration of undissociated forms at the same pH (Lindgren and Dobrogosz, 1990). Therefore, while *Salmonella* inhibition occurs at pH lower than 4.4 for lactic acid, the same inhibition effect can be achieved with acetic acid already at pH 5.4 (Goepfert and Hicks, 1969). Jin (2000) obtained similar results with enterotoxigenic *E. coli*. At the same pH acetic acid was more effective inhibitor on the growth of pathogenic *E. coli* than lactic acid. It has been also reported that inhibition of *Salmonella* is most likely due to the synergistic function of lactic and acetic acids, where increase of undissociated forms of acetic acid is a consequence of the strong acidic effect of lactic acid (Adams and Hall, 1988). The high intake of lactic acid by FLF-fed sows together with the highest production of acetic acid as well as other SCFA in the colon could be another possible reason of more efficient elimination of coliforms from the sow's gut. The very low concentration of butyric acid in the faeces can be explained by the fact, that butyric acid is almost completely used as a direct energy source by the colonic cells as well as for the regulation of cell growth and differentiation (Berggren *et al.*, 1993; Salminen *et al.*, 1998a). Despite feeding high concentration of lactic acid to the FLF-fed sows there was no lactic acid present in their faeces. Macfarlane *et al.*, (1992) reported that this metabolite of carbohydrates breakdown is usually further oxidised by other species in the gut, and only with the occasional exception, it does not accumulate there to any significant extent. The very efficient utilisation of lactic acid by

pigs was shown also in the study of Everts *et al.*, (2000). However, Johansson *et al.*, (1998) observed a significant increase in the concentration of faecal lactic acid in men fed a rose-hip drink containing oats fermented with *Lactobacillus plantarum*. The concentration was very low ($\sim 5 \mu\text{mol g}^{-1}$ wet faeces) but they were able to detect even such low amounts of faecal lactic acid due to using more sensitive capillary GLC method.

In addition to production of organic acids and concomitant pH reduction there are many other factors, which could also contribute to the inhibition of coliforms by LAB. LAB are known to produce different bacteriocins, hydrogen peroxide, cause nutrient depletion or low redox potential (Lindgren and Dobrogosz, 1990). Olsen *et al.*, (1995), who screened 241 lactic acid bacteria obtained from maize fermentation showed, that almost half of the *L. plantarum* investigated inhibited other Gram-positive and Gram-negative bacteria due to production not only of organic acids but also bacteriocins as well as other compounds with antimicrobial activity. Recently, Delgado *et al.*, (2001) isolated two antimicrobial proteinaceous compounds produced by *Lactobacillus plantarum*, which have been found to inhibit Gram-negative human pathogens as well as natural competitors of *L. plantarum* from olive fermentation brines. Similar suppression of faecal coliforms by using a diet fermented with LAB was observed in the human studies of other workers (Chen *et al.*, 1999; Kingamkono *et al.*, 1999).

In spite of feeding very large numbers of LAB, the faecal LAB counts did not increase significantly. This is in agreement with results obtained by Paul and Hoskins (1972), Muralidhara *et al.*, (1977), Jensen and Mikkelsen (1998), du Toit *et al.*, (1998) and Donnet-Hughes *et al.*, (1999). The reason for this was not described in any of the studies mentioned. Muralidhara *et al.*, (1977) however, speculated that it may be that the

lactobacilli fed were colonising the small intestine, and only those in excess were voided in the faeces. Many authors have suggested that survival of LAB in the faeces following oral administration reflects large bowel colonisation. This suggestion indicates that enumeration of faecal bacterial strains may not be an accurate reflection of those in the gut. A confirmation of this suggestion could be also found in the study of Murphy *et al.*, (1999) who administered a *L. salivarius* strain to mice. The *L. salivarius* was not longer detectable in mouse faeces, even though the marked strain was found to have persisted in the ileo-cecal region of the small intestine for 7 days after termination of dosing. Since individual intestinal compartments were not examined in the current study, there is some probability that despite not reporting any significant increases of LAB in the faeces, the high numbers of lactic acid bacteria in the FLF diet may probably lead to a more stable and 'friendly' microflora throughout the sow's GI tract. In addition, by feeding high doses of LAB helps sows to avoid significant decreases of these bacteria after farrowing compared with those numbers at 1 week before parturition, which was observed in other two treatments.

In order to determine whether the *L. plantarum* in the starter culture can be transferred from the feed to the sow and from the sow to the piglets, rifampicin resistant mutant of the parent *L. plantarum* (PC-81-11-02, Alltech Inc., Kentucky) was generated. This technique not only facilitated enumeration of the administered *L. plantarum* from faeces but also allowed its easy differentiation from the indigenous population. The power and stability of antibiotic selection in tracking exogenous organisms introduced into the host gastrointestinal tract has been demonstrated by many other workers (Pedersen *et al.*, 1992; Pedersen and Jørgensen, 1992; Rada, 1995; Murphy *et al.*, 1999; Simpson *et al.*, 2000; Fujiwara *et al.*, 2001a; Fujiwara *et al.*, 2001b). However, Pedersen and Tannock (1989) pointed out that there is a possibility of *in vivo* transfer of antibiotic resistance genes from

marker strain to other bacteria. To avoid complications with detection of a marked strain it is better to use a more selective technique based on the combination of double antibiotic resistance and identification of resistant bacterial colonies by using molecular methods such as RAPD-PCR or DGGE (Simpson *et al.*, 2000; Fujiwara *et al.*, 2001a; Fujiwara *et al.*, 2001b). However, other workers have described rifampicin resistance as stable trait (Pedersen *et al.*, 1992; Pedersen and Jørgensen, 1992; Rada, 1995). Naturally occurring resistance to rifampicin among porcine lactobacilli and enterococci is negligible (Pedersen *et al.*, 1992). This was also confirmed by our preliminary experiments where no naturally rifampicin resistant LAB were found in sows' faeces. The strain was easily detectable on antibiotic-selective plates, at a level of 10^4 - 10^5 CFU g⁻¹ (wet weight). This count indicates that despite ingesting high numbers of rifampicin resistant *L. plantarum* from the feed (10^8 - 10^9 CFU ml⁻¹), the faecal counts of the introduced strain constituted less than 1% of the total LAB. On the other hand, according to these data, the introduced strain survived passage through porcine GI tract, but unfortunately, because of the limited monitoring time (no data present after termination of dosing), it is hard to conclude whether colonisation or death of the introduced strain occurred in the sow's gut. In addition, study by (Alander *et al.*, 1999) demonstrated that analysis of faecal samples alone is not sufficient in evaluating colonisation by a probiotics. *Lactobacillus rhamnosus* GG persisted in colonic mucosa even after its disappearance from faecal samples. Intestinal colonisation is defined as a bacterial population in the GI tract that is stable in size over time, without the need for periodic re-introduction of the bacteria by repeated oral doses (Freter, 1992). The succession of exogenous bacterial strain to colonise the host's gut depends on many external and internal factors (Conway, 1997). The colonisation of human *Lactobacillus plantarum* was observed in the study of Johansson *et al.*, (1998), who reported that an ingested strain was still present on the human mucosal surface 11 days after feeding of

oatmeal soup was stopped. According to the literature, the strain origin and the ability to adhere are very important factors in terms of successful colonisation (Naidu *et al.*, 1999). Our introduced *L. plantarum* was not of pig origin, as it was selected only on the basis of very good fermentative abilities and no screening was performed to investigate its colonisation potential. However, studies to investigate this possibility need to be conducted.

During parturition, the piglet encounters numerous microorganisms in the maternal vagina, faeces and in the environment into which it is born. Consequently, it would be expected that similar bacterial floras in piglets and sows faeces during the early stage of the piglets' life. However, not all of the bacteria encountered will become established in the newborn's gut. Gut conditions define which bacteria will be able to colonise and which organisms fail. Age, diet, body secretions, and many others factors make microbial succession an ongoing process (Mackie *et al.*, 1999). This means that a strain, which is predominant in newborn's gut day after birth, does not remain predominant after 2 weeks and a strain, which is predominant in the stomach, is not the same one as in the colon. This explains why Katouli (1995) did not find much similarity between the dominant *E.coli* strains in piglets and those of their dams in samples collected during the first week of the piglets' life. On the other hand, similar metabolic fingerprinting and fermentative capacity of the intestinal flora of sows and their newborns suggested that the sow was the initial source of bacterial flora for her litters (Katouli *et al.*, 1997). However, this similarity was lost in week 2 and piglets developed a flora, which differed from that of sows (Katouli *et al.*, 1995). Colonisation of the piglet's gut occurs very rapidly after birth. Naito *et al.*, (1995) reported that total counts of faecal bacteria of all newborn piglets tested in their study reached $10^{9.4} - 10^{10.6} \text{ g}^{-1}$ faeces (wet weight) 24 hours after birth. He also observed that the

composition of piglets' faecal flora, with special reference to species of the *Lactobacillus* strain, changed with piglets' growth. While *L. reuteri* colonised the piglet's gut on the first day of birth, in one-week's time it was *L. acidophilus* which became the predominant strain. In the study reported here, 7 day old piglets in all experimental groups excreted considerable numbers of coliforms ($10^{7.6} - 10^{8.5} \text{ g}^{-1}$) and LAB ($10^{7.8} - 10^{8.2} \text{ g}^{-1}$). However, the piglets from FLF-fed mother excreted significantly less coliforms and higher numbers of LAB compared with other two treatments, which was reflected in a higher LAB: coliforms (L: C) ratio. According to Muralidhara *et al.*, (1977), higher L: C ratio is usually associated with bacterial flora that contributes to improved animal growth and performance. The reason for having a higher L: C ratio is not known but we can suggest several possibilities. It could be a result of ingesting colostrum with significantly higher mitogenic activity. The functional and structural maturation of gut epithelium was quicker and ingested LAB could possibly have more binding sites for adherence and colonisation. A consequence of having higher numbers of LAB could be a higher probability of lower pH in the piglets' GI tract and/or greater production of antimicrobial substances. In addition, the more mature epithelial cells could be more efficient in eliminating coliforms by producing a variety of innate antimicrobial defences such as mucins, lysozyme and other antimicrobial compounds (McCracken and Lorenz, 2001). Lower numbers of coliforms could also be due to increased activity of lymphocytes, which could activate other parts of the immune system more rapidly.

The test strain *L. plantarum*^{rif} was detected in all faecal samples collected from the piglets, but it constituted less than 1% of the total LAB. Despite such low numbers we can conclude that the transfer of the strain from the feed to the mother and from the mother to the piglet did occur, which is in accordance with the results of other workers (Tannock *et*

al., 1990; Pedersen *et al.*, 1992). That means that the mother animal really represents source of piglets' lactic acid bacteria flora and by appropriate nutritional regimes there is an opportunity to beneficially influence the sows' bacterial excretion, which could be reflected in more 'friendly' bacterial flora in the neonate GI tract. This approach would represent a very natural way of protecting piglets during this short but critical period after birth.

Mitogenic activity of sow colostrum on IEC-6 and blood lymphocytes

There are many possible targets in the GI tract of suckling piglets for bioactive molecules in sow colostrum. Structurally and functionally underdeveloped epithelium as well as mucosal immune system are of great importance in terms of maintaining piglet's health and survival during the postnatal period. The growth promoting effect of sow colostrum and/or milk on the gastrointestinal tissue development, especially in the small intestine, has been well documented (Cera *et al.*, 1987; Simmen *et al.*, 1990; Kelly and King, 1991; Wang and Xu, 1996; Zhang *et al.*, 1997; Zhang *et al.*, 1998). This was confirmed also in the current study, where all colostrum samples had mitogenic potential on the intestinal epithelial cells (IEC) compare with the control (cells without colostrum), but there were significant differences between the experimental groups. Colostrum produced from sows fed liquid feed had significantly higher growth promoting activity compare with DF fed sows. However, the exact molecular mechanisms regulating this process in pigs are unclear. In order to demonstrate and specify the growth factors in pig colostrum Cera *et al.*, (1987) performed one of the first experiments *in vitro*. In this study the fractionation of colostrum, on gel-filtration columns, revealed the presence of three factors (208.6, 66 and 4.6 kDa) with fibroblast and epithelial cell mitogenic activity. There were no similarities

between these pig factors and colostral specific growth factors in ruminants. Furthermore, detailed analyses of porcine colostrum showed the presence of trophic factors such as epidermal growth factor (EGF), insulin-like growth factor I and II (IGF-I, IGF-II) and insulin (Jaeger *et al.*, 1987; Donovan *et al.*, 1994; Xu *et al.*, 1994). All these factors are present in much higher quantity (3-to 100-fold) in colostrum than mature milk (Cera *et al.*, 1987; Xu *et al.*, 1994) and despite their protein structure, all of them are stable in the gastrointestinal lumen of suckling piglets (Shen and Xu, 1998; Shen and Xu, 2000b; Shen and Xu, 2000a). Therefore, it has been speculated that these colostral factors may play an important role in GI growth and adaptation during the neonatal period. In addition, Mubiru and Xu (1997) reported that EGF and IGFs might also stimulate pancreatic development in newborn animals. Porcine colostrum contains 10 to 20 nM of EGF, 10 nM of insulin (Jaeger *et al.*, 1987) and 200 to 1000 ng ml⁻¹ of IGF-I and IGF-II (Xu *et al.*, 1994). In addition to the growth factors mentioned above, there are other specific molecules present in colostrum that may have a potent growth stimulating effect on gut epithelium. They include nitrogen compounds - polyamines (spermidine and spermine) (Kelly and King, 1991; Motyl *et al.*, 1995), iron binding protein -lactoferrin (Hashizume *et al.*, 1983; Hagiwara *et al.*, 1995), glutamine (Ko *et al.*, 1993), platelet-derived growth factor (PDGF) cytokines (Hagiwara *et al.*, 2000) and leptin (Zabielski *et al.*, 2003).

Intestinal epithelium represents tissue with rapid cellular turnover. The lining of the small intestine is maintained through the proliferation of stem cells that are found protected at the base of the crypts of Leiberkuhn. The new epithelial cells migrate from the bottom of the crypt up to the top of the villus where they are shed. An increase of the cell proliferation rate in the crypts will result in an overall increase of the epithelial cell population and associated increases in villus height (Lipkin, 1985). Thus colostrum with higher mitogenic

activity has the potential to speed up the maturation of newborn's GI tract and provides the piglet with better protection by keeping the intestinal mucosa sealed and impermeable to toxins and bacteria. In addition, it is well known that intestinal epithelial cells produce a variety of innate antimicrobial substances such as lysozyme and defensins, acting directly against bacterial growth by disrupting their cell membranes (Quellette, 1997). Finally, by having the ability to act as antigen presenting cells (APC) (Bromander *et al.*, 1993), and producing different types of cytokines (Seydel *et al.*, 1997), intestinal epithelial cells can no longer be considered as only a mechanical barrier, but '*as an integral component of a communication network that involves interactions between epithelial cells, luminal microbes and host local mucosal immune system*' (Kagnoff and Eckmann, 1997). By realising the importance of proper function of epithelial cells, the significance of having colostrum of higher mitogenic activity becomes very clear. Therefore, good management strategies for efficient pig production should aim to achieve not only to produce a greater quantity of colostrum but also, and this is even more important, colostrum of improved quality.

The increased mitogenic activity of colostrum produced by sows fed FLF was confirmed also in the next experiment with pig blood lymphocytes. However, the fact, that there was also a significant difference between FLF and NFLF colostral samples leads to speculation, that it is most likely the fermentation process that gives the feed some factors the presence of which are needed to produced colostrum of higher mitogenic potential in terms of lymphocyte proliferation. Lessard and Brisson (1987) demonstrated that supplementation of a pig's diet with a *Lactobacillus* fermentation product improved growth and feed intake, but as they concluded, that the product also had a stimulating effect on the immune system. However, the nature of the factor(s) responsible for those

effects has not been identified. Lymphocytes represent key cellular components responsible for the specificity of the immune system. The actions of T-lymphocytes are collectively termed cell-mediated immunity, while immunity mediated by B cells is referred to as humoral immunity. Cell mediated immunity in newborn pigs is at a very immature stage and the piglets depend almost entirely on passive protection acquired through ingesting immunoglobuline-rich colostrum (Allen and Porter, 1973). Immune responses depend not only on the activation of lymphocytes, but also on the ability of lymphocytes to proliferate. Some factors responsible for proliferation of epithelial cells are also responsible for proliferation of lymphocytes. IGF-I represents one of the factors involved in the proliferation of both T and B cells (Clark *et al.*, 1993). EGF was also shown to stimulate lymphocyte proliferation (Acres *et al.*, 1985). Other factors that may be involved in influencing the proliferation of lymphocytes are colostral lymphokines such as interferon- γ (Lawton *et al.*, 1979) and possibly interleukin-1 β (Juto, 1985) produced by colostral leukocytes *in vitro*. *In vivo* studies by Stephens *et al.*, (1986) supported this hypothesis of lymphokine involvement in cell proliferation. However, in the current experiment it is not possible to conclude which type of lymphocytes proliferated, as there was a mixture of both B and T lymphocytes in the blood samples. More detailed experiments are planned, which may answer this question.

Three immunoglobulin classes are recognised in the pig, of which IgG is quantitatively the most important (80%), following by IgA (14%) and IgM (Porter, 1975). The internal absorption of these colostral antibodies by the piglet's gut is of tremendous importance as newborns do not synthesise any appreciable level of antibody before the age of 3-4 weeks (Brown *et al.*, 1961). Immunoglobulin G has an important role in protection of the young pig against *E. coli* enteritis by neutralising heat labile enterotoxin and decreasing the rate

of multiplication of the bacterium (Brandenburg and Wilson, 1973). Although protection against many systemic pathogenic agents will be provided by IgG, immunity is further limited by the fact that many pathogens encountered by the young pigs are found at mucosal surfaces where IgG antibodies are rarely found and are largely ineffective (Gaskins and Kelley, 1995). In terms of mucosal protection, secretory IgA (sIgA) plays the most important role. Porcine secretory IgA shows considerably greater resistance to enzymatic degradation than does IgG and its main role is neutralising viruses, inhibiting the attachment of bacteria to enterocytes, and opsonizing bacteria, which facilitates their destruction (Porter, 1973; Abraham and Ogra, 1994). Bactericidal activity of IgA against *Escherichia coli* was demonstrated by Hill (1974). Therefore, despite not being the main Ig in sow colostrum, IgA seems to have an important function in the protection of neonatal pigs against enteric infections.

The major reason for the large differences in the concentrations of colostral immunoglobulins and proteins was the parity numbers of the experimental sows, which ranged from 2 to 9. Klobasa and Butler (1987) demonstrated a pronounced tendency for older sows to produce higher IgA, IgM and IgG concentrations than did sows with fewer gestations. The Igs that the neonate receives are a reflection of the immunisation history and pathogen exposure of the dam. Thus older sows may provide better immunity for the young piglets by having more protective Igs both in quantity and quality. Similar results were also reported in the studies of other investigators. Inoue *et al.*, (1980) analysed the relationship between 13 possible factors and concentration of IgG in sow colostrum. Number of parturitions was found as one of the most influencing factors together with kind of feed, type of farming and number of sows. Colostral concentration of IgA was also most influenced by number of parturition and breed factor (Inoue *et al.*, 1980).

The second reason for having large differences in ELISA values was the time of milking. The colostral samples in our experiment were obtained in the time range from 0 to 12 hours after parturition. This means that sows were milked at different times after parturition, which makes big difference to the quality of their colostrum. Klobasa and Butler (1987) demonstrated lactation stage-dependent changes in the concentration of all three immunoglobulins IgG, IgA, and IgM. The most rapid decrease in the concentration of colostral Ig(s) was observed during the first 24 hours. Colostrum taken at 6 hours after parturition contained approximately 40% less IgG and IgA than colostrum taken at parturition. At 12 hours after parturition the decrease in Ig(s) levels was already 55-60%.

In addition to these two major factors, there are also other items, which influence the amount of lacteal Ig(s) in sows. According to Klobasa and Butler (1987) individual sow variation seems to have the largest influence.

Despite not having statistically significant results, the data are still interesting and noteworthy. The sows in FLF group had numerically higher concentration of both Igs than DF fed sows, although FLF sows represented the youngest group of sows in the experiment in terms of number of lactation. On the other hand, despite almost the same parity numbers, NFLF group of sows produced considerably higher levels of IgG and IgA than sows on DF. These trends suggest that in terms of colostral Ig(s), liquid feeding could be more beneficial for farrowing sows than DF. The reasons and mechanisms by which that occur remained to be determined. FLF may have an immunostimulatory effect due to the presence of high numbers of LAB, namely *Lactobacillus plantarum*. The ability of LAB to modulate various pathways of immune system has been reviewed by Naidu *et al.*, (1999). Although the mechanisms by which LAB are able to modulate the immune system

are not clear, it has been shown that live cultures are more efficient at enhancing certain aspects of immune function than the killed cultures (Eddie *et al.*, 1971; Vesely *et al.*, 1985; De Simone *et al.*, 1986). In addition, lactic cultures delivered in fermented products induce a greater response compared to cultures given in non-fermented products (Perdigon *et al.*, 1986b). However, as demonstrated by Maassen *et al.*, (1998) *Lactobacillus* strains affect the immune system differently by inducing different cytokine profiles, which determines the direction and efficacy of the humoral response. In other words, lactobacilli are able to enhance or inhibit the development of disease. This can be supported also by study of Perdigon *et al.*, (1990a), who carried out an experiment with four different LAB to demonstrate if the oral administration of viable bacteria increases the local immune response in intestinal secretions. The results showed that only one strain (*L. casei*) had the potential to increase the IgA secreted into the intestinal lumen, while the others were not effective.

It is possible that during fermentation, the metabolites of *L. plantarum* may also contribute to the biological effects observed, but we have not specifically addressed this point in this study. In the study of (Lessard and Brisson, 1987) a *Lactobacillus* fermentation product slightly increased the concentration of serum IgG in weaned piglets. Laffineur *et al.*, (1996), tested 10 culture supernatants of lactobacilli in order to investigate their immunomodulatory activity. In their study only a *L. helveticus* supernatant allowed the modulation of lymphocyte proliferation in vitro. They concluded that the culture supernatant activity might be related to interaction with monocyte-macrophage and T helper cells.

Bloksma *et al.*, (1979) reported that a dose 10^8 lactobacilli was optimal for adjuvant effects and demonstrated the immunostimulation effect of *L. plantarum* by using mouse model. According to Donnet-Hughes *et al.*, (1999) the minimum daily dose of viable *Lactobacillus* strains, which will significantly modulate nonspecific immunity, is 10^9 CFU. This level also represents a dose of *L. plantarum* in FLF, which sows received daily in our experiment.

This means that proper strain selection could be helpful in directing the immune responses in sows, which will subsequently be reflected in the production of higher quality colostrum. However, more detailed studies are needed to confirm immunostimulation effect of *L. plantarum* used in this experiment. Although the strain was not recovered from the sow faeces in high amounts, the study of Donnet-Hughes *et al.*, (1999) showed that for a certain forms of immune reactivity, a high recovery in the faeces is not prerequisite. As we have already mentioned, the faecal strains are a reflection of large bowel colonisation, but the target for immunological modulation does not necessarily reside in the colon. The target may be a structure of the small intestine such as the Payer's patches of the gut-associated lymphoid tissue (GALT) (Takahashi *et al.*, 1991). Consequently, if a strain is able to colonize the small intestine, the faecal numbers will be lower but immunomodulation can still take place through the GALT.

2.4. Conclusion

This study showed that FLF has the potential to beneficially modify the microflora the lactating sows introduces into the environment, which is consequently reflected to a more friendly gut microflora of their litters. This study also indicated that enhanced neonatal

defense can be made available by manipulating the immune status of farrowing sows. This in turn will be reflected in the production of colostrum / milk of higher immunological and nutritional quality. The combined effects of enhanced maternal / passive immunity and the reduction in the level of environmental contamination with coliforms, achieved by FLF, may be important in achieving improved health status for both sows and piglets.

Chapter 3

Selection of porcine *Lactobacillus* isolates as a starter culture to control the fermentation of liquid feed

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3.1. Introduction

Lactic acid bacteria (LAB) in food can colonize the intestine transiently and exert beneficial effects. The use of specific cultures of LAB is standard in the production of many fermented foods and feeds (Lindgren and Dobrogosz, 1990). LAB inoculants are used to ensure a more rapid drop in pH, a higher level of lactic acid and LAB and concomitant lower numbers of enterobacteria, clostridia and yeasts. Previous work at our laboratory included a large screening programme in order to find suitable LAB starter cultures for fermentation of liquid feed (Moran, 2001). The selection criteria were based purely on the fermentative ability of screened bacterial strains to ensure a rapid decrease of pH in the pig feed. As a result of this screening programme, *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) was selected and used to control the fermentation of liquid feed (Moran, 2001). The aim of the screening programme, described in this chapter, was to select a suitable starter culture from LAB of porcine origin, which would have similar fermentative abilities to *L. plantarum*, but could exert possible 'probiotic' effects on the host (sow). Therefore, the previous screening programme set up by Moran (2001) was expanded and included some additional criteria used to select new probiotics.

Stringent criteria have been set by most workers for the selection of probiotic bacteria (Havenaar and Huis in't Veld, 1991; Huis in't Veld and Havenaar, 1993; Huis in't Veld and Shortt, 1996; Schiffrin *et al.*, 1997; Dunne *et al.*, 2001; Mercenier *et al.*, 2003). Table 3.1 shows that expected beneficial characteristics of potential probiotic strains encompass physiological, immunological, metabolic and genetic traits, and in addition important technological properties (Mercenier *et al.*, 2003).

Table 3.1. Characteristics expected of potential probiotic strains (Mercenier *et al.*, 2003).

<ul style="list-style-type: none"> • Non toxic and non pathogenic • Accurate taxonomic identification • Normal inhabitant of the targeted species • Capable of survival, proliferation and metabolic activity in the target site, which implies: <ul style="list-style-type: none"> - Resistance to gastric and acid and bile - Ability to persist, albeit for short periods, in the GI-tract - Adherence potential preferred - Ability to compete with the resident flora • Production of antimicrobial substances • Antagonism (<i>in vivo</i>) towards pathogenic bacteria • Ability to modulate immune responses • Ability to exert at least one clinically documented health benefit • Genetically stable • Amenability of the strain and stability of the desired characteristics during processing, storage and delivery • Viability at high populations • Desirable organoleptic and technological properties when included in fermentation process
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The succession of criteria and experiments are often chosen in such a way that the microorganisms can be submitted to tests which grow gradually more and more complex. The screening programme usually starts with the origin and safety issues. The potential beneficial bacterial strains must be non-toxic and non-pathogenic. Choice of origin of bacterial strain will mainly be determined by the specific purpose of the probiotic strain (Havenaar *et al.*, 1992). For example, if adherence/colonisation is not required the question of origin is not very important. However, if colonization is essential to achieve the ultimate aim of the probiotic application, species and location specificity represent significant selection characteristics. Since viable and biologically active microorganisms are usually required at the target site in the host it is essential that the probiotics will not be killed by the host's defence mechanisms. Therefore, intrinsic microbiological properties, such as tolerance to gastric acid and bile represent one of the next selection criteria, which must be

screened. However, this doesn't exclude a possibility that non-viable products can also express health-promoting properties. To ensure prolonged survival of probiotic bacteria in the body of the host, the organism should be able to colonise the internal body surfaces. In general, bacterial adhesion without *in vivo* invasion of mucosal epithelial surfaces would aid in establishing colonization and place the probiotic strains in the closest proximity to the mucosal immune system. Schiffrin *et al.*, (1995) have suggested that adhesion of lactobacilli to mucosal epithelial cells may be a critical factor in immune stimulation. In addition, the ability to inhibit opportunistic pathogens through colonization resistance would also be facilitated by adhesion and masking of attachment sites. However, it is not clear whether daily consumption of non-colonizing, viable bacterial are without any health-promoting value. Hood and Zottola (1988) showed that viability itself is not prerequisite for adherence as killed *L. acidophilus* cells were still able to adhere to intestinal cells as effectively as viable bacterial cells. However, viability is essential for growth and colonisation.

The production of antimicrobial peptides (bacteriocins), that target different pathogens without toxic or other adverse effects represent another selection factor for suitable probiotics. The recent progress in LAB bacteriocin research was recently reviewed by Cleveland *et al.*, (2001). In addition to antimicrobial activity, screening for antifungal activity of LAB could also be of importance. In the study of Magnusson *et al.*, (2003) approximately 10% of 1200 screened LAB isolated from different environments, showed antifungal activity and 4% showed strong activity against the indicator mould *Aspergillus fumigatus*. The majority of the fungal inhibitory isolates were identified as *L. coryniformis*, however *L. plantarum* and *Pediococcus pentosaceus* were also identified frequently among the active isolates. Chemical analyses by HPLC revealed the presence of antifungal cyclic

dipeptides, as well as several other active fractions, suggesting a highly complex nature of the antifungal activity.

At present, many *in vitro* methods are suitable for screening some of the above mentioned criteria. However, it is important to realise that results from such studies may not be predictive of the actual *in vivo* situation. For example, the survival of bacterial strain at low pH in a test tube is not a reflection of what happens in the stomach, where more complex physiological conditions exist. Also, the results from experiments performed on cell lines should be interpreted cautiously because of the absence from these models of mesenchymal and lymphoid cells, cytokines and other luminal factors normally present in the intestine (McCracken and Lorenz, 2001). A recent study by Ibnou-Zekri *et al.*, (2003) clearly demonstrated that despite similar *in vitro* probiotic properties (similar growth, survival, and adherence properties), distinct *Lactobacillus* strains colonized the gut differently and generated divergent immune responses. Therefore, it would be more appropriate to have a dynamic model that mimics *in vivo* GI conditions as closely as possible. Such a model has been developed by researchers at TNO Nutrition and Food Research at Zeist in the Netherlands (Minekus *et al.*, 1995; Minekus *et al.*, 1999). This multicompartmental *in vitro* model reproduced very closely the dynamic conditions based on the *in vivo* situation in monogastric animals and human.

The aim of this study was to select a LAB inoculant of porcine origin for the production of fermented liquid feed.

The main objectives were as follows:

- to compare fermentative properties of new strain with *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA)
- to investigate the *in vitro* colonization potential of the new strain
- to investigate the *in vitro* immunomodulatory potential of the new strain
- to identify the strain using microbiological and molecular approaches

3.2. Materials and methods

All microbiological media were obtained from Oxoid Ltd. (Hants, England) and were prepared according to manufacturer's recommendations or altered accordingly based on the requirements of the experiment. All chemicals were obtained from Sigma-Aldrich Ltd., (Dorset, England).

3.2.1. Microorganisms

In total, 87 faecal *Lactobacillus* strains were isolated from sows, pigs and piglets. Details of the bacterial strains used and their sources are shown in Table 3.2. Stock cultures were maintained at -80°C in 1 ml aliquots of MRS broth with 20% v/v glycerol and were subcultured twice in MRS broth for 'activation' prior to experimental use. Faecal lactobacilli were isolated on Rogosa agar (containing 100 µg ml⁻¹ cycloheximide and 0.04% w/v chlorophenol red). Acid producing colonies were purity plated on MRS agar and Gram-positive and catalase negative rods were presumptively taken as lactobacilli and used for further screening.

Lactobacillus plantarum (PC-81-1-06; Alltech Inc., Kentucky, USA), which was previously selected as a suitable starter culture for the preparation of fermented liquid feed (Moran, 2001), was used as control strain in this screening program.

E. coli K99 isolated from pig faeces was obtained from Veterinary Laboratories Agency (VLA) (Starcross, Exeter, England). Prior to use the strain was subcultured twice in Brain Heart Infusion (BHI) at 37°C for 24 hours.

Table 3.2. Faecal porcine bacterial strains used in screening programme.

No.	Culture number	Strain name	Growth temp.	Source
1	SHCM FC1	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
2	SHCM FC2	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
3	SHCM FC3	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
4	SHCM FC4	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
5	SHCM FC5	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
6	SHCM FC6	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
7	SHCM FC7	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
8	SHCM FC8	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
9	SHCM FC9	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
10	SHCM FC10	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
11	SHCM FC11	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
12	SHCM FC12	<i>Lactobacillus</i> spp.	37°C	Sow (farrowing)
13	SHCM FC13	<i>Lactobacillus</i> spp.	37°C	Sow (farrowing)
14	SHCM FC14	<i>Lactobacillus</i> spp.	37°C	Piglet (5 weeks)
15	SHCM FC15	<i>Lactobacillus</i> spp.	37°C	Sow (farrowing)
16	SHCM FC16	<i>Lactobacillus</i> spp.	37°C	Piglet (5 weeks)
17	SHCM FC17	<i>Lactobacillus</i> spp.	37°C	Piglet (5 weeks)
18	SHCM FC18	<i>Lactobacillus</i> spp.	37°C	Piglet (5 weeks)
19	SHCM FC19	<i>Lactobacillus</i> spp.	37°C	Piglet (5 weeks)
20	SHCM FC20	<i>Lactobacillus</i> spp.	37°C	Sow
21	SHCM FC21	<i>Lactobacillus</i> spp.	37°C	Sow
22	SHCM FC22	<i>Lactobacillus</i> spp.	37°C	Sow
23	SHCM FC23	<i>Lactobacillus</i> spp.	37°C	Sow
24	SHCM FC24	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
25	SHCM FC25	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
26	SHCM FC26	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
27	SHCM FC27	<i>Lactobacillus</i> spp.	37°C	Sow
28	SHCM FC28	<i>Lactobacillus</i> spp.	37°C	Sow
29	SHCM FC29	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
30	SHVD FC1	<i>Lactobacillus</i> spp.	37°C	Sow
31	SHVD FC2	<i>Lactobacillus</i> spp.	37°C	Sow
32	SHVD FC3	<i>Lactobacillus</i> spp.	37°C	Sow

Table 3.2. (cont.) Faecal porcine bacterial strains used in screening programme.

No.	Culture number	Strain name	Growth temp.	Source
33	SHVD FC4	<i>Lactobacillus</i> spp.	37°C	Sow
34	SHVD FC5	<i>Lactobacillus</i> spp.	37°C	Sow (gestating)
35	SHVD FC7	<i>Lactobacillus</i> spp.	37°C	Sow (gestating)
36	SHVD FC8	<i>Lactobacillus</i> spp.	37°C	Sow (gestating)
37	SHVD FC23	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
38	SHVD FC24	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
39	SHVD FC25	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
40	SHVD FC26	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
41	SHVD FC27	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
42	SHVD FC28	<i>Lactobacillus</i> spp.	37°C	Pig (grower)
43	SHVD FC29	<i>Lactobacillus</i> spp.	37°C	Pig (fattening)
44	SHVD FC30	<i>Lactobacillus</i> spp.	37°C	Pig (fattening)
45	SHVD FC31	<i>Lactobacillus</i> spp.	37°C	Pig (fattening)
46	SHVD FC32	<i>Lactobacillus</i> spp.	37°C	Pig (fattening)
47	SHVD FC33	<i>Lactobacillus</i> spp.	37°C	Pig (finisher)
48	SHVD FC34	<i>Lactobacillus</i> spp.	37°C	Pig (finisher)
49	SHVD FC35	<i>Lactobacillus</i> spp.	37°C	Pig (finisher)
50	SHVD FC36	<i>Lactobacillus</i> spp.	37°C	Pig (finisher)
51	SHVD FC39	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
52	SHVD FC40	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
53	SHVD FC41	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
54	SHVD FC42	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
55	SHVD FC43	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
56	SHVD FC44	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
57	SHVD FC45	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
58	SHVD FC46	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
59	SHVD FC47	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
60	SHVD FC48	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
61	SHVD FC49	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
62	SHVD FC50	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)

Table 3.2. (cont.) Faecal porcine bacterial strains used in screening programme.

No.	Culture number	Strain name	Growth temp.	Source
63	SHVD FC51	<i>Lactobacillus</i> spp.	37°C	Piglet (preweaning)
64	SHVD FC52	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating) ¹
65	SHVD FC53	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
66	SHVD FC54	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
67	SHVD FC55	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
68	SHVD FC56	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
69	SHVD FC57	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
70	SHVD FC58	<i>Lactobacillus</i> spp.	37°C	Sow D (gestating)
71	SHVD FC59	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating) ²
72	SHVD FC60	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
73	SHVD FC61	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
74	SHVD FC62	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
75	SHVD FC63	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
76	SHVD FC64	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
77	SHVD FC65	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
78	SHVD FC66	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
79	SHVD FC67	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
80	SHVD FC68	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
81	SHVD FC69	<i>Lactobacillus</i> spp.	37°C	Sow F (gestating)
82	SHVD FC70	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating) ³
83	SHVD FC71	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating)
84	SHVD FC72	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating)
85	SHVD FC73	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating)
86	SHVD FC74	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating)
87	SHVD FC75	<i>Lactobacillus</i> spp.	37°C	Sow N (gestating)

Key: SH-Seale-Hayne Microbiology culture collection

¹ Sow on Dry feed (D)

² Sow on Fermented liquid feed (F)

³ Sow on Non-fermented liquid feed (N)

3.2.2. Bacterial growth, acid and gas production of faecal lactobacilli

The key to a good fermentation of pig feed is establishing a homofermentation rather than a heterofermentation. Therefore the homo/heterofermentation test was performed on all 87 porcine faecal *Lactobacillus* isolates. All strains were cultivated at 30°C in Man-Rogosa-Sharpe (MRS) broth and plates were incubated in anaerobic jars (Gas Pak Anaerobic System, Oxoid, Basingstoke, UK). Colonies were tested for acid and gas production from glucose by inoculating 10 ml of a modified glucose-MRS (containing no citrate or meat extract; and addition of 10 ml l⁻¹ chlorophenol red (0.4% solution)) broth in test tubes containing inverted Durham tubes and incubating at 30°C (Muller, 1990). Tubes were examined after 24 h and 48 h. Gas production was considered as an indication of heterofermentative metabolisms. A colour change (chlorophenol red indicator) from red to yellow indicated acid production and turbidity indicated growth of the culture. *L. brevis* was used as a positive control for gas and acid production.

3.2.3. Aggregation and coaggregation experiments

It has been suggested that lactobacilli, which aggregate with pathogens, may constitute an important host defence mechanism against infection. Moreover, aggregating bacteria had stronger adhesion ability to the intestinal cells than non-aggregating one, indicating that aggregation can increase substantially the colonisation potential of lactobacilli in environments with short residence time. Therefore, all selected homofermentative lactobacilli were tested for aggregating ability. Aggregation test was performed as described by Kmet and Lucchini (1999). Briefly, each strain was grown overnight at 37°C in MH broth (*E. coli*) or MRS broth (lactobacilli) in 5% CO₂ atmosphere. Bacteria were

centrifuged for 10 minutes at 10 000 x g, washed three times with sterile PBS, resuspended in PBS at a concentration of 10^9 CFU ml⁻¹ and incubated at room temperature in the presence of 10% (v/v) freshly prepared filter-sterilized own supernatant fluid. The total volume of aggregative mixture was 1 ml. Aggregation was scored positive when clearly visible sand-like particles, formed by the aggregated cells, gravitated to the bottom of the tubes, leaving a clear supernatant fluid, within 1 hour. The reaction time was recorded for the measuring of aggregating intensity (rapid, 15 min (+ + +); normal 15-30 min (+ +); slow, 30-60 min (+)).

Coaggregation experiments were performed according to Drago *et al.*, (1997). 500 µl of each *Lactobacillus* suspension was mixed with 500 µl of *E. coli* K99 suspension for at least 10 sec on a vortex mixer and then incubated in 24-well microtrays (Corning, Italy) at 37°C under agitation. After 4 hours the suspension were observed by scanning electron microscope (SEM). SEM was also used to observe autoaggregation of pure lactobacilli.

3.2.4. Scanning electron microscopy (SEM)

Ten µl of each bacterial suspension was placed onto a slide (Thermanox, Nunc, Inc.), air dried, fixed with 2.5% glutaraldehyde for 2 hours at room temperature, washed three times in 0.1% (w/v) sodium cacodylate buffer pH 7. Samples were dehydrated through a graded series of ethanol and acetone mixtures, critical point dried at 35°C and 1250 psi for 15 min, coated with gold and observed by SEM under high vacuum with a JSM-6100 electron microscope (JOL, Ltd. Akishima, Japan). Six randomized fields were evaluated in each sample.

3.2.5. Acid resistance

As potential candidates for inoculum will be delivered to sows in a feed, the high acidity in the stomach and high concentration of bile components in the proximal small intestine are the first host attributes that affect the strain selection. Therefore selected homofermentative and aggregating *Lactobacillus* strains were further tested for acid and bile tolerance. Growth at pH 3.5 was tested in MRS broth adjusted to pH 3.5 with 1 N HCl. Cultures were diluted 1:10 000 in fresh MRS broth to obtain a bacterial concentration of approximately 1×10^5 CFU ml⁻¹. Diluted cultures, 400 µl, were added to individual wells, in triplicate, of a sterile Bioscreen (Life Sciences International, Basingstoke, UK) honeycomb plate. The inoculated honeycomb plates were then placed in the reading chamber of a Labsystems Bioscreen (Life Sciences International) and incubated at 30°C for 48 hours. Measurements of the OD of wells were at a wavelength of 600 nm at 60 min intervals for 48 hours. The data generated were then converted into Microsoft Excel (Microsoft, Seattle, USA) format and, where appropriate, processed into growth curves.

3.2.6. Bile-salt tolerance

Lactobacillus cultures were diluted 1:10 000 in fresh MRS broth supplemented with 0.3% (w/v) oxgall (Sigma-Aldrich, Dorset, UK) to obtain a bacterial concentration of approximately 1×10^5 CFU ml⁻¹. Diluted cultures, 400 µl, were added to individual wells, in triplicate, of a sterile Bioscreen (Life Sciences International, Basingstoke, UK) honeycomb plate. The inoculated honeycomb plates were then placed in the reading chamber of a Labsystems Bioscreen (Life Sciences International) and incubated at 30°C for 48 hours. Measurements of the OD of wells were at a wavelength of 600 nm at 60 min

intervals for 48 hours. The data generated were then converted into Microsoft Excel (Microsoft, Seattle, USA) format and, where appropriate, processed into growth curves.

3.2.7. Fermentation of pig feed

In order to determine the activity of the candidate organisms in pig feed, all 13 aggregating porcine lactobacilli were tested for fermentation potential. The initial stages involved examining the growth of these organisms in a standard sow diet that had been sterilized by irradiation.

3.2.7.1. Feed and feed preparation

A lactation diet (BOCM Pauls, Ltd) in pelleted form was used for fermentation experiments. The composition of the diet was described in chapter 2 (Table 2.1). The feed was divided into 10 x 200 g aliquots and placed in sealed bags for γ -irradiation at 25 kGy from a Cobalt⁶⁰ source (Becton and Dickenson, Plymouth, England). Irradiated feed was tested for sterility by enriching 10 g of feed in 90 ml of each of the following: nutrient broth, glucose broth and MRS broth for 72 hours. Due to the nature of the feed in broth-feed mixture it was difficult to estimate visually if microbial growth was present. Therefore, 1 ml samples were removed daily and pour-plated in Plate Count Agar (PCA); and 100 μ l samples were spread plated on MacConkey agar and Rose-Bengal Chloramphenicol agar. Plates were incubated at the required temperature for enumeration of organisms selected by media.

3.2.7.2. Fermentation of feed

Liquid feed fermentations were prepared by mixing 200 g of sterile feed with 500 ml of sterile deionised water (20°C) (1 : 2.5 w/w ratio) in sterile 1 L jars (Nalgene, Milton Keynes, England). All fermentation studies were carried out in triplicate. Feed was inoculated with approximately 0.7 ml of an overnight culture (MRS broth, 30°C, $\sim 9 \log_{10}$ CFU ml⁻¹) of the individual inoculants to give a final concentration of $6 \log_{10}$ CFU ml⁻¹ liquid feed. Control fermentations did not contain an inoculant. Feed was steeped for 96 hours at 30°C and 10 ml samples were removed aseptically every 24 hours for measurement of pH (Hannah Checker, Hannah Instruments Ltd., Leighton Buzzard, Bedfordshire, UK) and organic acid concentrations. Samples of liquid feed were serially diluted in Maximum Recovery Diluent (MRD) (1 ml sample: 9 ml MRD).

3.2.8. Analysis of organic acid production and pH

High levels of lactic acid are required to be produced quickly in order to reduce the pH of the feed and to inhibit the growth of spoilage organisms, such as coliforms. Therefore, ability of selected homofermentative, aggregating lactobacilli to produce sufficient amount of lactic and acetic acid was tested in the following test. The concentrations of lactic and acetic acids in the fermented feed samples were determined by the HPLC method. Sampling times were set at 0, 24 and 48 hours from inoculation. 1 ml of sample was removed at each sampling time and added to a centrifuge tube. After the addition of sulphuric acid (at a final concentration of 0.1 g l⁻¹) as the internal standard, a proportion of the processed sample was centrifuged (10000g for 15 min) and filtered (filter pore size 0.45 µm). HPLC separations were performed using a P580 HPG pump and GINA 50

autosampler (Dionex, Cheshire, UK) with an Animex HPX-87H (30 cm x 6.8 mm) cation exchange column (Bio-rad, Herts, UK) and refractive index (RI) detector. The column temperature was maintained at 55°C and the flow rate at 0.7 ml min⁻¹. Determinations were based upon retention time in relation to authentic reference compounds. All calibrations were produced from peak height determinations utilising integration software (Chromeleon, Dionex-Softron GmbH, Gemering, Germany). Ten mls of sample was also removed at each sampling time to measure the pH of the feed using an electronic pH meter (W.G. Pye & Co. Ltd., Cambridge, UK).

3.2.9. Adhesion assays

The colonization of the GI-tract can occur in three phases: (1) adhesion to the mucus layer, the first step in colonization, (2) adhesion to the epithelial surface and finally (3) sub-epithelial adhesion, which is utilized mainly by pathogenic bacteria in invasion. Adhesion or close association of the *Lactobacillus* starter culture to these intestinal compartments could be another very useful characteristic as it may contribute to competitive exclusion of pathogens by occupying the adhesion receptors. For this reason, the following adhesion assays were conducted:

3.2.9.1. Porcine mucus binding assay

In the experiment with porcine mucin (Sigma-Aldrich, Dorset, UK) the Nunc-Immuno microtitre 96-well plates with Maxi Sorp surface (Invitrogen, UK) were coated with mucus solution (100 µl) at a concentration of 1000 µg mucin proteins per ml and subsequently incubated overnight at 4°C. Mucin solutions were removed and plates were washed three

times with PBS. Then bacterial suspensions ($100\ \mu\text{l}$; $10^9\ \text{CFU ml}^{-1}$) of individual strains were added and the plates were incubated on an orbital platform shaker for 2 hours at 37°C . All unbound bacteria were removed by washing the wells three times with PBS. Bacteria in the wells were then fixed at 60°C for 20 minutes and stained with crystal violet ($95\ \text{ml}$ per well) for 45 minutes. Wells were subsequently washed six times with PBS to remove excess stain. After adding $100\ \mu\text{l}$ of citrate buffer ($\text{pH } 4.3$) to each well and 45 min incubation at room temperature to release the stain bound to bacteria, the absorbance values ($A_{550\text{nm}}$) were determined by ELISA reader and the averages of 10 absorbance values were calculated. Each batch of assays included also blank wells (only mucin and PBS without bacteria) and *L. reuteri* (NCIB 11951) as a positive control (Aleljung, 1994). Lactobacilli were classified as strongly adherent ($A_{550\text{nm}} > 0.3$), weakly adherent ($0.1 < A_{550\text{nm}} < 0.3$), or nonadherent ($A_{550\text{nm}} < 0.1$) (Styriak *et al.*, 1999a).

3.2.9.2. Caco-2 cell adhesion assay

Caco-2 cells were used in order to characterize the adhesion ability of lactobacilli. Caco-2 cells were obtained from the European collection of cell cultures (ECACC, Salisbury, UK) cat. No. 860102002. Cells were maintained as stock cells (passage 30-45) in $75\ \text{cm}^3$ tissue culture flasks in $20\ \text{ml}$ of standard medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Poole, UK) containing 10% (v/v) foetal calf serum, $2\ \text{nM}$ glutamine, $2\ \text{mM}$ penicillin/streptomycin and $1\times$ non-essential amino acid solution. Incubation conditions were maintained at 37°C , $5\%\text{CO}_2$, 95% humidity in an incubator. The cells were routinely subcultured weekly. The monolayer was washed three times with $20\ \text{ml}$ Hanks Balanced Salt Solution (HBSS) which was then completely removed. $10\ \text{ml}$ of 0.05% trypsin-EDTA heated to 37°C was added and the cells were incubated for 10

minutes at 37°C, 5%CO₂, 95% humidity. They were then resuspended in standard medium and centrifuged at 200g for 5 minutes. The supernatant was removed and the pellet resuspended in standard medium and the cells counted using a haemocytometer (Improved Neubauer, Weber Scientific Ltd.). The cells were then seeded onto new flasks at a concentration of 1×10^6 cells/dish in 2 ml standard media on 35 mm dishes (Corning Costar, High Wycombe, UK). After 5 days, half of the culture media was removed and replaced with fresh standard media. Thereafter, half of the culture media was replaced every 2 days with fresh standard media.

The epithelial cell concentration was adjusted to 8×10^5 CFU ml⁻¹. The bacterial cells were harvested in mid log phase by centrifugation at 14000g for 10 min, washed twice in sterile MRD and resuspended in the same buffer to yield a final density of 1.0×10^9 CFU ml⁻¹. Before any adhesion assay was performed, Caco-2 monolayers were washed twice with sterile PBS buffer (pH 7.0) at room temperature. Then, a volume (2.0 ml) of selected individual *Lactobacillus* strains were added to the cell culture and incubated for 4 hours at 37°C, 5%CO₂, 95% humidity. After incubation, monolayers were washed three times with HBSS, using a plastic Pasteur pipette, in order to remove non-adherent bacteria. Finally, monolayers were harvested and solubilised in 1% triton- X 100 (2 mls) by vortexing. Adherent bacteria were counted by plating serial 10-fold dilutions of the cell suspensions on Rogosa agar plates and incubated at 37°C, 5% CO₂ atmosphere. Each adhesion assay was conducted in triplicate.

3.2.9.3. Collagen-I (Cn-I) binding assay

Cn-I binding capacity of the potential new FLF inoculant might be useful in order to antagonize the colonization of Cn-I binding sites by pig's GI pathogens. High incidence of

Cn-I binding was reported for all EAEC and EHEC strains. Therefore, all homofermentative, aggregating lactobacilli were further tested for Cn-I binding ability.

Nunc-Immuno microtitre 96-well plates with Maxi Sorp surface (Invitrogen, UK) were coated with type I collagen solution (100 μ l) at a concentration of 100 μ g per ml subsequently incubated overnight at 4°C. Protein solutions were removed and plates were washed three times with PBS. Then PBS with BSA (200 μ l of a 2% solution in PBS) was added to each well to prevent non-specific bacterial binding. After 2 hours incubation at 25°C, BSA was removed and wells were washed twice with PBS. Finally, bacterial suspensions (100 μ l; 10^9 CFU ml⁻¹) of individual strains were added and the plates were incubated on an orbital platform shaker for 2 hours at 37°C. All unbound bacteria were removed by washing the wells three times with PBS. Bacteria in the wells were then fixed at 60°C for 20 minutes and stained with crystal violet (95 μ l per well) for 45 minutes. Wells were subsequently washed six times with PBS to remove excessive stain. After adding 100 μ l of citrate buffer (pH 4.3) to each well and 45 min incubation at room temperature to release the stain bound to bacteria, the absorbance values ($A_{550\text{nm}}$) were determined by ELISA reader and the averages of 10 absorbance values were calculated. *Lactobacillus reuteri* (NCIB 11951) was used as a positive control (Aleljung *et al.*, 1994). This strain was obtained from the National Collection of Industrial and Marine Bacteria Ltd (NCIB, Aberdeen, Scotland). Blank wells were used as negative control. Lactobacilli were classified as strongly adherent ($A_{550\text{nm}} > 0.3$), weakly adherent ($0.1 < A_{550\text{nm}} < 0.3$), or nonadherent ($A_{550\text{nm}} < 0.1$) (Styriak *et al.*, 1999a). The specificity of binding was tested by 1-hour preincubation of bacteria with an equal volume of type-I collagen solution at concentration of 100 μ g ml⁻¹ and subsequent washing followed by examination of bacterial binding to the same protein.

3.2.10. Cytokine gene expression in Caco-2 cells after *Lactobacillus* exposure

The one way by which ingested bacteria can directly influence immune system is by modulating intestinal cytokine profile. Depending on the type of cytokines produced after the stimulation, lactobacilli are able to enhance or inhibit the development of disease (Maassen *et al.*, 1998). Therefore, the ability of 2 lactobacilli strains to induce cytokines IL-12 and IL-8 after exposure to Caco-2 cells was tested in the following experiment.

3.2.10.1. Extraction of total RNA from Caco-2 cells

Differentiated Caco-2 monolayers were challenged for 5 hours with 2 *Lactobacillus* strains (10^7 CFU ml⁻¹) *L. salivarius* and *L. plantarum* (SHVD FC 36) and *Salmonella enteritidis* (NTCC, Colindale, UK) (10^7 CFU ml⁻¹) as a positive control. Caco-2 cells with no bacterial challenge were used as a negative control. Then the cell supernatant was discarded and the cell layer washed with three aliquots of 6 ml HBSS using a plastic Pasteur pipette. The cells were then harvested using a cell rake in 5 ml DTS media (no antibiotics) and transferred to a 50 ml centrifuge tube. The cells were pelleted by centrifugation at 200g for 5 minutes and after supernatant aspiration the pellets was washed in 20 ml PBS (pH 7.4) and centrifuged at 200g for another 5 minutes. Finally, the supernatant was removed and the cell pellet was ready for RNA purification. Total RNA (tRNA) from Caco-2 cells was extracted using the RNeasy[®] mini kit (Qiagen Ltd., West Sussex, UK) and following the manufacture's instructions. All solutions and plasticware used in the isolation were supplied with the kit unless stated. The cell pellet was resuspended in the buffer RA1 (350 µl) by vortexing. The buffer and cells were then transferred to a 1.5 ml RNase free eppendorf and passed four to six times through a 19

guage needle (Sherwood Medical, Hampshire, UK). 350 μ l of 70% ethanol was then added to the cleared lysate and mixed by vortexing. The tissue lysate was loaded on the RNeasy Nucleospin column placed into a 2 ml collection tube and centrifuged for 30 sec at 8000g and the flow through was discarded. Then 350 μ l of MDB buffer was added to the RNeasy column and samples were centrifuged at 11000g for 1 min. The column was then removed from the microcentrifuge tube and the liquid inside the microcentrifuge tube discarded. The RNeasy column was transferred into a new collection tube and 95 μ l DNase 1 reaction mix was added to each Nucleospin column and incubated at room temperature for 15 minutes. After incubation 200 μ l of RA2 buffer was added and the Eppendorfs were centrifuged at 8000g for 30 sec. The column was placed into a new 2 ml collection tube and after adding 600 μ l of buffer RA3 the Eppendorfs were again centrifuged at 8000 g for 30 sec. The flow-through was discarded. Another 250 μ l of buffer RA3 was added to the column and centrifuged at 11000g for 2 min. The flow-through was again discarded and the nucleospin column was placed into a 1.5 ml centrifuge tube. In order to elute RNA 50 μ l of nuclease free water was pipetted directly onto the RNeasy silica gel membrane of the column and sample was centrifuged at maximum speed for 1 min. The elution step was repeated with another 50 μ l of RNase-free water.

The concentration of RNA was quantified by spectrophotometric analysis. Total RNA samples were usually diluted 1:100 using 5 μ l of tRNA sample and 495 μ l of DEPC H₂O in a sterile, 1.5 ml, microcentrifuge tube. 500 μ l of diluted tRNA was then added to an ultra-microvolume spectrophotometer cell (Amersham Pharmacia Biotech, Buckinghamshire, UK). The optical density was measured at 260 nm and 289 nm against the same volume of DEPC H₂O (blank), using a spectrophotometer (GeneQuant II, Amersham Pharmacia Biotech). The concentration was calculated assuming $1\text{OD}_{260\text{nm}} = 40$

µg of RNA per ml (Sanbrook *et al.*, 1989). The ratio of 260 nm / 280 nm reading indicated the purity of the sample; a ratio of 2.0 indicating pure RNA (Sanbrook *et al.*, 1989).

Finally, tRNA samples were examined by electrophoresis using a 1.5% agarose denaturing gel (1.5% (w/v) agarose, 0.89 M formaldehyde, 1x MOPS buffer). The volume of sample corresponding to 20 µg of tRNA was added to a sterile 0.5 ml microcentrifuge tube. To estimate the size of RNA 3 µg of RNA size markers (range 0.28-6.58 kb, Promega UK, Southampton, UK) were added to a 0.5 ml microcentrifuge tube. The volumes of the samples and RNA size markers were equalized to 10 µl using DEPC H₂O. Then 14.2 µl of formamide was added to each sample and the samples were incubated at 45°C for 5 minutes on a heating block. The samples were then removed from the heating block and 10.7 µl of RNA load buffer was added and then the samples were incubated at 65°C for another 5 minutes. The samples were then loaded onto the gel and run at 50V for 20 minutes (PSU-400/200, Origo) in formaldehyde gel running buffer (1x MOPS buffer and 2.8% (v/v) formaldehyde in ultra pure H₂O). After 20 minutes the voltage was increased to 65V and allowed to run for 5 hours. Bands were then visualised using a UV transilluminator.

3.2.10.2. Reverse Transcription (RT) of total (t)RNA

Total RNA prepared from Caco-2 cells was reverse transcribed to single-stranded complementary DNA (cDNA) after incubation with Superscript II reverse transcriptase and random hexanucleotide primers then scaled up to a final volume of 20 µl. The RT was performed at 42°C for 50 min, followed by heating at 70°C for 15 min. 20 µl of the reaction contained 50 U MuLV reverse transcriptase, 5 mM MgCl₂, 10 mM Tris-HCl (pH

8.3), 50 mM KCl, 1.25 μ M random hexadeoxyribonucleotide (pd(N₆)) primers (random hexamer primer, Amersham Pharmacia Biotech), 0.5 U/ml Rnase inhibitor (GeneAmp[®] RNA PCR kit, Applied Biosystems), and 1mM dNTPs (Amersham Pharmacia Biotech). The resulting cDNA was stored at -20°C until use.

3.2.10.3. PCR amplification of cytokine cDNA

Cytokines were detected by the PCR method using the PCR primers (for human IL-12, IL-8). The universally expressed housekeeping gene for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an amplification control in order to verify initial equal quantities of RNA and the integrity of the RNA preparation. All the primers were purchased from Sigma-Genosys (Pampisford, UK) and their sequences (5'-3') were as follows:

IL-12_{Forward} CCTTCACCACTCCCAAAC

IL-12_{Reverse} ATCAGCTCATCAATAACTGCC

IL-8_{Forward} CTGCAGCTCTSTGTGARGSTGC

IL-8_{Reverse} CTSYACAACCYTYTGYACCCA

GAPDH_{Forward} ACCACAGTCCATGCCATC

GAPDH_{Reverse} TCCACCACCCTGTTGCTGTA

All the reagents needed for PCR reaction were supplied with the PCR kit (Qiagen, Ltd., West Sussex, UK). The PCR reaction was performed in a final volume of 50 μ l using a

thermal cycler (PTC-200 PCR system, MJ Research, Inc. Waltham). 50 μ l of the PCR mixture contained Qiagen PCR buffer (Tris-HCl, KCl, $(\text{NH}_4)_2\text{SO}_4$, pH 8.7 at 20°C), 1.5 mM MgCl_2 , Q solution, 50 pmol primer “forward”, 50 pmol primer “reverse”, 25 ng cDNA and 1.25 U Taq DNA polymerase. The program was then run as follows:

95°C/30 sec	}	35 cycles
54°C/30 sec		
72°C/60 sec		

PCR products were then separated by electrophoresis on a 1.5% agarose nondenaturing gel, stained with ethidium bromide (1.5% (w/v) agarose, 1xTBE, ethidium bromide 0.5 μ g ml^{-1} final concentration). To prepare the samples for electrophoresis 5 μ l of saturated orange G solution was added to the 50 μ l PCR product. To estimate the size of the PCR products DNA size markers were also prepared by adding 650 ng of 100 base pair markers (100-1500 base pair range, Promega UK, Southampton, UK) to a 0.5 ml microcentrifuge tube. The volume was equalized to 50 μ l by adding 45 μ l DEPC H_2O . Then 5 μ l of saturated orange G was added. The gel was run at a constant voltage of 100 V for 2 hours and the bands were visualized using a UV transilluminator. Relative amounts of cytokine mRNA were estimated by dividing the densitometric area of the cytokine PCR band by the densitometric area of the housekeeping gene GAPDH.

3.2.11. Identification of lactobacilli

Lactobacillus strains were identified using API CHL kit analysed by API LAB Plus software version 4.0 database (BioMérieux, France). 16sRNA PCR was used to confirm the

species identification as demonstrated in previous studies.

3.2.11.1. API50 CHL fermentation assay

Overnight cultures of lactobacilli isolates grown in 10 ml MRS broth at 30°C were washed twice with sterile physiological saline (0.9% sodium chloride) and pellets were suspended in API 50 CHL medium (API systems, BioMérieux (UK), Ltd.). Using sterile Pasteur pipettes, homogenized suspensions of the cells in the medium, with subsequent vortex mixing, were transferred into each of the 50 wells on the API 50 CH strips (API systems, BioMérieux (UK), Ltd.). This was done for all isolates and type strains. All wells were overlaid with sterile mineral oil (BioMérieux (UK), Ltd.) to maintain anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 30°C. Changes in colour from violet were monitored after 1, 2 and 3 days. Results for each of the 49 strips were graded from 0 to 5, where 5 denoted a complete change to yellow and 0, no change at all. The first strip served as a control well. Aesculin hydrolysis (revealed by a change to a darker colour or black) was represented by a positive sign (+) while a negative sign (-) represented no change. The biochemical profiles obtained from individual strains were recorded on the results sheets and sent to BioMérieux (UK), Ltd. for identification by identification software.

3.2.11.2. 16S rRNA PCR

Bacterial DNA extractions from 5 candidate lactobacilli were performed by using Wizard® Genomic DNA Purification kit (Promega, Southampton, UK) and following the manufacturer's instructions (Appendix 4). Briefly, the method is based on a four-step

process. The first step in the purification procedure lysed the cells. The cellular proteins were then removed by a salt precipitation step. Finally, the genomic DNA was concentrated and desalted by isopropanol precipitation. Just 1 ml of an overnight bacterial broth culture is sufficient for DNA extraction with this system. Bacterial suspensions were firstly centrifuged for 2 minutes at 13,000-16,000g. The culture medium was removed and pellet was resuspended thoroughly in 480 μl of 50 mM EDTA (pH=8). Then 120 μl of Lysozyme solution (10 mg ml^{-1}) (Sigma-Aldrich, Product code L 7651) was added to the resuspended cell pellet and incubated for 30-60 minutes at 37°C. After 2-min centrifugation the supernatant was removed. Then bacterial cells were incubated with appropriate enzymes provided in the kit to ensure efficient cell lysis and DNA release from the cells. Isolated DNA was stored at 4°C. DNA purified with this system is suitable for immediate PCR applications.

The universal 16S rRNA gene forward and reverse primers (5' to 3') were AGAGTTTGATCCTGGCTCAGG and ACGGCAACCTTGTTACGAGTT, respectively. The primers were purchased from Sigma-Genosys (Pampisford, UK). PCR master mix contained: 5 μl 10 x Qiagen PCR buffer, 5 μl 10 x dNTP's, 10 μl 5 x Q solution (Qiagen Ltd., West Sussex, UK) 5 μl forward primer (10 pmol μl^{-1}), 5 μl reverse primer (10 pmol μl^{-1}), 5 μl enzyme mix, 5 μl DEPC H_2O . Enzyme mix contained: 1 μl *Taq* DNA polymerase (5 units / μl), 2 μl 10 x Qiagen PCR buffer, 17 μl H_2O . The PCR reaction was set up in 0.2 ml microcentrifuge tubes as follows: 35 μl of PCR buffer master mix was added to each tube. Note that PCR buffer and Q solution are supplied with *Taq* DNA polymerase enzyme (Qiagen Ltd, West Sussex, UK). Then 10 μl of cDNA from the species to be amplified was added to each tube, mixed by flicking then centrifuged at 10000 g / 30 seconds and placed in the thermal cycler (PTC-200 PCR system, MJ Research, Inc.

Waltham). The reaction was denaturated at 95°C for 2 minutes then held at 70°C while 5 µl of enzyme mix (pre-warmed to approximately 70°C using heating block) was added. The reaction was mixed by flicking, centrifuged at 10000g for 30 seconds, then replaced in the thermal cycler. The program was then run as follows:

95°C/30 sec	}	35 cycles
57°C/30 sec		
72°C/120 sec		

The PCR products were then resolved by electrophoresis on 1.5% agarose non-denaturing gel (1.5% (w/v) agarose, 1x TBE, ethidium bromide 0.5 µg ml⁻¹ final concentration). 3 g of agarose was dissolved in 200 ml of 1 x TBE by boiling in a microwave. After the solution had cooled to approximately 45-55°C, ethidium bromide (10 mg ml⁻¹) was added to 0.5 µg ml⁻¹, mixed and the gel poured into the casting tray and left to set. To prepare the samples for electrophoresis 5 µl of saturated orange G solution were added to the 50 µl PCR product. To estimate the size of the PCR product, DNA size markers were also prepared by adding 650 ng of 100 base pair markers (100-1500 base pair range, Promega UK, Southampton, UK) to a 0.5 ml microcentrifuge tube. The volume was equalized to 50 µl by adding 45 µl DEPC H₂O. Then 5 µl of saturated orange G were added. The PCR product and DNA size markers were loaded onto 1.5% agarose gel. The gel was run at a constant voltage of 100V for 1 hour. The gel bands were visualised using a UV transilluminator.

3.2.11.3. Sequencing of PCR products

Purification of DNA from agarose gel

The DNA contained in the band was eluted using QIAquick gel extraction kit (Qiagen Ltd., West Sussex, UK). All solutions and reagents used were supplied with the kit unless otherwise stated. The weight of the excised gel piece was determined and assuming that $1 \mu\text{l} = 1 \mu\text{g}$, 3 μl of buffer QG was added for each μg of excised gel. The gel piece was dissolved by incubation on a heating block at 50°C for 10 minutes. Then 1x gel volume of 100% isopropanol was added and mixed by inversion. To bind the extracted DNA, the sample was applied to a column, placed in a 2 ml microcentrifuge tube and centrifuged for 1 minute at 10000g. The eluant collected was discarded and 0.5 ml of buffer QG was added to the column and centrifuged at 10000g for 1 minute. The flow through was discarded and 0.75 ml buffer PE was added to the column. The column was allowed to stand for 5 minutes, and then centrifuged at 10000g for 1 minute. The flow through was discarded and the column re-centrifuged at 10000g for 1 minute. The column was then placed in a sterile 1.5 ml microcentrifuge tube and 50 μl of buffer EB added to elute the DNA. The column was allowed to stand for 1 minute, then centrifuged at 10000g for 1 minute. The flow through, collected in the 1.5 ml microcentrifuge tube, contained the extracted DNA and was stored at -20°C .

Sequencing reaction

Sequencing reactions of the PCR products were performed using a fully automated capillary DNA sequencing system (Beckman CEQ2000, Beckman Coulter Inc.) at the Rowett Research Institute in Aberdeen. Samples were prepared for sequencing using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (applied Biosystem,

Perkin-Elmer) according to the manufacturer's protocol. The retrieved sequences were compared to those available in nucleic acid databases using BLAST (NCBI) on the search browser Pub Med in order to determine the closest relatives.

3.2.12. Study of carbohydrate preference of selected *Lactobacillus salivarius*

In order to assess the sources of carbohydrates, which are preferred by the selected candidate and to determine which substrates would be more suitable to include in the fermentations, an experiment was carried out involving MRS broth containing different carbohydrate sources. A modified version of MRS broth was prepared as described for the homo/heterofermentative tests, but with the chlorophenol red solution omitted. The carbohydrate source was altered, to include the sugars glucose and sucrose, at a concentration of 2% (w/v). All the following steps were exactly the same as described for acid and bile resistance. The bioscreen machine assessed the growth in the media. This was carried out at 30°C. *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) was used as a control and the growth rates were compared using MicroFit[®] software (version 1.0; Norwich, UK).

3.2.13. Total starch and proteins assays of liquid feed

Fermentation of pig feed is assumed to be a complex process and the decrease in dry matter of the diet after the fermentation has been reported in several studies (Moran, 2001; Geary, 1997). In order to investigate the fermentation effect on the starch and proteins concentrations of the pig diet, the following tests were conducted.

Total starch assay of FLF and NFLF was determined using standard methods (AOAC method 996.11; AACC Method 76.13; ICC Standard method No. 168) using a commercial 'total starch assay' kit (Megazyme International Ireland Ltd., Bray, Ireland) and following the manufacturer's instructions. This kit was design to allow the measurement of total starch in most cereal products (natural and processed).

Crude protein assay of FLF and NFLF was determined by Dumas combustion method (AOAC Nr. 96806) on a LECO[®] FP-2000 nitrogen/protein combustion analyzer (LECO Corporation, Michigan, USA) (AOAC, 1995) according to the manufacture's instructions. The LECO[®] FP-2000 analyzer is a microprocessor based, software-controlled instrument that determines the nitrogen content in variety of biological materials. The instrument was calibrated using ethylenediaminetetraacetic acid (EDTA 9.56% nitrogen). Dumas combustion analysis for total nitrogen in biological sample is based on the transformation to gas phase by extremely rapid and complete flash combustion of the sample material. Nitrogen-bearing combustion products include N₂ and various oxides of nitrogen NO_x; these pass through a reduction column filled with chopped Cu wire (600°C), in which the nitrogen oxides give up their oxygen to the copper and emerge as N₂. Final results are displayed as weight percentage of nitrogen. A factor 6.25 was used to convert nitrogen values to proteins. Both total starch assay and crude proteins assay were performed in triplicates.

3.3. Results

Bacterial growth, acid and gas production of faecal lactobacilli

All 87 lactobacilli tested were positive for acid production. Only one strain (SHVD FC48) showed poor growth in both MRS broth and MRS agar. Therefore it was not used further in this study. Among all lactobacilli tested, 35 strains were negative for gas production and therefore, only those homofermentative strains were used for further screening.

Aggregation experiments

From the 35 homofermentative *Lactobacillus* sp. strains autoaggregative activity was found in 13 isolates (Table 3.3). The addition of saline-washed *Lactobacillus* cells to their own supernatant caused rapid autoaggregation, in six strains within 15-20 minutes (+ + +); the effect was also observed by SEM (Figure 3.1). Two others strains (SHCM 27 and SHCM 28) had a normal reaction (+ +) and the rest of the strains revealed only weak (+) autoaggregation activity. Mixed cultures of lactobacilli, with porcine pathogen *E. coli* K99 showed co-aggregation and as an example Figure 3.2 shows the microscopic (SEM) appearance of the aggregates formed between *E. coli* and *Lactobacillus* cells.

Acid resistance

Figures 3.3 shows the bioscreen curves for the growth of all 13 aggregating lactobacilli strains at 30°C at pH 3.5. It is quite clearly seen that lactobacilli expressed different level of acid resistance. Six *Lactobacillus* strains (SHVD FC 36, SHVD FC 51, SHVD FC 23,

SHVD FC 75, SHCM FC 28, SHVD FC 28) performed well and may be thus regarded as acid tolerant. SHCM FC 15, SHCM FC 27 and SHVD FC 71 could be considered as acid tolerant however, their growth in an acid environment was clearly affected, as they were not able to reach the stationary phase even after 72 hours. The rest of the tested strains (SHCM FC 26, SHVD FC 57, SHVD FC 73, SHVD FC 72) were not able to grow at the pH 3.5.

Table 3.3. Autoaggregation patterns of porcine lactobacilli

<i>Lactobacillus</i> sp.	Autoaggregation
SHCM FC 15	+
SHCM FC 26	+
SHCM FC 27	++
SHCM FC 28	++
SHVD FC 23	+++
SHVD FC 28	+
SHVD FC 36	+
SHVD FC 51	+++
SHVD FC 57	+++
SHVD FC 71	+++
SHVD FC 72	+++
SHVD FC 73	+++
SHVD FC 75	+

Autoaggregation (+++ rapid, ++ normal, + slow)

Figure 3.1. Scanning electron micrographs (SEM) of non-aggregating *L. plantarum* and some examples of aggregating porcine *Lactobacillus* sp. (Magnification: A,B,C,E 5000X, D 2000X; F 13000X; bar 1µm).

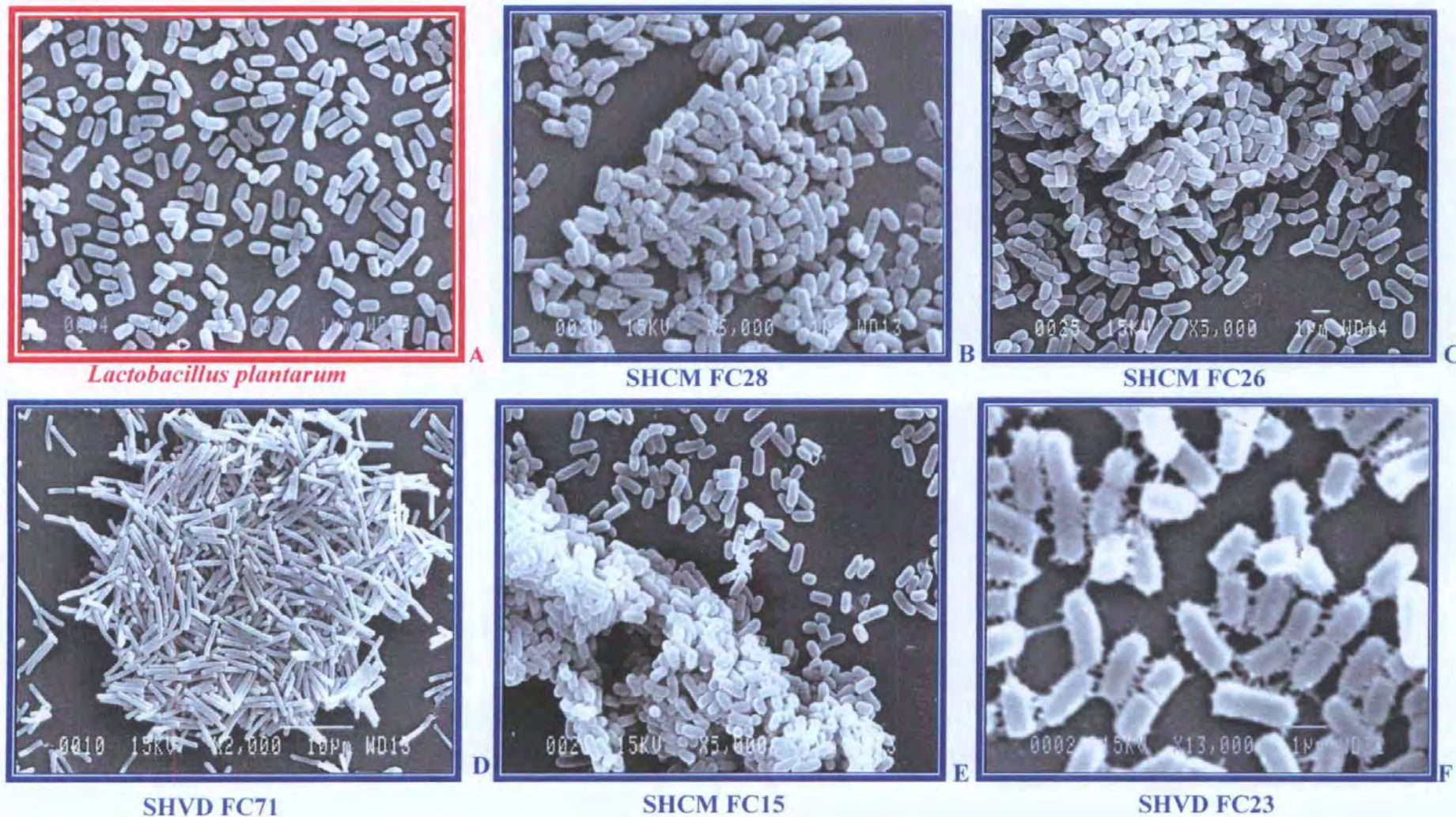
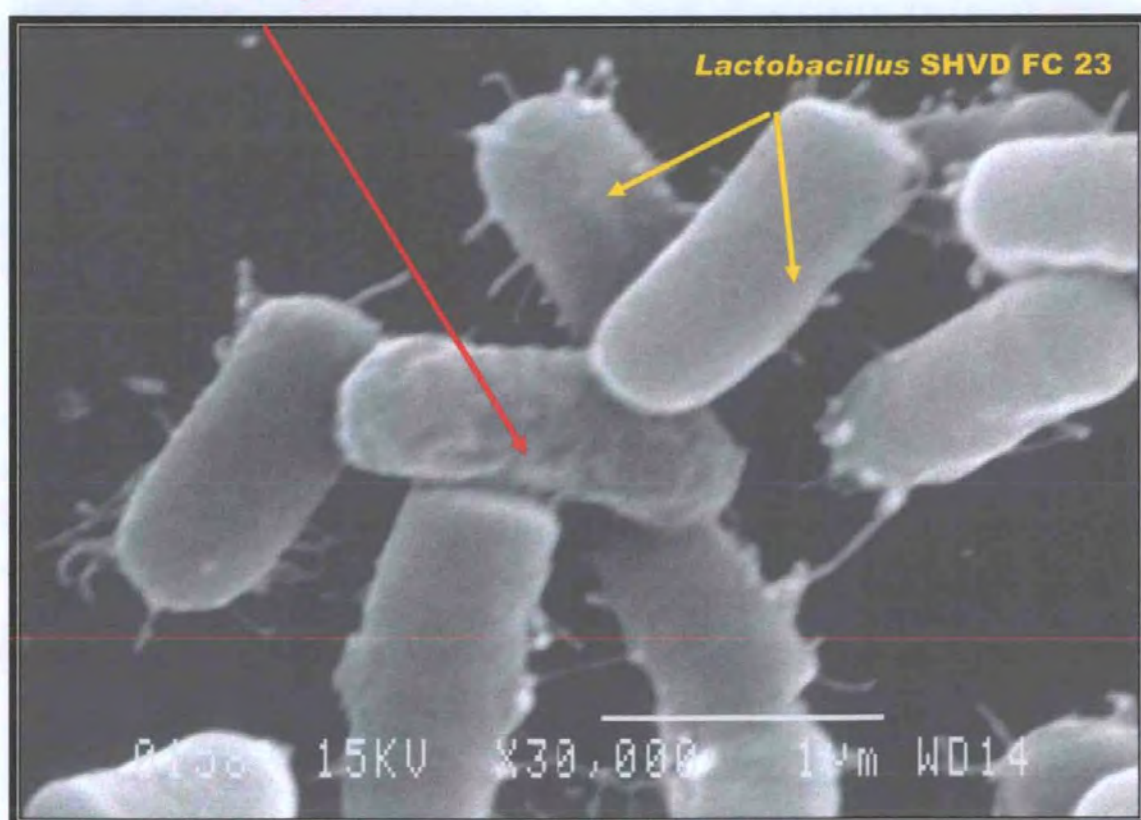
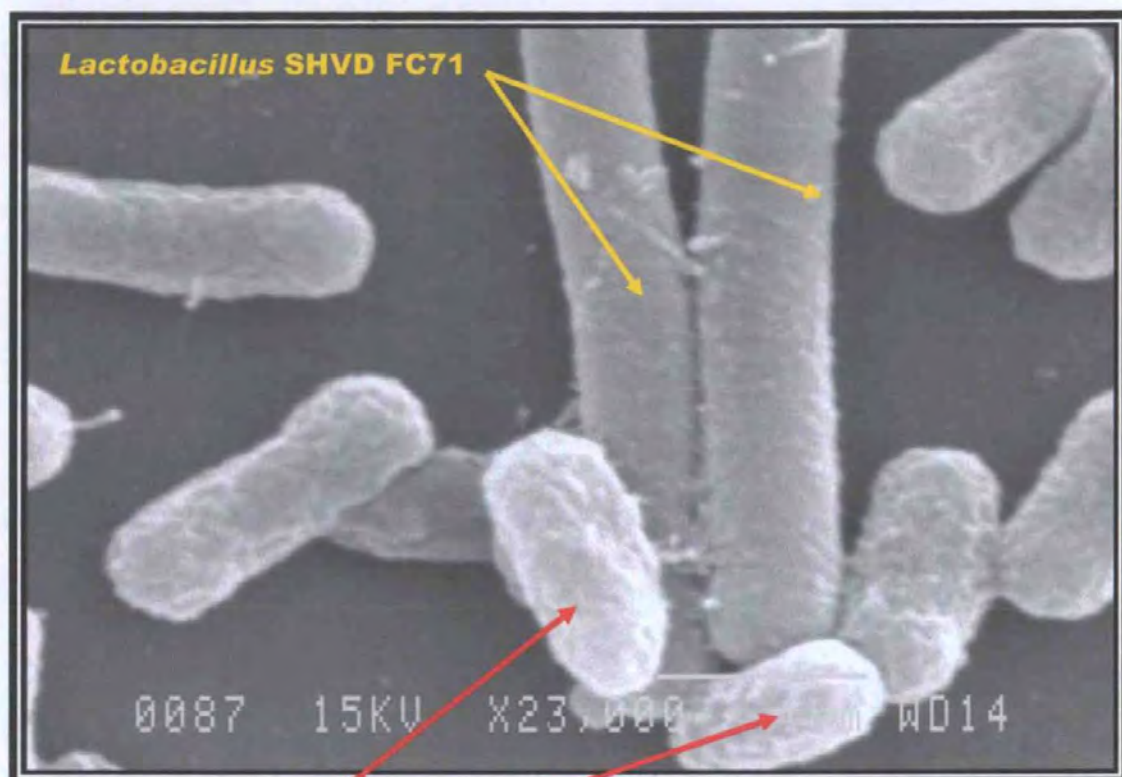


Figure 3.2. Example of *Lactobacillus* sp. – *E. coli* K99 coaggregation by scanning electron microscopy (SEM). (Magnification: A-23 000X; B-30 000X; bar = 1 μ m).



Bile-salt tolerance

The bile-salt tolerance varied among the porcine *Lactobacillus* isolates (Figure 3.4). Two strains SHVD FC 28 and SHVD FC 23 showed the highest degree of bile resistance, followed by SHVD FC 36, SHCM FC 28 and SHCM FC 15. On the other hand, five strains (SHVD FC 73, SHVD FC 57, SHCM FC 27, SHVD FC 71, SHCM FC 26) were not able to growth in the presence of porcine bile (0.3%). The remaining three strains (SHVD FC 75, SHVD FC 51, and SHVD FC 72) expressed some degree of bile tolerance however their growth was delayed and significantly affected.

Analysis of organic acid production and pH

The in-feed lactic acid production data at 30°C are given in Tables 3.4. Changes in pH after 24, 48 and 72 hours of fermentation are shown in Table 3.5.

Figure 3.3. Resistance of aggregating bacteria to pH = 3.5 at 30°C.

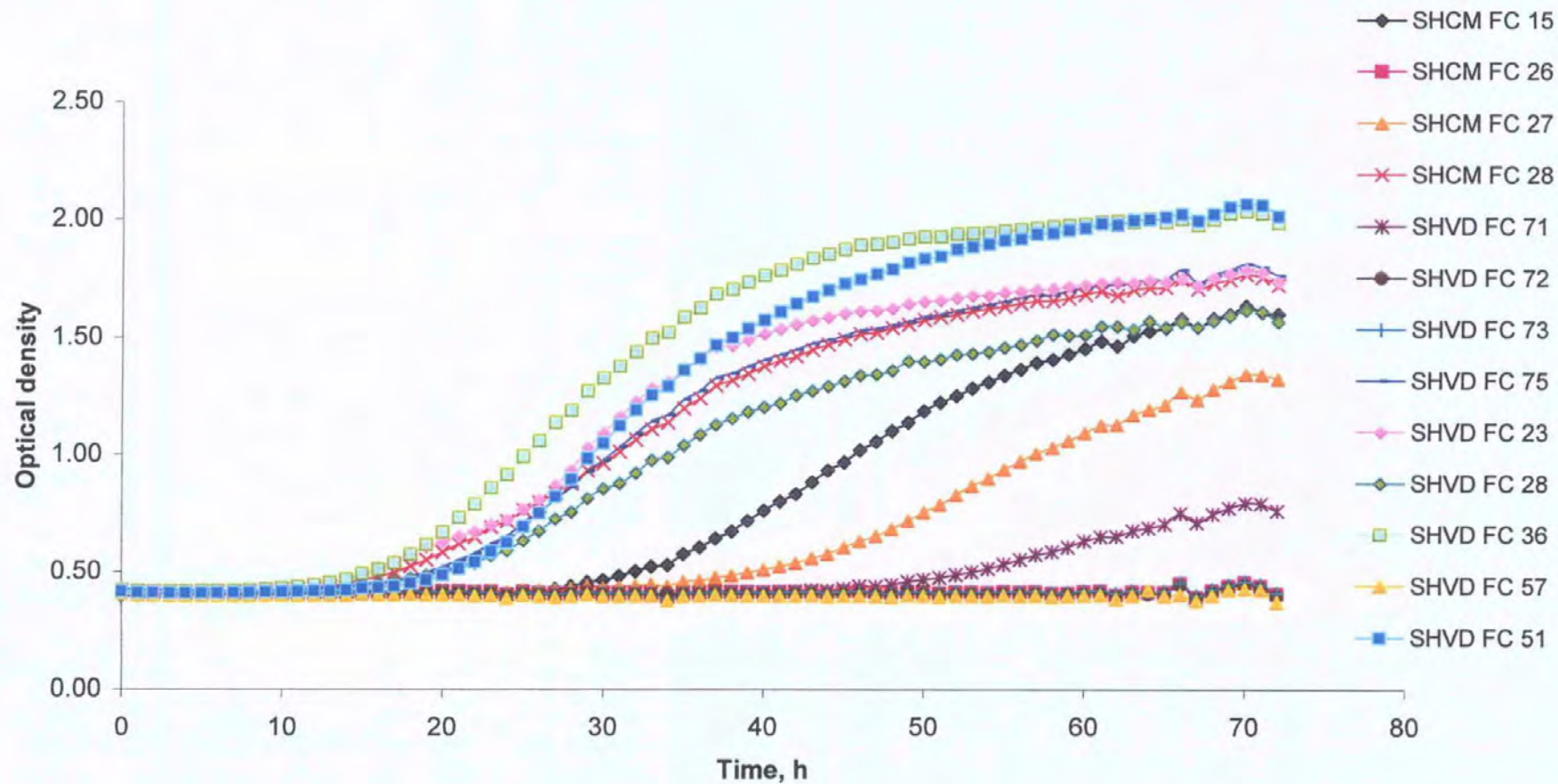


Figure 3.4. Bile-salt tolerance of porcine aggregating bacteria at 30°C.

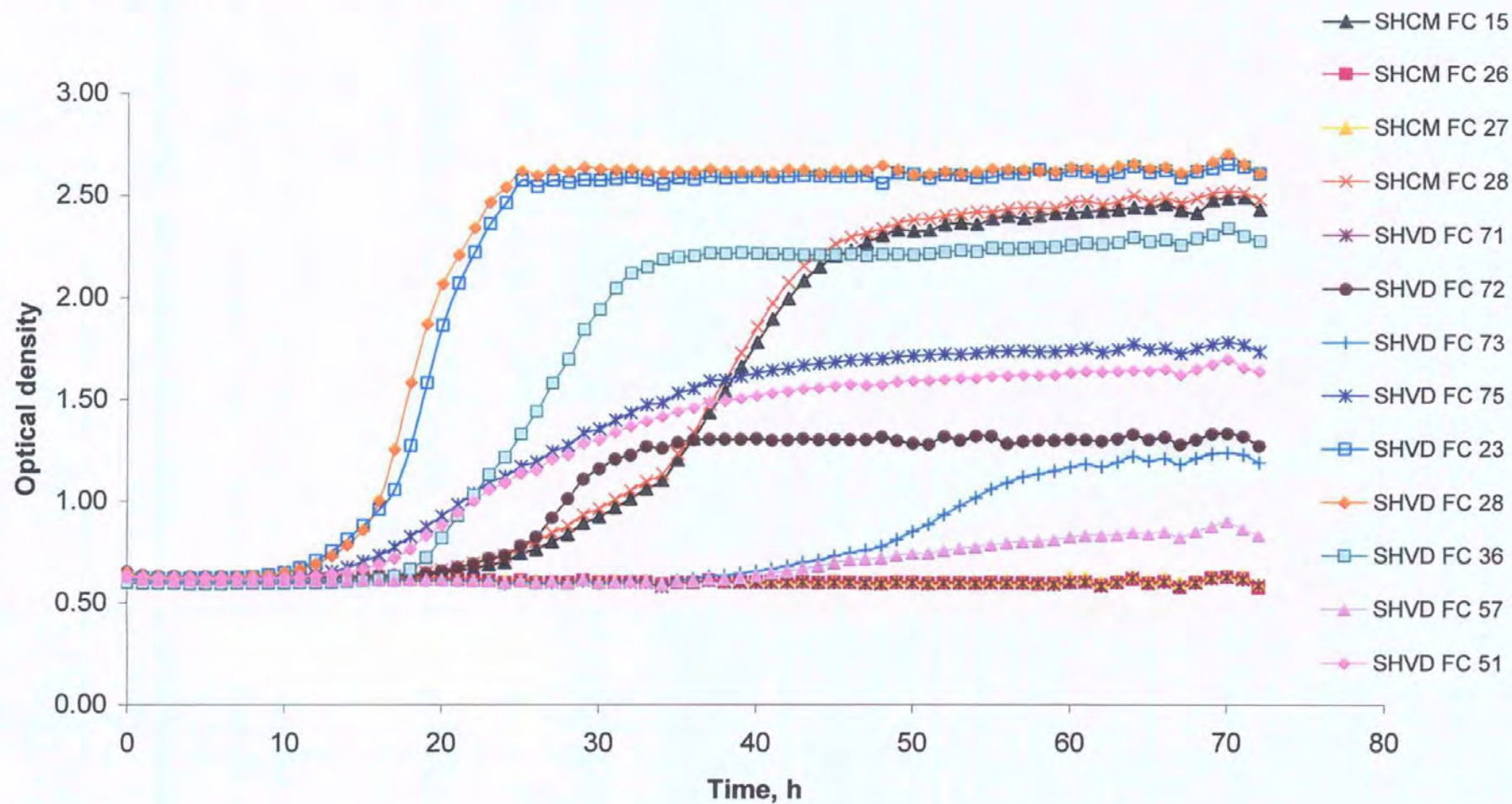


Table 3.4. Concentrations (mmol L⁻¹) of lactic and acetic acids produced by aggregating porcine lactobacilli (1% inoculum) after 24, 48 and 72- hour fermentation (30°C) of commercial sow feed.

LACTIC ACID						
Strain	24h	SD	48h	SD	72h	SD
SHCM FC 15	166.62	13.22	238.01	2.51	255.07	5.06
SHCM FC 26	222.86	4.43	273.43	3.09	272.23	15.82
SHCM FC 27	234.93	3.09	257.24	9.26	295.65	15.82
SHCM FC 28	234.68	7.76	282.92	0.82	267.91	11.46
SHVD FC23	210.8	1.62	248.1	36.8	281	41.9
SHVD FC 28	239.12	4.16	271.8	9.65	262.22	3.03
SHVD FC 36	223.56	1.71	257.91	6.57	287.93	1.12
SHVD FC 51	211.5	5.91	257.02	3.83	280.19	8.41
SHVD FC 57	230.18	2.04	282.8	8.05	287.53	8.65
SHVD FC 71	206.55	2.64	264.49	9.35	267.27	5.11
SHVD FC 72	231.48	1.41	278.96	6.58	270.92	1.75
SHVD FC 73	212.12	1.87	271.93	2.62	274.93	6.4
SHVD FC 75	229.3	2.49	259.79	0.12	268.08	3.93
<i>L. plantarum</i>	225.55	4.8	266.03	8.42	317.9	17.4

ACETIC ACID						
SHCM FC 15	3.82	0.23	4.81	0.15	5.49	0.063
SHCM FC 26	8.36	0.36	9.81	0.12	9.75	0.59
SHCM FC 27	9.47	1.18	10.75	0.29	9.79	0.93
SHCM FC 28	8.49	0.41	10.08	0.11	9.99	0.71
SHVD FC 23	10.17	0.14	11.26	0.17	12.2	1.75
SHVD FC 28	9.77	0.15	11.25	0.45	11.18	0.10
SHVD FC 36	6.23	0.23	9.52	0.22	9.57	0.24
SHVD FC 51	3.14	0.10	4.61	0.34	5.47	0.08
SHVD FC 57	10.11	0.11	11.46	0.28	11.69	0.47
SHVD FC 71	9.43	0.19	10.89	0.39	10.98	0.17
SHVD FC 72	3.93	0.10	5.084	0.11	5.35	0.13
SHVD FC 73	10.58	0.08	14.23	0.11	15.04	0.44
SHVD FC 75	11.48	0.33	14.94	0.13	15.79	0.42
<i>L. plantarum</i>	10.98	0.03	14.05	0.49	18.09	0.18

Table 3.5. pH of the sow feed fermented by porcine aggregating lactobacilli after 24, 48 and 72 hours.

<i>Lactobacillus</i>	0h	24h	48h	72h
SHCM FC 15	5.46	4.04	3.81	3.7
SHCM FC 26	5.47	3.85	3.76	3.71
SHCM FC 27	5.48	3.82	3.78	3.75
SHCM FC 28	5.47	3.86	3.77	3.70
SHVD FC23	5.47	3.91	3.80	3.72
SHVD FC 28	5.46	3.81	3.76	3.68
SHVD FC 36	5.47	3.85	3.74	3.65
SHVD FC 51	5.47	3.84	3.77	3.69
SHVD FC 57	5.47	3.80	3.76	3.70
SHVD FC 71	5.46	3.89	3.83	3.74
SHVD FC 72	5.47	3.80	3.75	3.67
SHVD FC 73	5.47	3.82	3.76	3.68
SHVD FC 75	5.47	3.88	3.76	3.69
<i>L. plantarum</i>	5.47	3.94	3.83	3.73

Type-I Collagen and porcine mucus microtitre plate binding assay

Screening procedures to investigate Collagen-I (Cn-I) and porcine mucin binding of 13 aggregating lactobacilli were performed. None of the *Lactobacillus* strain tested expressed strong adherence ability ($OD_{550nm} > 0.3$) to Cn-I or mucus (Figure 3.5). Three porcine lactobacilli (SHCM FC 28, SHVD FC 71, SHVD 36) were shown to have low to moderate ($0.1 < OD_{550nm} < 0.3$) Cn-I adherence while the remaining 10 strains did not bind to Collagen-I at all. Two strains (SHCM FC 28 and SHVD FC 71) binding to Cn-I also bound to porcine mucus (Figure 3.6).

Figure 3.5. Collagen-I (Cn-I) binding by 13 aggregating porcine lactobacilli, *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA), and control strain *Lactobacillus reuteri* NCIB 11951.

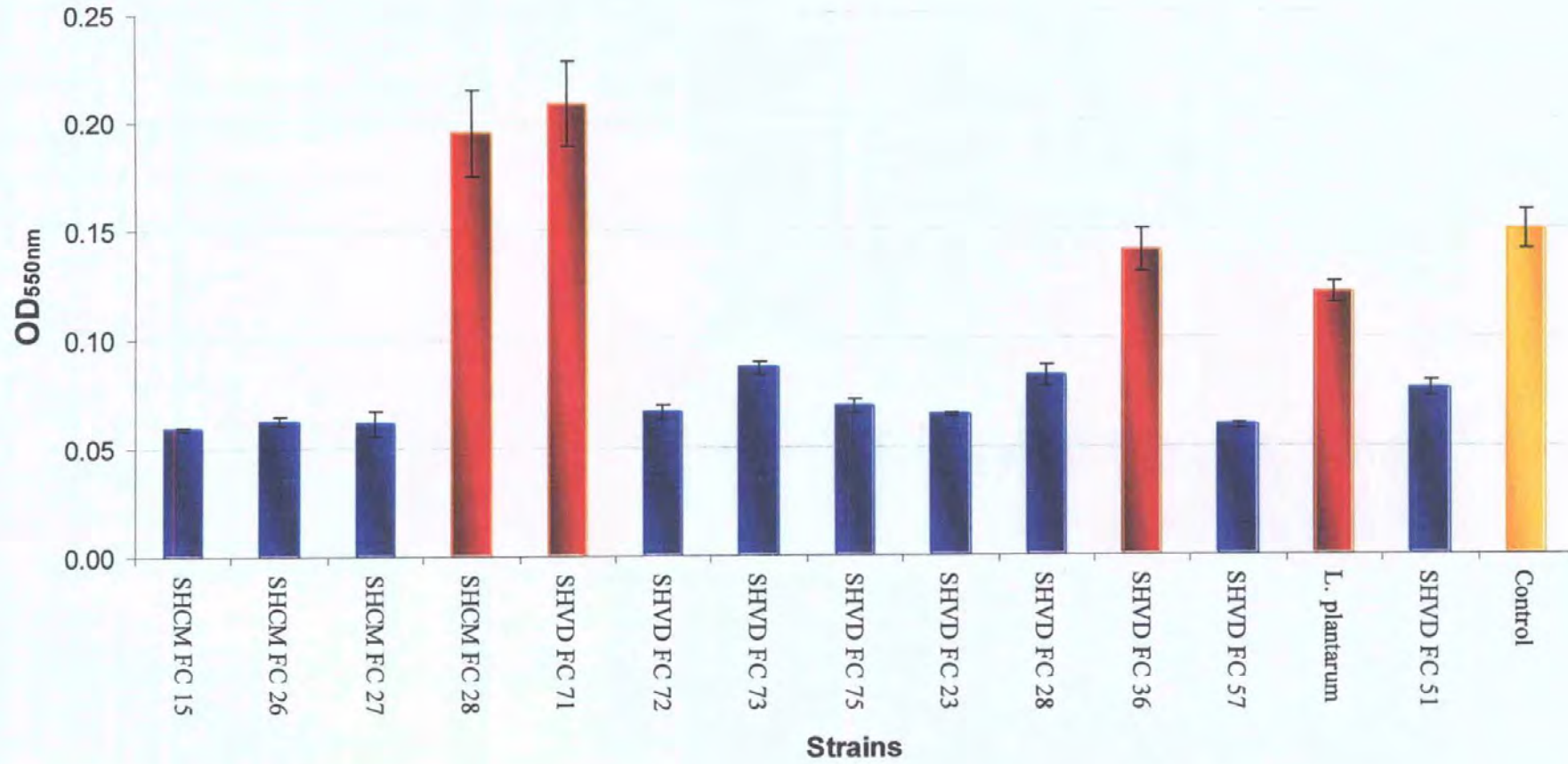
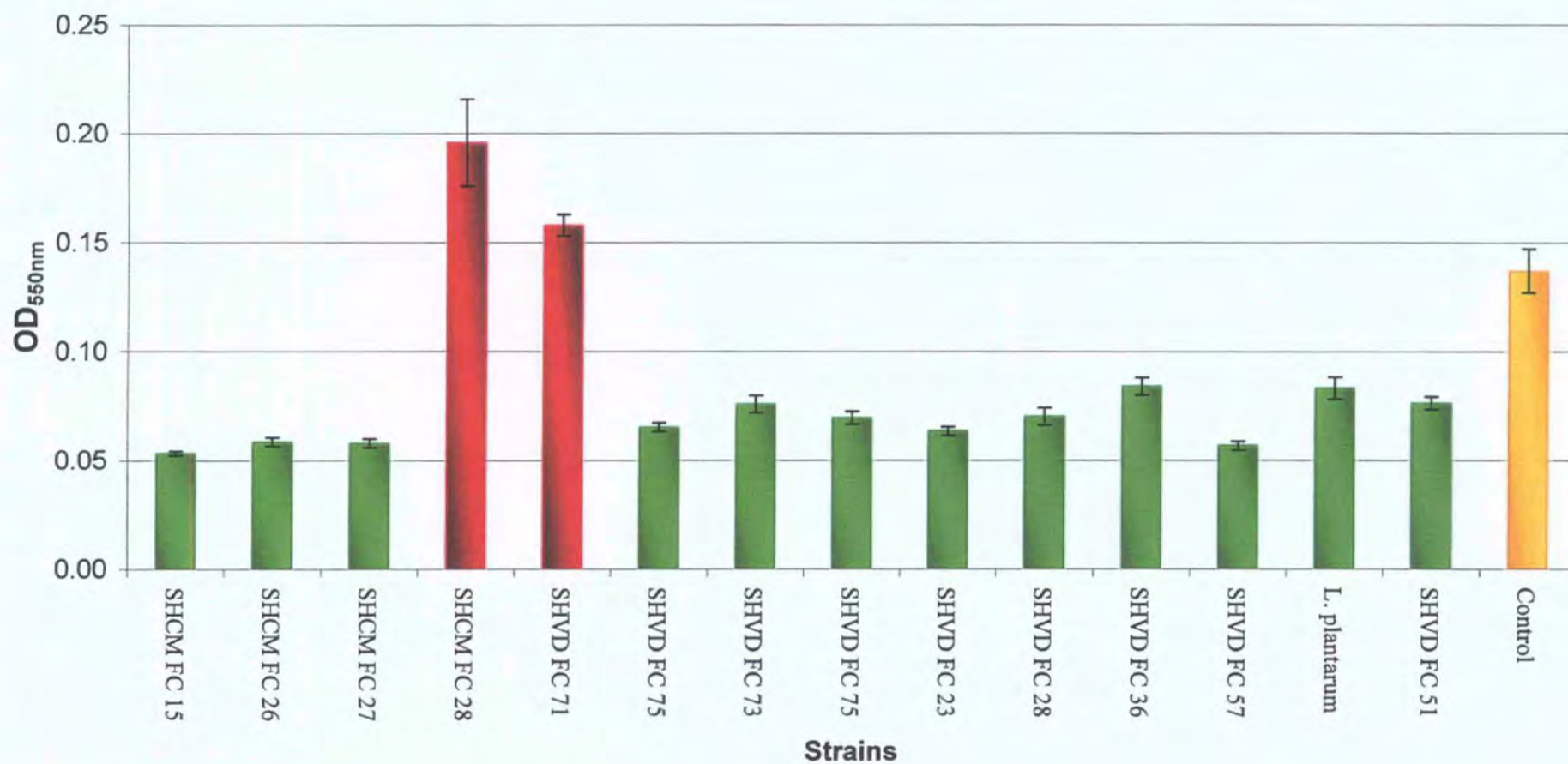


Figure 3.6. Mucus binding by 13 aggregating porcine lactobacilli, *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA), and control strain *Lactobacillus reuteri* NCIB 11951.



Silage isolate *Lactobacillus plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) also showed low affinity to (Cn-I) (Figure 3.5) however, it did not bind to porcine mucus (Figure 3.6).

Adhesion assay to Caco-2 cells

The four *Lactobacillus* candidates from previous screening (SHCM FC 28, SHVD FC 28, SHVD FC 71, and SHVD FC 36), which expressed good fermentative ability in addition to acid and bile tolerance and adherence properties to Cn-I and porcine mucus, were evaluated for adherence to human Caco-2 cell culture (Table 3.6). All lactobacilli tested adhered to some extent to Caco-2 cells. Among all of them *Lactobacillus* SHCM FC 28 showed the highest percentage of adherence (Table 3.6), followed by *Lactobacillus* SHVD FC 28. Compared with SHCM FC 28 *Lactobacillus* SHVD FC 36 and SHVD FC 71 were only weakly adherent.

Table 3.6. Adhesion of 4 porcine *Lactobacillus* strains to Caco-2 cell monolayers.

<i>Lactobacillus</i> sp.	Initial numbers (Log ₁₀ CFU ml ⁻¹)	Adherent cells (Log ₁₀ CFU ml ⁻¹)	% of binding
SHCM FC 28	9.1 ± 0.07	6.43 ± 0.14	71.44
SHVD FC 28	9.2 ± 0.05	5.24 ± 0.29	56.96
SHVD FC 71	9.0 ± 0.01	4.40 ± 0.05	48.89
SHVD FC 36	9.2 ± 0.06	4.30 ± 0.09	46.74

Data are expressed as a mean (Log₁₀CFU ml⁻¹) ± SD.

Cytokine gene expression in Caco-2 cells induced by lactobacilli

To assess the effect of porcine lactobacilli on modulation of the cytokine response by enterocyte-like Caco-2 cells, mRNA expression of two different cytokines (IL-12 and IL-8) was evaluated by the RT-PCR method. The strain with the highest (SHCM FC28) and the lowest percentage (SHVD FC36) of binding to Caco-2 cells were chosen for this test in order to see whether different adherence potential will result in different level of cytokine production. The results showed that both lactobacilli upregulated mRNA expression of IL-12, but did not induce mRNA expression of the proinflammatory cytokine IL-8 (Figure 3.7).

Figure 3.7. Differential cytokine mRNA expression in Caco-2 cells. Reverse transcription-PCR (RT-PCR) analysis was used to determine interleukin (IL)-12 and IL-8 mRNA expression in Caco-2 cells after lactobacilli exposure (5 hours, 10^7 CFU ml⁻¹). *Salmonella enteritidis* and culture medium (no bacteria) were used as controls. The signal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize for differences in RNA extractions and for different efficiencies of cDNA synthesis.

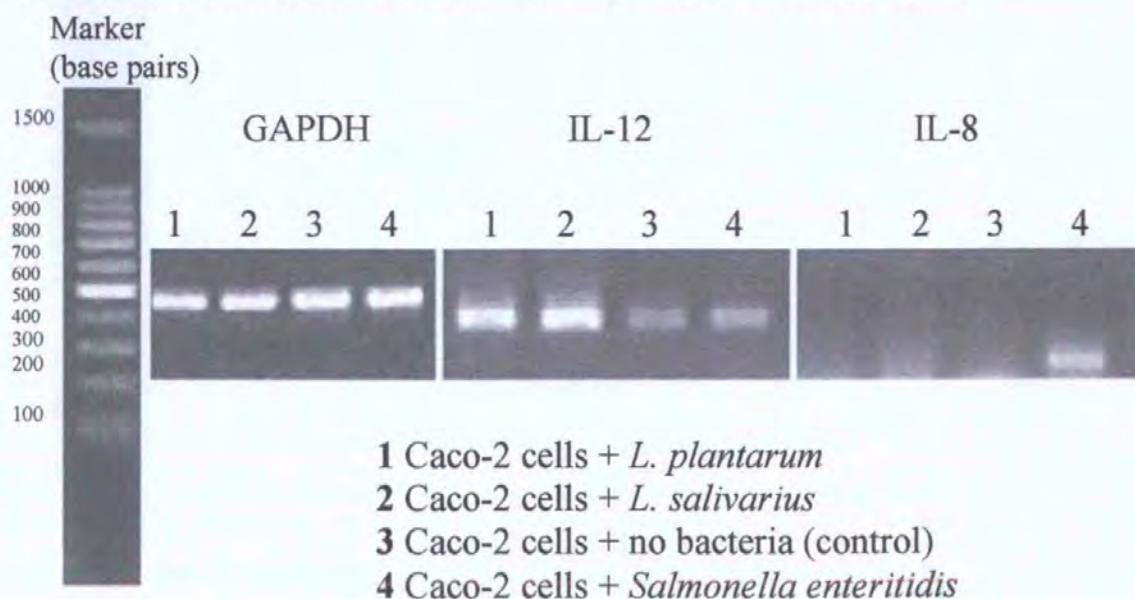
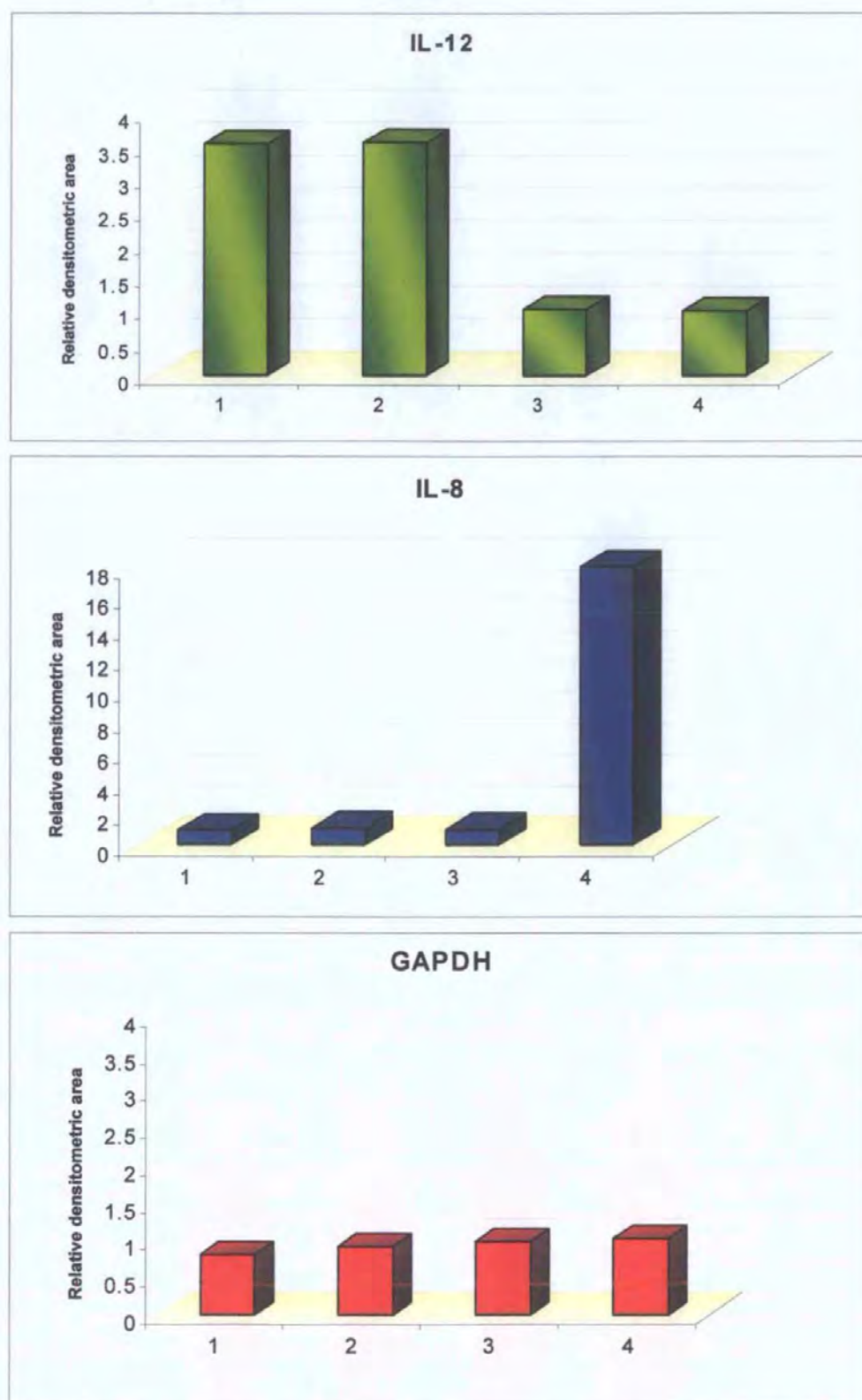


Figure 3.8. Cytokine (IL-12 and IL-8) mRNA levels in Caco-2 cells after *Lactobacillus* and *Salmonella* exposure, all normalized to mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results represent n-fold increase of cytokines over unstimulated Caco-2 cells, which served as a calibrator.



(1 Caco-2 cells + *L. plantarum*; 2 Caco-2 cells + *L. salivarius*; 3 Caco-2 cells + no bacteria (control); 4 Caco-2 cells + *Salmonella enteritidis*).

In contrast, *Salmonella* challenge did not affect IL-12 level, however significantly up-regulated expression of IL-8 (Figure 3.7). Analyses of densitometric area of the cytokine PCR bands showed that both lactobacilli elevated IL-12 mRNA expression approximately 3.5 fold compared with unstimulated Caco-2 cells (Figure 3.8). The signals of GAPDH mRNA, which verifies initial quantities of RNA and the integrity of the RNA preparation, were equal in all samples (Figure 3.7; Figure 3.8).

Identification of lactobacilli

In order to identify the 4 *Lactobacillus* strains (SHCM FC 28, SHVD FC 28, SHVD FC 36 and SHVD FC 71) which were used in all screening assays (except the cytokine test where only 2 lactobacilli were tested), API identification kit and 16S rRNA identification method were used. Together with these 4 strains, *Lactobacillus* SHVD FC 51 was also identified as it exerted the strongest autoaggregation potential and very good acid and bile tolerance and fermentation characteristics. Table 3.7 shows that identification of 5 candidate lactobacilli based on API 50 CH fermentation data were not in agreement with genetic identification. According to fermentation profiles all the *Lactobacilli* tested were identified as a *L. plantarum*. Determination of 16S rRNA gene sequences identified 3 x *L. plantarum* / *paraplantarum* (SHVD FC 28 and SHVD FC 51, SHVD FC 36), 1 x *L. salivarius* (SHCM FC 28), and 1 x *L. farciminis* (SHVD FC 51).

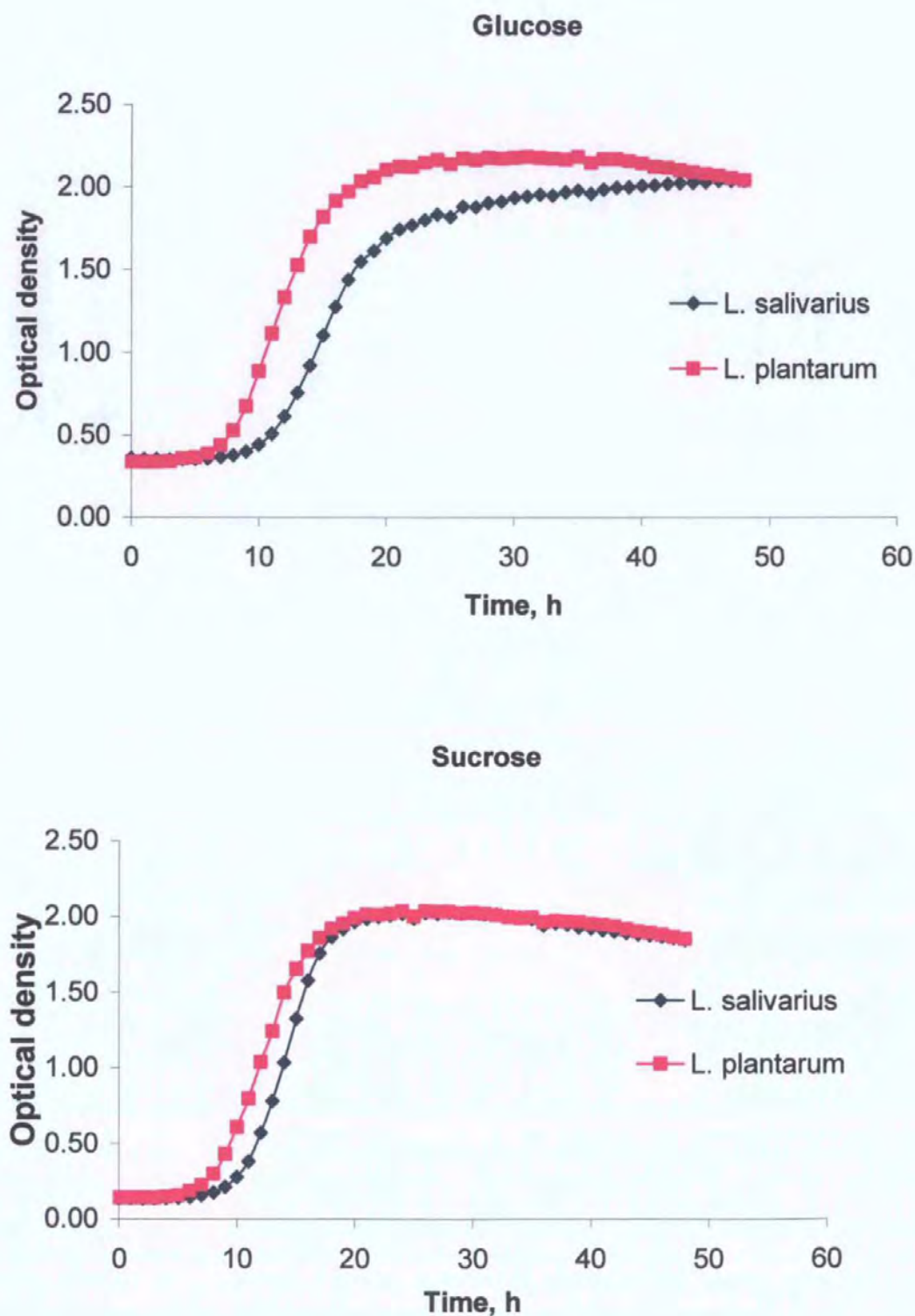
Table 3.7. Identification of candidate strains by API 50 CHL and 16S rRNA PCR.

Strain	API 50CHL	%Id	16S rRNA PCR
SHCM FC 28	<i>Lactobacillus plantarum</i>	99.9	<i>Lactobacillus salivarius</i>
SHVD FC 28	<i>Lactobacillus plantarum</i>	99.7	<i>Lactobacillus plantarum</i> / <i>paraplantarum</i>
SHVD FC 36	<i>Lactobacillus plantarum</i>	99.9	<i>Lactobacillus plantarum</i> / <i>paraplantarum</i>
SHVD FC 51	<i>Lactobacillus plantarum</i>	99.8	<i>Lactobacillus farciminis</i>
SHVD FC 71	<i>Lactobacillus plantarum</i>	99.9	<i>Lactobacillus plantarum</i> / <i>paraplantarum</i>

Carbohydrate preference of selected Lactobacillus candidate

Based on the previous screening tests, the *L. salivarius* was selected for the last 2 experiments. In order to assess the sources of carbohydrates which are preferred by the selected *L. salivarius* strain an experiment was carried out involving MRS broth containing different carbohydrate sources. The above strain was compared with the FLF inoculum *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) currently used in our laboratory.

Figure 3.9. Growth of *L. salivarius* and *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) in the presence of different carbohydrates (glucose and sucrose)



The results showed that *L. plantarum* possessed significantly better growth characteristics compared with *L. salivarius* in the presence of both carbohydrates tested (Figure 3.7, Table 3.8).

Table 3.8. Comparison of estimated growth parameters for *L. salivarius* and *L. plantarum* in the presence of different sugars.

	<i>Lactobacillus plantarum</i>	<i>Lactobacillus salivarius</i>
Glucose		
t-lag (h)	8.39	11.45
t-d (h)	1.10	1.51
Mumax (h ⁻¹)	0.63	0.46
Sucrose		
t-lag (h)	9.09	11.41
t-d	0.84	1.0
Mumax (h ⁻¹)	0.82	0.69

t-lag (the lag time); t-d (the doubling time), Mumax (the maximum specific growth rate)

Total starch and protein assays of liquid diets

Fermentation (30°C) of sterile pig feed with *L. salivarius* did not have any significant effect on the crude protein level of the diet. However, approximately 2% loss in total starch concentration was observed (Table 3.9).

Table 3.9. Starch and proteins of sterile commercial sow diet before and after fermentation with *L.salivarius* (SHCM FC 28) at 30°C.

TOTAL STARCH (%)		
Non-fermented liquid feed	Fermented liquid feed	<i>P</i>
9.983 ± 0.163	7.963 ± 0.037	***
CRUDE PROTEINS (%)		
5.59 ± 0.13	5.76 ± 0.29	ns

Data are expressed as a mean (%) ± SD

3.4. Discussion

Bacterial growth, acid and gas production of faecal lactobacilli

When fermenting carbohydrates the lactic acid bacteria will utilize one of two pathways, with the resulting products being dependent on the pathway taken. The two pathways are denoted as homofermentative and heterofermentative, and are based on the fermentation of glucose. The key to a good fermentation of pig feed is establishing a homofermentation (dominance of organisms that produce mostly lactic acid) rather than a heterofermentation (Mayrhuber *et al.*, 1999). Lactic acid is preferred because it is a stronger acid and will reduce pH faster (Mayrhuber *et al.*, 1999). Although heterofermentation also leads to the production of lactic acid, the other end products such as acetic acid and carbon dioxide are produced as well, which are responsible for malfermentation and leads to the feed becoming unpalatable for pigs as they do not like the pungent smell of acetic acid. In addition, the development of CO₂ that occurs during heterofermentation indicates potential dry matter loss. Using an inoculant that provides a homofermentative process should result in less dry matter loss and higher feed intake due to lower content of acetic acid. Therefore, only those organisms that utilize the homofermentative pathway were selected for further analysis.

Autoaggregation and coaggregation experiments

Aggregation or 'clumping' of bacterial cells belonging to the same (autoaggregation) or to different (coaggregation) bacterial cell lineages is a well-documented example of bacterial interactions (Kolenbrander *et al.*, 1990; Rickard *et al.*, 2003). It was once thought that such

adhesion features occurred exclusively between dental plaque bacteria (Kolenbrander *et al.*, 1993). However, aggregation was also observed among vaginal (Kmet and Lucchini, 1997; Boris *et al.*, 1998; Styriak *et al.*, 2001; Bujnakova and Kmet, 2002) and intestinal lactobacilli (Vandevoorde *et al.*, 1992; Kmet *et al.*, 1995; Gusils *et al.*, 1999; Bujnakova and Kmet, 2002) and bifidobacteria of human, porcine, bovine and chicken origin (Del Re *et al.*, 1998; Del Re *et al.*, 2000). In this study, from the total 35 porcine lactobacilli tested only 13 expressed autoaggregating potential, which confirms the results of Kmet and Lucchini (1997) that efficient aggregation activity is found in 20-30% of *Lactobacillus* isolates. Vandevoorde *et al.*, (1992) showed that the autoaggregating mechanism enabled chicken lactobacilli to maintain higher numbers in fed-batch reactors simulating the gastrointestinal tract. In addition, co-aggregation, possibly together with lactic acid and/or bacteriocin production, may prove to be an important factor in the establishment and maintenance of 'healthy' GI and urogenital microflora. It has been suggested that lactobacilli, which aggregate with pathogens, may constitute an important host defence mechanisms against infection (Reid *et al.*, 1988; Kmet and Lucchini, 1997; Boris *et al.*, 1998). The co-aggregation effect of porcine lactobacilli with enterotoxigenic *E. coli* K88 (Spencer and Chesson, 1994; Kmet *et al.*, 1995; Kmet and Lucchini, 1999), chicken and bovine lactobacilli with enterohemorrhagic O157 *E. coli* (Bujnakova and Kmet, 2002) and human vaginal lactobacilli with uropathogenic *E. coli* (Reid *et al.*, 1988; Boris *et al.*, 1998) have been reported. Moreover, there is an association between the ability of bacteria to adhere to epithelial cells and aggregation activity (Boris *et al.*, 1998; Del Re *et al.*, 1998). Aggregating bacteria had stronger adhesion ability to porcine ileum and colon tissues than non-aggregating one, indicating that aggregation can increase substantially the colonisation potential of lactobacilli in environments with short residence time. *Lactobacillus*

aggregation may also speed up the transit of pathogenic *E. coli* through the piglet's gut by the formation of *Lactobacillus* – *E. coli* aggregates (Kmet and Lucchini, 1999).

The presence of the F4 (K88) fimbrial antigen was found to be an active factor in co-aggregation between *E. coli* and some strains of lactobacilli (Hillman *et al.*, 1994). The presence of an aggregation phenotype in human *L. gasseri* 4B2 was demonstrated to be mediated by a secreted 32 kDa protein APF (Aggregation-Promoting Factor) with function of aggregation mediator (Reniero *et al.*, 1992). It was suggested that these proteins are able to establish a bridge between two bacterial cells containing, as a binding substance, lipoteichoic or teichoic acids. Two primer pairs APF3-APF4 were designed for simultaneous amplification of a specific fragment of the APF gene and a highly conserved region of the 16S rRNA gene by PCR (Lucchini *et al.*, 1998). By using this simple and fast method, the presence of APF was confirmed in many other aggregating lactobacilli of different origin (Kmet and Lucchini, 1997; Kmet and Lucchini, 1999; Styriak *et al.*, 2001; Bujnakova and Kmet, 2002). The secretion of APF, however of different size (2 kDa), by aggregating *L. gasseri* 2459 was confirmed also in the study of Boris *et al.*, (1997). More detailed analysis revealed that it is a hydrophilic peptide active at pH 3-4 and stable at neutral and acid pH. The protein activity was resistant to heat, chymotrypsin, chelating agents, triton X-100 and reducing agents, but sensitive to other proteases and SDS.

Recently, Ventura *et al.*, (2002) identified and sequenced the genes encoding the aggregation-promoting factor (APF) protein from six different strains of *L. johnsonii* and *L. gasseri*. They showed that both species harbor two *apf* genes (*apf1* and *apf2*), which are in the same orientation and encode proteins of 257 to 326 amino acids. Northern blot analysis showed that both genes are transcribed, reaching their maximum expression

during the exponential phase. Western blot analysis demonstrated that APF proteins are located on the cell surface and it was suggested that they might belong to an S-layer-like family. The cell surface location of APF(s) was also demonstrated in the study of Boris *et al.*, (1998).

Therefore, both autoaggregation and co-aggregation may favour the colonisation of the mucosal epithelia through the formation of a bacterial film that may contribute to the exclusion of pathogens.

Acid resistance and bile-salt tolerance

The stomach and intestinal tract represent a hostile environment that can easily discourage growth or survival of microorganisms. Therefore, screening experiments were conducted to determine the degree of acid and bile resistance exhibited by the 13 selected homofermentative, aggregating lactobacilli strains. Berrada *et al.*, (1991) reported the time from entrance to release of bacteria from stomach to be 90 min. However, further digestive processes have longer residence times; hence there is need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, which contains bile. In terms of acid resistance, Ibrahim and O'Sullivan (1998) demonstrated that although some strains can survive very low pH directly, others could tolerate it only if they were first primed at higher pH, which means that potential isolates for probiotic cultures need not necessarily be directly resistant to pH 3 as long as they tolerate it after prior priming at higher pH. Exposing organism to mild stresses can induce tolerances, which can enable the organism to withstand greater stresses (O'Sullivan, 2001). This could be achieved when yogurt or

other fermented food is used as the delivery vehicle for the probiotic, as the mild acid conditions may be sufficient to prime many isolates effectively.

Bile acids are physiological detergents, synthesized in the liver from cholesterol, that facilitate excretion, absorption, and transport of fats and sterols in the intestine and liver (Chiang, 2003). They are secreted from the gall bladder into the duodenum in the conjugated form, and only afterwards they undergo extensive chemical modification (deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation) in the colon almost solely as a result of microbial activity. Both conjugated and deconjugated bile acids exhibit antibacterial activity, inhibiting the growth of pathogens such as *E. coli*, *Klebsiella* sp., and *Enterococcus* sp. *in vitro*. However, the deconjugated forms are more inhibitory and Gram-positive bacteria are found to be more sensitive than Gram-negative bacteria (Floch *et al.*, 1972). Therefore, bile tolerance is considered to be an important characteristic of lactobacilli strains that enables them to survive, grow, and exert their action in the small intestine. Strains that are able to grow and metabolize in the presence of physiological levels of bile should logically be more likely to survive intestinal transit. Klaenhammer (1982) and Park *et al.*, (2002b) reported that lactic acid bacteria vary considerably in their level of bile tolerance, and this could be also seen in the present study. Although the mechanism of tolerance and degree of bile tolerance required for maximum growth of the organism in the intestinal tract is not known, it is important to select one that has a high degree of bile resistance. The results presented here (Figure 3.3 and 3.4) show that 4 (SHCM FC 28, SHVD FC 23, SHVD FC 28, SHVD FC 36) from the 13 aggregating *Lactobacillus* strains were able of growing in acid conditions (pH 3.5) as well as in bile presence, suggesting that these porcine isolates could transit the stomach

successfully and may be capable of reaching the intestinal environment and functioning effectively there.

Analysis of organic acid production and pH

High levels of lactic acid are required to be produced quickly in order to reduce the pH of the feed and to inhibit the growth of spoilage organisms, such as coliforms. In fact, production of organic acids has been widely used in poultry industry, to sanitise the feed and reduce *Salmonella* contamination (Iba and Berchieri, 1995; Thompson and Hinton, 1997; Hinton *et al.*, 2000). However, it has been shown that subsequently a low pH diet could have an inhibitory effect on potential pathogens in the GI-tract, which would be of benefit with respect to animal health. In the study of Cole *et al.*, (1968) the addition of lactic acid, in concentrations of 0.8% to a control weaner diet, effectively reduced the levels of *E. coli* in the duodenum and jejunum of 8 week old piglets. Moreover, in contrast to the control-fed animals, piglets fed the acid-added diets had only non-haemolytic *E. coli*. Another study (Maribo *et al.*, 2000) demonstrated that dietary acidification of pigs diet with 0.7, 1.4 or 2.8% lactic acid resulted in changes of gastrointestinal characteristics. The pH in the GI-tract was reduced, and the lactobacilli density was lower in the small intestine (1.4% lactic acid) and higher in the caecum and colon (0.7% lactic acid) of pigs fed the diet supplemented with lactic acid. Furthermore, lactic acid decreased the counts of coliforms and increased the counts of yeast along the GI-tract.

In addition to pathogen inhibition, dietary acidification increases gastric proteolysis and protein and amino acid digestibility. The acid anion has also been shown to complex with Ca, P, Mg and Zn, which results in an improved digestibility of these minerals.

A lactic acid concentration of 70 mMol was found to be bacteriostatic, but higher levels (> 100 mMol) are needed in order to be bactericidal. For example, studies by Rubin *et al.*, (1982) and van Winsen *et al.*, (2001a) estimated that a minimum of 150 mmol L⁻¹ lactic acid is needed in order to inhibit growth of *Salmonella typhimurium*. In addition, combinations of lactic acid (200 mmol L⁻¹) and acetic acid (10, 20, 30 mmol L⁻¹) with the same pH (4.2) showed an acetic acid concentration-dependent reduction of *S. typhimurium* (van Winsen *et al.*, 2001a). Unfortunately, natural fermentations cannot be relied upon to produce these concentrations of acid. For example, in samples of wheat from across the UK fermented for 24 h at 30°C the lactic acid level varied from 0-50 (8.7 ± 12.2) mMol. After 72 hours the range was from 0.14 to 135 (48 ± 38) mMol lactic acid. Only circa 10% of natural fermentations achieved the threshold level of 150 mMol lactic acid needed to eliminate *Salmonella*. However, this problem can be overcome by inoculating liquid feed with LAB that produce lactic acid rapidly, and to a high concentration. In the current study all the porcine lactobacilli screened were capable of producing in excess of 150 mMol lactic acid with less than 12 mMol acetic acid in 24 hours. Based on this criterion only, the best candidates for inoculation of liquid feed would be strains SHVD FC51 and SHVD FC72 as they produced the less amount of acetic acid (< 4 mMol).

Different aspects of the working mechanism of organic acids with respect to their antibacterial activity are given in the review articles of Cherrington *et al.*, (1991) and Russell *et al.*, (1992). In general, the antibacterial activity of organic acids is related to the reduction of pH, as well as their ability to dissociate, which is determined by the pK_a-value of the respective acid and pH of the surrounding milieu. The antibacterial activity increases with decreasing pH value. The antimicrobial effect of lactic and acetic acid in fermented pig feed on *Salmonella* reduction was demonstrated by van Winsen *et al.*, (2001a). The

high concentration of lactic acid in the feed should not represent any problem for a pig as it has been shown that L^+ and D^- lactic acid are utilized as efficiently as wheat starch and has no negative effects on animal health and performance (Everts *et al.*, 2000). In fact, Roth *et al.*, (1993) and Roth and Kirchgessner (1998) showed that a significant improvement of growth rate and feed conversion rate of weaning pigs can be achieved by the inclusion of lactic acid in the diet. The suggested mode of action was thought higher protein and energy digestibility and retention in the presence of acid, an alteration of bacterial populations and metabolites in the gastrointestinal tract and possibly an effect on metabolism (Roth and Kirchgessner, 1998).

Unlike Gram-negative bacteria, *Lactobacillus* species can tolerate low pH conditions by maintaining a pH difference between their cytoplasm and the environment by their unique pH homeostasis (McDonald *et al.*, 1990; Hong *et al.*, 1999). Despite the importance of pH homeostasis in the lactic acid bacteria, the mechanism for acid tolerance by this group of microorganisms is not fully understood. McDonald *et al.*, (1990) showed that regardless of the growth medium, growth of *L. plantarum* stops when a specific internal pH of the cell is reached. In the case of *L. plantarum* the growth limiting internal pH_{in} was estimated to be 4.6-4.8 (McDonald *et al.*, 1990). It was further demonstrated that external pH alone cannot define the conditions which will prevent bacterial growth. The composition of the growth medium and more importantly the presence and type of organic acid, determines the limiting lower external pH at which an organism terminates its growth. It was shown that *L. plantarum* can initiate their growth even when the external pH is 3.0 and with lactic acid concentration of 160 mmol L⁻¹. Recently Hong *et al.*, (1999) demonstrated that acid tolerance of *L. plantarum* was found to be closely related to the gross membrane damage

and proton permeability of whole cells as well as the specific activity of membrane ATPase.

Adhesion assays

Although lactobacilli survive the selective pressures of the hostile environment of the stomach and upper intestine, flow rates of digesta through the small intestine would wash out any organism unable either to multiply rapidly enough to avoid dilution or to maintain their residence by physical attachment to the intestinal epithelium (Percival, 1997). Therefore, one factor that has been generally used as a selection criterion for probiotic formulation is the ability of strains to adhere to the host's gut epithelia. It is well recognized that intestinal attachment is an important prerequisite for colonization of the GI tract by many bacterial pathogens. More specifically, the ability to adhere to intestinal cells and mucus is an essential stage in the pathogenesis of intestinal infections, especially with diarrhoea of bacterial origin (Conway and Ronald, 1988), which has led to studies aimed at creating vaccines that block adhesion events. Arguably, the use of commensal bacteria to inhibit pathogens has even greater potential than vaccine use, because these bacteria are natural competitors of pathogens and their action does not require host immune stimulation (Reid *et al.*, 2001). In fact, the role of adhering commensal bacteria in protection against enteric pathogens was recognized already by Fuller (1973).

The colonization of the GI-tract can occur in three phases: (1) adhesion to the mucus layer, the first step in colonization, (2) adhesion to the epithelial surface and finally (3) sub-epithelial adhesion, which is utilized mainly by pathogenic bacteria in invasion (Patti and Höök, 1994).

(1) Adhesion assay to porcine mucus

In this study, lactobacilli SHCM FC 28 and SHVD FC 71 adhered in greater numbers to porcine intestinal mucus than other strains tested. LAB vary greatly in their adherence to mucus as it was shown in the study of Kirjavainen *et al.*, (1998). The exact composition of mucin molecules can vary greatly and there are clear differences not only between animal species but also within localized regions of the intestinal tract (Karlsson *et al.*, 1997; Cone, 1999; Cornberg *et al.*, 2001). In addition, marked changes in the composition of mucin occur with development. Kirjavainen *et al.*, (1998) showed that all LAB strains tested adhered better to the mucus of adults than to that of infants which indicates that the age of the target group may be worth of consideration when planning a schedule for probiotic therapy. The mucus in our study was isolated from the porcine stomach of unspecified age. Therefore, it would be interesting to see whether non-adherent strains from this study would show different adherent potential if mucus from different region of porcine GI-tract would be used.

As the outermost luminal layer mucus is the first intestinal component or surface that bacteria are likely to contact before they reach epithelial cells. Hence, it can have a substantial role in the colonisation of intestinal surfaces. This protective mucus layer in the GI-tract is composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells (Deplancke and Gaskins, 2001). Intestinal mucus acts as a medium for protection, lubrication, and transport between the luminal contents and the epithelial lining. The physical barrier of mucus contains protease inhibitors, lysozyme, lactoferrin and antibodies (Cone, 1999). Thus, any damage or disturbance of this mucus layer will compromise the host's mucosal defence function.

Many infections are initiated by bacterial colonisation of the host mucosa as the carbohydrate moieties that make up most of the mucus structure could represent pathogen binding receptors. The presence of protein and glycolipid receptors, specific for K88 fimbriae in porcine mucus, has been demonstrated (Blomberg *et al.*, 1993c; Grange *et al.*, 1998; Francis *et al.*, 1999). However, *Lactobacillus* strains can also adhere to mucus, as has been shown in many *in vitro* studies (Henriksson and Conway, 1996; Rojas and Conway, 1996; Roos *et al.*, 2000; Jonsson *et al.*, 2001; Juntunen *et al.*, 2001; Ouwehand *et al.*, 2001; Zhou *et al.*, 2001; Edelman *et al.*, 2002). Unlike pathogens, LAB adhere to GIT mucus without its degradation, which highlights their non-invasiveness and non-toxicity (Zhou *et al.*, 2001). The adherence was shown to be mediated by producing mucus-binding adhesins of proteinaceous structure (Blomberg *et al.*, 1993b). More detailed studies of mucus-binding *L. reuteri* 1063 (pig intestinal isolate) identified a large (385 kDa) surface protein, Mub, which mediates binding of this strain to pig intestinal mucus (Roos *et al.*, 2000). The presence of Mub was demonstrated on the cell surface of *Lactobacillus* strains as well as in the growth medium (Roos and Jonsson, 2002). Different adhesion-promoting protein involved in the binding of *L. fermentum* strain 104R to porcine mucus was isolated and characterized by Rojas *et al.*, (2002). The protein was released to the culture supernatant fluid after 24 h of growth and had affinity for both small intestinal mucus and gastric mucin. In the native state this protein was variable in size, and it had a molecular mass of 29 kDa when denatured.

This mucus adherence potential of many LAB could be another very useful characteristic of potential probiotic strains. Some *Lactobacillus* strains, either the cells alone or in combination with their spent culture supernatant, have been shown to inhibit adhesion of pathogens. The *in vitro* protective effect of certain *Lactobacillus* strains with potential to

prevent the adherence of K88 fimbriae (Blomberg *et al.*, 1993b) and *Salmonella typhimurium* (Craven and Williams, 1997) to intestinal mucus have been reported. The adhesion of *E. coli* K88ab and *E. coli* K88ac to 35- day old piglet ileal mucus was reduced in the presence of spent culture fluid porcine lactobacilli, by approximately 50% (Blomberg *et al.*, 1993b). However, adherence does not always give *Lactobacillus* strains a protective effect. For example, the attachment of *Salmonella typhimurium* was significantly reduced by prior exposure of mucus to cultures of a *L. salivarius* strain and a *L. delbrueckii* strain but not to a strain of *Lactobacillus* for which the species had not been determined. Similar negative results were obtained by Jin *et al.*, (2000), where 14 adherent *Lactobacillus* strains did not affect the attachment of *E. coli* K88 to the small intestinal mucus of piglet.

It was also suggested that the mucus adherence mechanism for some strains differs from the one that is used for the intestinal epithelia (Henriksson and Conway, 1996; Kirjavainen *et al.*, 1998). This could be seen also in our study as strain SHVD FC 28 showed to have good adhering ability to Caco-2 cells, however in terms of mucus it is considered to be non-adhering. This suggests that receptor molecules for bacterial adhesins on mucus may be different, or present in different numbers than on enterocytes. It is possible that some strains adhering well to intestinal cells are valuable in treating hosts with a damaged mucus layer allowing adhesion to the epithelial cells. However, such strains may not be as suitable for healthy subjects, or for hosts with extensive mucus secretions, which is the consequence of many enteric infections. In the future, it may be possible to plan probiotic treatments that will be targeted individually for bacterial populations in the mucus or in the epithelial cells. However, more information on these different subpopulations and their interaction with probiotic bacteria *in vivo* are still needed.

(2) Adhesion assay to Caco-2 cells

Adhesion or close association of potentially probiotic LAB to epithelial cells may contribute to competitive exclusion of pathogens by occupying/masking the adhesion receptors on the mucosal surfaces (Stavric *et al.*, 1987). This is of course a valid principle only if pathogens and LAB have parallel attachment mechanisms. Studies by (Bibiloni *et al.*, 1999) demonstrated that *Salmonella arizonae* adhered to Caco-2 cells in a density dependent way until a saturation point was reached, which indicates that Caco-2 cells may have a limited number of available sites where bacteria could bind. In addition to competitive exclusion, an adherent capacity may be important in other respect. Schiffrin *et al.*, (1995) have suggested that adhesion of LAB to mucosal epithelial cells may be a critical factor in immune stimulation as adhesive LAB strains can have prolonged contact with gut-associated lymphoid tissue (GALT).

Adhering bacteria have been defined as those that are not readily removed from the epithelium by specified washing procedure (Stavric *et al.*, 1987). In order to study the microbial adherence *in vitro*, different adherence assays have been developed for many bacteria with the use of cultured cells, tissue sections or plastic surfaces coated with proteoglycans or glycosaminoglycans (Rostand and Esko, 1997). Caco-2 cell lines isolated from a human colonic adenocarcinoma have been commonly used in adhesion studies with LAB as they structurally resemble differentiated enterocytes at the intestinal level (Pinto *et al.*, 1983; Chauviere *et al.*, 1990). However, it needs to be point out that it is not a thorough test on the true adherence abilities of bacteria and positive attachment to this cell line should be viewed only as a good indicator of bacterial potential to attach. To draw negative conclusions about an isolate's adherence potential from a failure to attach to this *in vitro*

cell line is not scientifically correct (O'Sullivan, 2001). The results obtained in the current study showed that although all the tested strains showed some degree of adherence to Caco-2 cells, the adhesion rate was variable, which was also confirmed by other studies (Barrow *et al.*, 1980; Mäyrä-Mäkinen *et al.*, 1983; Jacobsen *et al.*, 1999). In a series of *in vitro* studies conducted with lactobacilli strains, several studies have demonstrated that these bacteria bind to human intestinal cell lines and inhibit the cellular adhesion of, and invasion by, Gram-negative pathogens. For example, the protective *in vitro* effect of porcine enteric lactobacilli on the adhesion of enteropathogenic *Escherichia coli* K88 were demonstrated by Ouwehand and Conway (1996). Heat-killed *L. acidophilus* in the study of Chauviere *et al.*, (1992), inhibited adhesion of diarrheagenic *E. coli* (ETEC) to Caco-2 cells in a concentration-dependent manner. Similar results were obtained by Coconnier *et al.*, (1993). Living and heat-killed *L. acidophilus* strain LB inhibited both cell association and invasion of Caco-2 cells by enterovirulent bacteria (*Salmonella typhimurium* and EPEC) in a concentration-dependent manner. Pre-treatment of *E. coli* O157:H7 with 2.5-fold concentrated cell-free culture supernatants from *L. acidophilus* HN017, *L. rhamnosus* DR20 and *Bifidobacterium lactis* DR10 reduced the culturable *E. coli* numbers and also reduced the invasiveness and cell association characteristics of this toxic strain (Gopal *et al.*, 2001). In contrast, study by Bibiloni *et al.*, (1999) demonstrated that high adhesiveness may not always guarantee protective properties in a selected strain. Two highly adherent bifidobacterial strains of human origin (*B. bifidum* CIDCA 537 and *B. bifidum* CIDCA 5310) were tested for their abilities to inhibit cell attachment and cell invasion of a diarrhoeagenic *Salmonella arizonae*. Both strains, which had adhered to Caco-2 monolayers before the addition of pathogen could not prevent adhesion of this pathogen. Similar results were obtained in the study of Spencer and Chesson (1994), where strongly adherent *L. fermentum* did not affect the attachment of enterotoxigenic *E. coli* to porcine enterocytes,

tested under the conditions of exclusion (lactobacilli added to the enterocytes before *E. coli*), competition (lactobacilli and *E. coli* added simultaneously) and displacement (*E. coli* added before lactobacilli). These studies highlighted the strain-dependent character of the 'probiotic effect' and the importance of *in vivo* experiments for the preparation of dietary adjuncts.

Although the molecular mechanism of adhesion is not well understood, the classical theories of adhesion suggest two to five stages in the process, involving passive van der Waals' attractive forces; electrostatic interactions; hydrophobic, steric forces; lipoteichoic acids; and frequently active adhesion through the production of specific structures by the bacteria, such as external appendages (lectins) and/or extracellular polymers (polysaccharides) (Busscher *et al.*, 1992; Gusils *et al.*, 2002). Gram-negative bacteria, for example pathogenic *E. coli* attach to the target cells via proteinaceous projections (pili), but LAB seem to adhere to the GI tract wall with extracellular substances, containing polysaccharides, proteins, lipids, and lipoteichoic acids (Wadström *et al.*, 1987; Coconnier *et al.*, 1992; Henriksson and Conway, 1992; Greene and Klaenhammer, 1994; Adlerberth *et al.*, 1996; Granato *et al.*, 1999; Neeser *et al.*, 2000; Gusils *et al.*, 2002).

(3) Adhesion assay to Collagen-I

The ability to adhere to specific host molecules enables a pathogen to target itself to a particular tissue within the host body. If the pathogen also produces toxins that cause tissue damage, destruction of host cells will expose the extracellular matrix (ECM), to which many pathogens can bind. This adherence step will help the pathogen to penetrate further into the body through activation of host cell signalling pathways (Finlay and Caparon,

2000). ECM is a stable structure underlying epithelia and surrounding connective tissue cells (Westerlund and Korhonen, 1993). Besides the structural support ECM is also involved in cellular development and function, cell adhesion as well as migration (reviewed in Preissner and Chhatwal, 2000). The composition of the ECM differs in various organs however, the main structural components of the mammalian ECM are collagens (Cn) type I-XV, fibronectin (Fn), laminin (Ln) and various glycosaminoglycans (e.g. heparin, heparin sulphate, chondroitin sulphate etc. (Preissner and Chhatwal, 2000). Collagen comprise one third of the animal proteins and in fact, more than 25 different collagen molecules have been defined. However, most (> 90%) of the body's collagens are of the fibril-forming types I-III (Preissner and Chhatwal, 2000). Type-I collagen (Cn-I) is the most abundant collagen molecule in soft body tissues (Aleljung, 1994). It has been shown that adhesion to extracellular matrix proteins is expressed by several pathogenic bacteria and is thought to contribute to their invasiveness (Westerlund and Korhonen, 1993). In fact, several genes encoding ECM-binding proteins, termed adhesins, have been cloned from pathogen such as *Staphylococcus aureus* (Flock, 1999) and shown to be important virulence factors that help initiate infections, particularly under conditions of injury or trauma (Ljungh *et al.*, 1996). High incidence (100%) of Cn-I binding was also reported for all EAEC and EHEC strains (Ljungh *et al.*, 1990). Exposure of ECM is also very often achieved by viral or bacterial infections of mucosal surfaces, through the action of proteolytic enzymes and toxins. In addition, the normal shedding of epithelia provides also the epithelial surface with ECM components. Finally, ECM molecules could help pathogens to escape certain antibiotics and by masking the microbial surfaces they might interfere with antigen presentation and thus provide an overall immune evasion strategy (Ljungh *et al.*, 1996). In light of these and other observations, it has been proposed that possible adherence of LAB to sub-intestinal ECM can represent a very useful probiotic

characteristic. Besides the fact that such an adherence may protect the host against bacterial invasion at damaged epithelia, ECM-LAB interactions might also provide direct stimulation of the lymphoid cells in the lamina propria (Miettinen *et al.*, 1993).

Adhesiveness of LAB to ECM proteins has been reported in many studies. Aleljung (1991) demonstrated that binding to solubilized collagen is frequently expressed among *Lactobacillus* strains of different origins; 75% of their LAB isolates bound solubilized type I collagen. Adherence of vaginal (Styriak *et al.*, 2001) and intestinal lactobacilli (Styriak *et al.*, 1999a) of porcine and bovine origin to collagen-I has also been demonstrated. In the current study only 3 porcine lactobacilli strains, from the total 13 were able to adhere to Collagen-I with SHVD FC71 expressing the greatest binding potential. This Cn-I binding capacity of the potential new FLF inoculant might be useful in order to antagonize the colonisation of Cn-I binding sites by pig's GI pathogens.

There is a substantial amount of evidence that bacteria-ECM interactions are based on specific molecular recognition and it was demonstrated that ECM binding proteins exhibit large variability in their ECM-target specificity (Holderbaum *et al.*, 1987; Aleljung *et al.*, 1991; Olusanya *et al.*, 1992; Patti and Höök, 1994; Toba *et al.*, 1995; Ljungh *et al.*, 1996; Styriak *et al.*, 1999b; Kapczynski *et al.*, 2000; Nallapareddy *et al.*, 2000; Nallapareddy *et al.*, 2003). Two surface adhesins of *L. reuteri* NCIB 11951 (29 kDa and 31 kDa), which binds collagen-I, have been identified and characterized (Aleljung *et al.*, 1994). Recently, Howard *et al.*, (2000) have attempted to identify other ECM-binding proteins that might be present within *Lactobacillus* biosurfactants by using recently developed Protein-Chip technology. This technique is capable of detecting proteins at picomolar to femtomolar levels in small biological mixtures with little or no preparations and indeed it was capable

of detecting several different sizes (2 to 48 kDa) of collagen binding proteins that bind to both collagen types III and VI. In addition, this study further demonstrated the strain differences in the levels of expression of Cn-binding proteins, indicating that not all probiotic lactobacilli are identical, and some may better inhibit pathogens binding to ECM proteins than the others.

Cytokine gene expression in Caco-2 cells induced by lactobacilli

Intestinal epithelial cells are constantly exposed to bacteria and bacterial products and are considered to participate in the initiation and regulation of the mucosal immune response by secretion of cytokines. Many recent studies demonstrated that intestinal microorganisms can influence this secretion (Morita *et al.*, 2002).

Recently, a number of *in vitro* techniques have become available to identify gene expression in normal and diseased tissues. However, it is necessary to realize that although the use of intestinal epithelial cell lines allows a simplified approach to understanding direct interaction between bacteria and the intestinal epithelium, results from experiments performed on cell lines should be interpreted cautiously because of the absence from these models of mesenchymal and lymphoid cells, cytokines and other luminal factors normally present in the intestine (McCracken and Lorenz, 2001).

The results presented here showed that both porcine lactobacilli were equally potent inducers of interleukin (IL)-12 with no effect on mRNA expression of proinflammatory IL-8. Interleukin-8 plays a key role in acute inflammation by recruiting and activating neutrophils (Iizasa and Matsushima, 2000). Morita *et al.*, (2002) studied 30 strains of lactic

acid bacteria for their adhesion to Caco-2 cells and their potential to stimulate proinflammatory cytokine secretion by this cell line. The bacteria adhered in a strain-dependent manner and their contact with Caco-2 cells did not result in the production of IL-6 or IL-8. In addition there was no correlation between adhesion and cytokine induction among the bacteria tested, which indicates that lactic acid bacteria, even those with strong adhesive properties, are not very likely to trigger an inflammatory response in human enterocytes. On the other hand, IL-12 represents an important immunoregulatory cytokine produced by cells of the innate defence system in response to bacteria. It represents the key messenger molecule, which bridges the gap between innate and acquired immunity. IL-12 activates proliferation, enhances cytotoxicity and interferon- γ (IFN- γ) secretion by T cells and NK cells. IL-12 also enhances resistance to a variety of infectious diseases and exhibits potent antitumor immunity (Esche *et al.*, 2000). Interferon- γ induced by IL-12 has a multitude of cellular biological functions e.g. it promotes phagocytosis, inhibits viral replication, upregulates microbial killing, upregulates endothelial cells and induces antibody class-switching by B-cells to complement fixing antibody isotypes such as the IgG2a (Billiau and Vandebroek, 2000). It has been demonstrated that non-pathogenic Gram-positive bacteria are strong IL-12 inducers with substantial differences among the strains (Hessle *et al.*, 1999; Kato *et al.*, 1999; Haller *et al.*, 2000; Hessle *et al.*, 2000; Christensen *et al.*, 2002). In addition to the effect on the epithelial cells it has been shown that certain lactobacilli are able to enhance IL-12 and IFN γ production by mouse peritoneal leukocytes, too (Tejada-Simon *et al.*, 1999b). Other strains of probiotic organisms tested in this study actually attenuated the cytokine response. Candidates for stimulation of IL-12 include the specific cell wall components especially lipoteichoic acid (LTA), which is found only in Gram-positive bacteria. However, some other components of bacterial cell membrane (e.g. lipopolysaccharides, peptidoglycans) could also be involved in the

stimulation process (Heumann *et al.*, 1994; Huang *et al.*, 1999; Tejada-Simon *et al.*, 1999a). Therefore, one of the reasons why lactobacilli hardly ever cause disease might be their potent ability to induce the production of IL-12 and thereby activate macrophages resulting in a more effective clearance of the bacteria, and thus decreasing their infectivity (Hessle *et al.*, 1999). Upon ingestion of feed fermented with either lactobacilli tested in the current study, the bacteria may be taken up via the Peyer's patches and trigger cytokine production. Thus, cytokines induced by lactobacilli colonizing the GI-tract may be important regulators of the gut associated immune system.

Identification of lactobacilli

Although phenotypic techniques such as the API system are still taken as powerful tools capable of discriminating between the species of *Lactobacillus*, the use of genetic methods for *Lactobacillus* taxonomy has become a necessity for reliable identification (Nigatu, 2000). The molecular method involving the PCR amplification of 16S ribosomal DNA genes represent one of the cornerstones of microbial taxonomy (Tannock, 2001; Heilig *et al.*, 2002). Alignment of the sequences with those stored in databanks permits reliable recognition of the bacterial species, including those that cannot be cultivated by conventional techniques. The disagreement between the molecular analysis and API 50 CH in our study showed the reduced accuracy of the phenotypic procedure to clearly identify *Lactobacillus* isolates, despite its claimed sensitivity. Nigatu *et al.*, (2001) suggested that one major reason for such discrepancy might be the loss and gain of plasmids, leading to inconsistency in metabolic traits of a strain, as most carbohydrate fermentation capacity is plasmid-encoded (Xanthopoulos *et al.*, 2000). While the interstrain phenotypic variability of *L. plantarum* group was demonstrated by Xanthopoulos *et al.*, (2000), the genetic

heterogeneity has been shown in the study of Dellaglio *et al.*, (1975). Particularly the species *L. plantarum*, *L. pentosus*, and *L. paraplantarum* can hardly be distinguished on the basis of their phenotypes (Curk *et al.*, 1996; Berthier and Ehrlich, 1998). Recently, Bringel *et al.*, (2001) investigated the diversity of 140 strains related to *L. plantarum* by using two molecular techniques (randomly amplified polymorphic DNA fingerprinting (RAPD) and Southern hybridisation) together with phenotypic characterisation. The results revealed four groups of profile with one group belonging to *L. paraplantarum*, the second to *L. pentosus* and the two remaining groups to *L. plantarum* G(LP1) and G(LP2). By comparing the fermentation patterns of the *L. plantarum* strains, three differences were found. Melezitose was not fermented by the G(LP2) group, unlike the G(LP1) group which included *L. plantarum* type strain NCIMB11974. Secondly, α -methyl-D-mannoside was fermented by a majority of the strains of the G(LP1) group but by none of the strains in the G(LP2) group. Thirdly, dulcitol was catabolized by nearly half of the strains of the G(LP2) RAPD group but by none of the strains in the G(LP1) RAPD group.

However, due to the high identity value of 16S ribosomal DNA (> 99%) shared by *L. plantarum* and *L. pentosus* (Collins *et al.*, 1991; Quere *et al.*, 1997), it is not possible to use 16S rDNA analysis for their identification. Therefore, Torriani *et al.*, (2001) came up with another useful approach, which could help to overcome even this problem. The method is based on the sequence comparison of *recA* gene, which permitted the unambiguous identification of these closely related strains in a single reaction.

Study of carbohydrate preference of selected Lactobacillus candidate

Carbohydrates such as glucose and sucrose are naturally present in cereals. *L. salivarius* utilised both glucose and sucrose, however with significant growth delay comparison to *L. plantarum*. This growth differences could be due to the fact that *L. plantarum* represent a strain used in silage fermentation, therefore the cell metabolism might be better adapted to a wheat based substrate comparison with *L. salivarius* isolated from porcine faeces. However, taken into account in-feed lactic acid production of *L. salivarius* and pH decrease in 24 hours, *L. salivarius* behave equally well than *L. plantarum*, as bactericidal level of lactic acid accompanied by pH drop below 4 is achieved in 24 hours.

Total starch and protein assays of liquid feed

Starch in pig diets is almost invariably provided from cereals, where it comprises between 600-800 g kg⁻¹ cereal dry weight, depending on factors such as cultivar, environment and growing season (Wiseman *et al.*, 2001). Cereal starch comprises 2 glucose polymers, amylose and amylopectin, which have different structure and properties. The actual fermentation of pig feed or food by-products is assumed to be a complex process (Oda *et al.*, 2000). Lactobacilli might first consume monosacharides and disacharides, then begin to synthesise amylase to hydrolyse starch. In the genus *Lactobacillus*, *L. amylovorus* (Oda *et al.*, 2000), *L. manihotivorans* (Ben Omar *et al.*, 2000) and certain strains of *L. fermentum* (Agati *et al.*, 1998; Calderon *et al.*, 2003) and *L. plantarum* (Johannson *et al.*, 1995) have been reported to hydrolyse starch by extracellular amylase. In the present study, fermentation of pig feed with *L. salivarius* did not affect protein content of the diet but it did affect total starch concentration as a 2% loss in total starch was observed.

However, it is difficult to conclude whether this is due to amylase production of the bacterial strain as no additional experiments, which would confirm amylase production by *L. salivarius* were performed. On the other hand, as starch granules are not totally impermeable to water, soaking pig feed for 24 hours at 30°C could be responsible for an increase in diameter of starch granules which would make starch molecules more digestible due to greater access area for α -amylase or other hydrolytic enzymes. Increased digestibility of starch is considered beneficial in terms of pig nutrition (Wiseman *et al.*, 2001).

In terms of dietary fibre, silage studies showed that microbial inoculation usually has little or no effect on the fibre content of silages because most LAB contain little or no ability to degrade plant cell walls (Woolford, 1984; Crittenden *et al.*, 2002). Decreases in fibre content may be due to partial acid hydrolysis of hemicellulose. In fact, some data suggests that certain microbial inoculants can increase fibre digestion (Rice *et al.*, 1990).

3.5. Conclusion

Based on the results of these screening experiments, a strain of porcine homofermentative aggregating *L. salivarius* was selected for further *in vivo* studies as it was able to resist bile and acid conditions, had good adherence abilities to all intestinal compartments (mucus, epithelial cells, ECM) and it had a potent IL-12 inducing potential. In addition, it had comparable fermentation properties as the *L. plantarum* that has been widely used to ferment FLF in this laboratory.

Chapter 4

The effects of liquid diets, fed in late pregnancy, on faecal microbiology and colostrum quality of primiparous sows: *in vivo* effect of porcine *Lactobacillus salivarius*.

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4.1. Introduction

Selection of microorganism for probiotic use is a difficult task. Targeted selection of microbial strains with health-promoting potential will only be possible when we have more understanding of the complex mechanisms involved. Experiments conducted using *in vitro* models have the advantage that they are relatively cheap, rapid, easily controlled and have no ethical constraints. However, it is important to realise that results from such studies may not be predictive of the actual *in vivo* situation. The liquid form of FLF provides a very suitable way of testing different bacterial inoculants for their *in vivo* health-promoting effects. Based on the *in vitro* results in Chapter 3, porcine *Lactobacillus salivarius* was selected as a potential new inoculant for FLF. This chapter examines its potential double role, namely: a) controlling the fermentation and b) expressing potential health benefit for the consumer (in this case the sow in late gestation). In order to minimise the differences between animals, especially on immunological parameters tested, all experimental animals in this study were of the same age, breed, disease and vaccination history. The aim of this study was to determine the effect of feeding sows a liquid feed fermented with *Lactobacillus salivarius* on:

- Faecal numbers of LAB
- Faecal numbers of coliforms
- Faecal numbers of *E. coli*
- Faecal LAB / coliform and LAB / *E. coli* ratio in piglet faeces

An additional aim was to examine the effect of FLF fermented with *L. salivarius* on the quality of colostrum (mitogenic activity, immunoglobulin and protein contents of colostrum samples).

4.2. Materials and methods

4.2.1. Experimental animals

A study was conducted according to a randomised block design with 2 replicates. Eighteen primiparous sows (Large White x Landrace) and their piglets were used in the experiment. They were selected on the basis of expected farrowing date. All piglets in the litter of each sow were tagged and weighed at birth, one, two and 3 weeks of age.

4.2.2. Dietary treatments

Gilts were randomly allocated to one of three dietary treatments, namely: fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF) in pelleted form. The piglets received maternal milk for the first 14 days of life. Feeding took place twice a day for a period of 2 weeks before farrowing date, and for 3 weeks after farrowing according to MLC's Stotfold Feeding Scale for lactating sows (MLC, 1999). In gestation the gilts were loose-housed in-groups of 6 in straw-bedded pens, which were provided with individual feeders. Water was provided *ad libitum*. Gilts were moved to farrowing crates 4 days prior to their anticipated farrowing date. Each of the gilts was individually penned in a farrowing crate with water provided *ad libitum*.

Preparation of feed

DF was supplied by BOCM Pauls Ltd. The feed had a specification designed to meet the nutritional requirements of the gestating and lactating gilts and to maintain normal health and vigour (diet specification in Table 1). The sows were given 3.0 kg of DPF/day.

NFLF: 3.0 kg of DPF was mixed with 6 kg of water, containing 300 ppm chlorine dioxide (ClO_2) (Sanitech 2%; Alltech Inc., Kentucky). Active chlorine dioxide was prepared by mixing 125.4 ml of ClO_2 concentrate and 12.5 g of citric acid in a 25-litre fermentation bin. The reaction was allowed to proceed for five minutes until the pH had dropped to approximately 2.6 and the activated solution turned yellow-green. 6 kg of water (30°C) was added to the fermentation bin and 3.0 kg of the feed added slowly and constantly mixed to ensure an homogenous product. The feed was then left to steep for 24 h at 30°C .

FLF: was prepared by mixing feed and water in the same ratio as for the NFLF. The diet was also sanitized with chlorine dioxide according to the same procedure as described for NFLF. After 24h steeping at 30°C , the feed was inoculated with liquid *Lactobacillus salivarius* starter culture to give a final concentration of between 6 and 7 \log_{10} CFU ml^{-1} liquid feed. The inoculated feed was fermented for 36 hours at 30°C .

Table 4.1. Declared composition of the experimental diet fed as either a liquid meal or in a dry pelleted form.

Ingredient	Inclusion g kg^{-1} dry matter
Oil	35
Protein	165
Fibre	55
Ash	57.5
Lysine	7
Moisture	138
Copper	20 mg kg^{-1}
Sodium selenite-selenium	0.25 mg kg^{-1}
Vitamin A –retinol	12 000 iu
Vitamin D3 –cholecalciferol	2 000 iu
Vitamin E. α -tocopherol	50 iu
Digestible energy (DE)	14 MJ Kg^{-1}

4.2.3. Monitoring of feed

The dry matter of fermented and non-fermented feed was determined daily by oven drying at 103°C for 3-4 days (Method: ISO 6469/NEN 3332). The results were expressed as percentage dry matter. Samples (~20ml) of liquid feed were also removed daily from each batch for pH measurement, for microbial and chemical analyses. pH of liquid feed was measured using an electronic pH meter (W.G. Pye & Co. Ltd., Cambridge, UK). Microbial analyses of the feed samples were conducted after sanitation of NFLF and FLF with ClO₂ and at the end of fermentation process. All selective media used were obtained from Oxoid, Basingstoke, UK. Representative samples were serially diluted 10-fold in Maximum Recovery Diluent (MRD) (1ml sample in 9 ml MRD). Relevant dilutions were plated out on selective media and plates were incubated at the recommended temperature. Coliforms were enumerated on VRBA agar using double-layered pour-plate technique and incubated aerobically for 24 hours at 37°C.

Lactic acid bacteria were enumerated on Rogosa agar and incubated anaerobically for 72 hours at 30°C.

Yeasts were enumerated on Rose Bengal Chloramphenicol agar (RBCA) and incubated aerobically for 72 hours at 30°C.

4.2.4. Collection of faecal samples and culture from samples.

Fresh faecal samples (approximately 100 g) were collected from the rectum of each sow before introducing experimental diets and weekly for the period 2 weeks before anticipated farrowing and for 3 weeks post-farrowing. Dry matter concentration of each faecal sample was determined by oven drying at 103°C for 3 days. Microbiological analyses were

performed immediately upon receipt of the samples. Faeces were suspended in sterile MRD as 10-fold dilutions (wt/vol) from which further 10-fold dilutions (vol/vol) were made. To evaluate LAB populations, relevant dilutions were plated out on Rogosa agar and plates were incubated anaerobically at 37°C for 48h. To evaluate coliform and *E. coli* populations, relevant dilutions were plated out on chromogenic *E. coli*-coliform medium. Chromogenic substrates of this medium provide simultaneous evaluation of *E. coli* and coliform numbers. Purple *E. coli* colonies are easily differentiated from pinky coliforms. Plates were incubated at 37°C for 24h.

4.2.5. DNA extraction from faecal samples

The commercial kit QIAamp DNA mini kit (Qiagen Ltd, West Sussex, UK) was used for DNA isolation from faecal samples and the extractions were performed according to the manufacturers' instructions (Appendix 3). Briefly, a faecal sample (100 µl) was lysed with buffer ATL (Qiagen) and proteinase K for 2 hours at 55°C and then with buffer AL for 10 min at 70°C. The samples were then centrifuged and anhydrous ethanol was added to the supernatant. The sample mixture was then passed through the QIAamp kit column, followed by the washes with buffers AW1 and AW2 (Qiagen). The DNA was eluted in a volume of 200 µl of elution buffer, which was passed through the same column twice.

4.2.6. PCR amplification

Specific identification of *Lactobacillus salivarius* was based on primers targeted against regions of the 16S rRNA gene. The universal primer was combined with the species-specific primers in order to amplify a 90 bp fragment of the very 3'-end of the 16SRNA

gene. The primers were purchased from Sigma-Genosys (Pampisford, UK) and their sequences (5'-3') were as follows: The universal primer: (AGA GTT TGA TCC TGG CTC AG) and *L. salivarius* specific primer (GAA TGC AAG CAT TCG GTG TA).

The PCR reaction was performed in a final volume of 50 µl using a thermal cycler (PTC-200 PCR system, MJ Research, Inc. Waltham). 50 µl of the PCR mixture contained Qiagen PCR buffer (Tris-HCl, KCl, (NH₄)₂SO₄, pH 8.7 at 20°C), 1.5 mM MgCl₂, Q solution, 50 pmol primer "1", 50 pmol primer "2", 25 ng cDNA and 1.25U Taq DNA polymerase. All the reagents needed for PCR reaction were supplied with the PCR kit (Qiagen, Ltd., West Sussex, UK). The following temperature profile was used: initial denaturation at 94°C for 3 min, addition of 1U Taq polymerase (hot start), followed by 35 cycles 94°C, 30s; 58°C, 30s; 72°C, 30s. The reaction was terminated at 94°C for 2 min and 72°C for 3 min. A 10 µl of each sample was separated by agarose gel electrophoresis.

4.2.7. Quantification of short-chain fatty acids (SCFA) and lactic acid

SCFA analyses of feed and faecal samples were conducted by the HPLC method as described in Chapter 2.

4.2.8. Collection and processing of colostral samples

Colostrum samples were collected on the day of parturition by manual milking. All samples were taken after the first piglet was born. These samples were centrifuged at 17 000g for 15 min to remove fat. The aqueous phase of whey samples was separated and stored at -20°C until used.

4.2.9. Mitogenic activity on intestinal epithelial cells (IEC-6)

The rat intestinal epithelial crypt cell line IEC-6 was obtained from the European Collection of Animal Cell Cultures (EAACC, Salisbury, UK) and they were cultured as described in Chapter 2. IEC-6 proliferation was determined using CellTiter 96® non-radioactive cell proliferation kit (Promega, Southampton, UK) according to the protocol (Appendix 2). Briefly, 5000 IEC-6 cells in 100 µl were pipetted into each well of a 96 well microtitre plate (Linbro/Costar, UK). Cells were allowed to adhere for 18 hours at 37°C in a humidified atmosphere of 5% CO₂ and were then washed 3 times in Hank's Balanced Salt Solution (HBSS) (Sigma, Poole, UK). Then 100 µl of DTS medium (Dulbeco's Modified Eagle Medium (DMEM) containing 25 mM Hepes, 2 mM glutamine, 100 units/ml penicillin, 100 µg ml⁻¹ streptomycin, 0.2 µg ml⁻¹ sodium selenite, and 5 µg ml⁻¹ transferin) was added to each well and incubated for another 18 hours at 37°C in a humidified atmosphere of 5% CO₂. The DTS medium was removed from the wells and replaced with 100 µl of colostrum sample. Each assay was done in triplicate and a negative control of DTS and a positive control of complete DMEM was also included. The composition of complete DMEM medium was as follows: DMEM (Sigma, UK), 25 mM Hepes 2 mM glutamine, 5% foetal calf serum (FCS) (Sigma, UK), 100 units/ml penicillin, 100 µg ml⁻¹ streptomycin. The plates with colostral samples were incubated for 29 hours and pulsed with 20 µl CellTiter 96 for the final 3 hours. The absorbencies were read at 540 nm with background subtraction at 630 nm.

4.2.10. Mitogenic activity on blood lymphocytes

Lymphocyte proliferation assay was performed according to the method described in Chapter 2.

4.2.11. Immunoglobulin analysis

The concentrations of IgG, IgA and IgM were determined by the sandwich ELISA method using commercial ELISA Quantitation Kits (Bethyl laboratories, Inc., USA) and following manufacturer's instructions (Appendix 1).

4.2.12. Total protein contents of colostrum samples

Protein content in defatted colostrum samples was determined by Lowry protein assay (Lowry *et al.*, 1951), using bovine serum albumin (BSA) (Sigma Aldrich, UK) as the standard. This method was described in Chapter 2:

4.2.14. Amino acid analyses of colostrum samples

The analysis of amino acids was performed by Amino Acid Analyser (AAA) ion chromatography unit (Dionex Inc, Sunnyvale, CA). Colostrum samples (100 µl) were first hydrolysed with 6 M HCl (900 µl) for 24 hours at 120°C. After hydrolysis samples were cooled to ambient temperature, transferred to 1.5-ml microcentrifuge tubes and the HCl was evaporated to dryness. Samples were then reconstituted in 1000 µl purified water and analysed by AAA. Tryptophan was destroyed by acid hydrolysis and therefore was not

measured. Separation was achieved using a AminoPac PA10 analytical column (i.d. 2 x 250mm), a guard column, AminoPac PA10 (i.d. 2 x 50 mm) was situated in front of the analytical column. The guard and analytical columns were maintained at a constant 30°C. Compounds were eluted using a water (A), 200 mM sodium hydroxide (B) (carbonate free) and 1M sodium acetate (C) (carbonate free) gradient system outlined in Table 4.2. Helium gas was used to pressurise the headspace of eluant bottles reducing carbon dioxide absorbance. Anion traps (ATC-1, i.d. 9 x 24 mm, Dionex Inc) were used to remove trace amounts of metal contamination in the eluants prior to use. The flow rate remained constant at 0.25 ml min⁻¹.

Table 4.2. Gradient conditions used in the elution of amino acids. A = water, B= 200 mM sodium hydroxide, C= 1 M sodium acetate.

	A (%)	B (%)	C (%)
0	80	20	0
2.0	80	20	0
12.0	80	20	0
16.0	68	32	0
24.0	36	24	40
40.0	36	24	40
40.1	20	80	0
42.1	20	80	0
42.2	80	20	0
62.0	80	20	0

Detection of amino acids was by a Dionex ED50 Electrochemical Detector (integrated amperometry). Samples introduction was via a Dionex AS50 Autosampler fitted with a 5µL sample loop. Amino acids were quantified using standard reference compound. Data capture was by Chromeleon software (Dionex Inc.) running on a personal computer. All samples were measured in triplicates.

4.2.15. Lysozyme analysis of colostrum samples

This assay is based on the decrease in absorbance (492 nm) of a cell suspension of *Micrococcus leisodeikticus* when the cells are digested with lysozyme in the presence of Na^+ ions. *Micrococcus leisodeikticus* suspension was prepared by dissolving 5 mg of *M. leisodeikticus* cells (Sigma, UK) in 5.5 ml of 0.1M Potassium phosphate buffer (pH=6.4). Lysozyme solution was prepared by dissolving 2 mg of lysozyme (Sigma, UK) in 1 ml of phosphate buffer. Then 1 to 8 dilution was made by mixing 125 μl of lysozyme solution with 1 ml of phosphate buffer. 96 microtitre plates (Immunolon-1, Dynatech Laboratory Inc., Virginia, USA) were used to perform the assay. 25 μl of 0.1M phosphate buffer (pH=6.4) was pipetted in each well of microtitre plates. Then 25 μl of lysozyme solution (1:8) was added to the first well of the strip and it was double diluted across 7 wells leaving the 8th well blank. 25 μl of colostrum samples were added to each first well of the strip and double diluted across 8 wells. All assays were performed in triplicate and repeated twice. 125 μl of *M. leisodeikticus* suspension was pipetted to each well of strips. The plates were read after 10, 30, 60 and 90 minutes at 492 nm with background subtraction at 0nm using a Dynatech, MR5000 plate reader. Between reading times the microtitre plates were put on the rocking table. Collected absorbance data were plotted on a graph, (absorbance (y-axis) vs. time in minutes (x-axis)) and the lysozyme concentration were calculated.

4.2.16. Animal performance

The biological performance of the sow was measured according to the following criteria; average daily dry matter feed intake, health status and reproductive performance.

Individual birth weight (IBW) of all piglets born alive was recorded within 24h after farrowing. At 7, 14 and 21 days after farrowing, the piglets were again weighed and average weekly live weight gain calculated.

4.2.17. Statistical analyses

All the original dilution and colony count data were recorded and calculated. The bacterial count per gram of faeces was further log transformed, tabulated, and statistically analysed by ANOVA. Mitogenic experiments were carried out in triplicate determinations and repeated at least twice. Significant differences between treatment means were compared by Tukey's HSD test (Zar, 1999). The statistical analyses were undertaken using Minitab v.10.2 (Minitab Inc., Pennsylvania, USA, 1994).

4.3. Results

Microbiology and analyses of the feed

Similarly to the previous feeding experiment (Chapter 2) it was impossible to maintain strict aseptic conditions during the mixing of FLF in the farm environment. As a consequence, the sanitation of liquid feed with chlorine dioxide eliminated coliforms successfully, but did not always eliminate LAB and yeasts. Most often, there were approximately 10^3 to 10^4 CFU ml⁻¹ LAB and yeasts still remaining after 24-hour steeping. The pH of the liquid feed after the sanitation process was around 5.2. After 36 hour fermentation at 30°C, the pH of the FLF dropped to 4.0 ± 0.2 due to the rather high concentration of lactic acid in the feed (220.57 ± 47.36 mmol kg⁻¹). The amount of acetic acid was found to be 38.74 ± 13.40 mmol kg⁻¹. The results from the microbial analysis of the fermented feed showed high numbers of LAB (around 10^9 CFU ml⁻¹) while coliform level was below the detectable limit in each feed sample tested (Table 4.3).

Table 4.3. Characteristics of FLF prepared using 36 hours fermentation time with *Lactobacillus salivarius* as an inoculant.

	Mean \pm SD	Range
pH	4.0 ± 0.2	(3.7 - 4.4)
Lactic acid (mmol kg ⁻¹)	220.57 ± 47.36	(140.11 - 341.57)
Acetic acid (mmol kg ⁻¹)	38.74 ± 13.40	(2.01 - 57.7)
Lactobacilli (log CFU ml ⁻¹)	9.0 ± 0.2	(8.50 - 9.40)
Coliforms (log CFU ml ⁻¹)	<3.0	(-)
Yeasts (log CFU ml ⁻¹)	4.0 ± 0.13	(3.7 - 4.4)
Dry matter (%)	27.2 ± 1.2	(24.8 - 29.4)

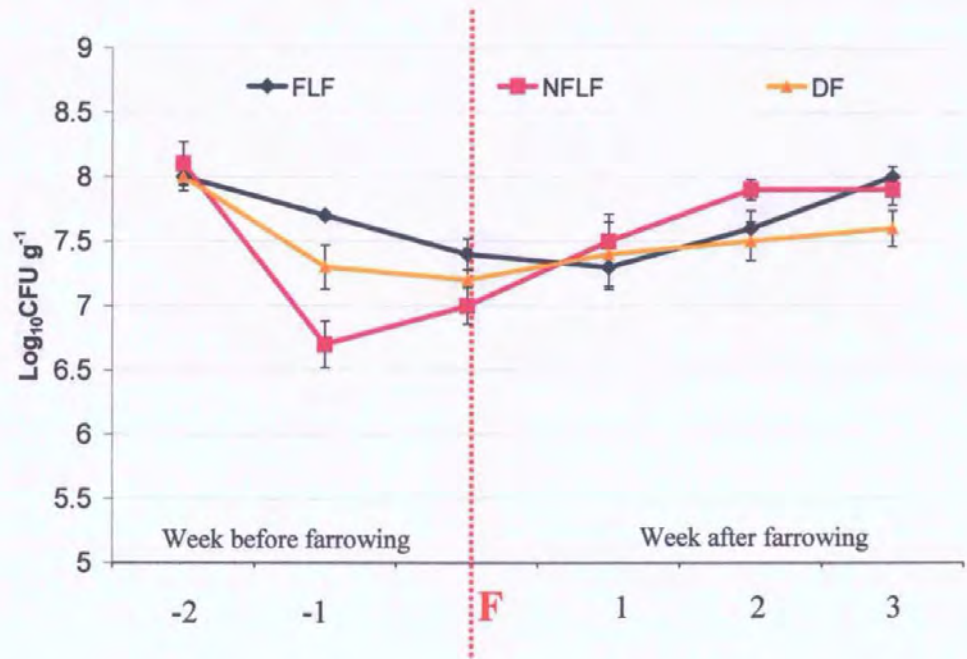
The dry matter (DM) concentrations of the FLF and NFLF were stable over the experimental period with no significant differences between replicates. The average DM concentration for FLF was $27.2 \pm 1.2\%$, which was significantly lower ($P < 0.001$) than the average DM concentration of NFLF ($29.5 \pm 1.6\%$) (Table 4.3). That means that approximately 8 % of the DM was lost through the fermentation process.

Microbiology of the sows' and piglets' faeces

Numbers of faecal LAB, coliforms and *E.coli* in the faeces of sows fed FLF, NFLF, and DF are shown in Figure 4.1. While the lactobacilli population was not affected by dietary treatment, significant differences in coliform and *E. coli* populations were observed in the sow faecal samples taken at parturition as well as post farrowing. In sows fed DF and NFLF, the coliforms and *E. coli* population increased considerably during the two weeks pre-partum and remained high throughout lactation (Figure 4.1). In contrast, the *E. coli* population declined for three weeks after the commencement of feeding FLF. The faeces excreted by sows fed FLF had significantly lower numbers of *E. coli* at parturition compared with sows fed NFLF ($P < 0.05$) and DF ($P < 0.0001$). These significantly lower numbers of *E. coli*, as well as coliforms in the faeces of FLF-fed sows, were maintained throughout lactation. The large increase in *E. coli* and coliforms around farrowing was usually accompanied by slight decrease of LAB population. The faecal LAB: *E. coli* / coliform ratio of piglets from liquid-fed mothers was significantly higher ($P < 0.01$) than for piglets born to DF-fed mothers (Table 4.4). No significant differences were observed between FLF and NFLF piglets.

Figure 4.1. Faecal counts ($\text{Log}_{10}\text{CFU g}^{-1}$ (dry weight)) of lactobacilli (A), coliforms (B), *E. coli* (C) in the sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF) for the period of 2 weeks before farrowing (F) till 3 weeks after parturition.

A) Lactobacilli



B) Coliforms

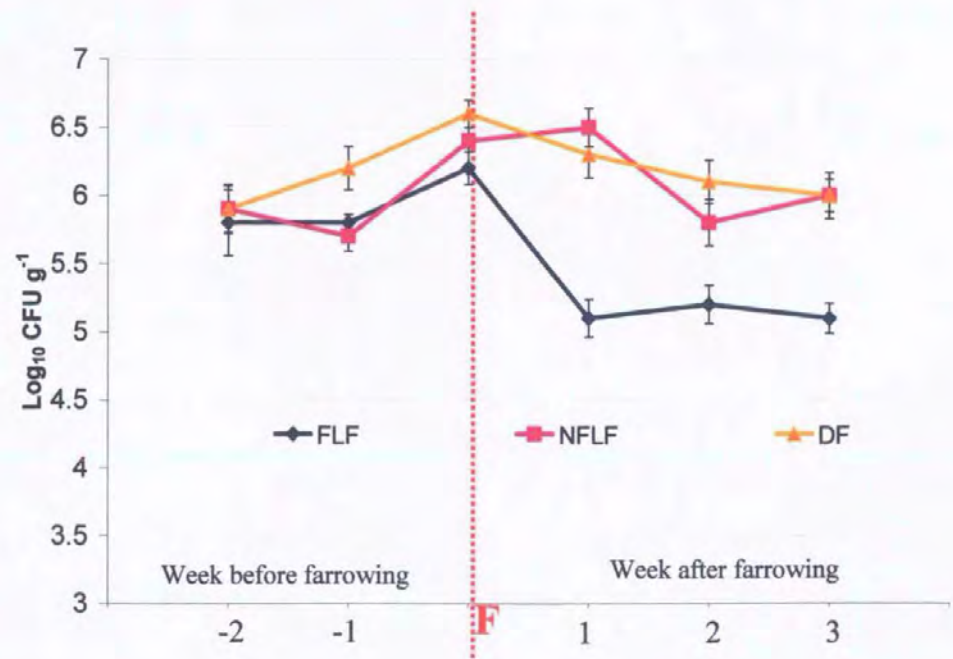


Figure 4.1 (cont.). Faecal counts ($\text{Log}_{10}\text{CFU g}^{-1}$ (dry weight)) of lactobacilli (A), coliforms (B), *E. coli* (C) in the sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF) for the period of 2 weeks before farrowing (F) till 3 weeks after parturition.

C) *E. coli*

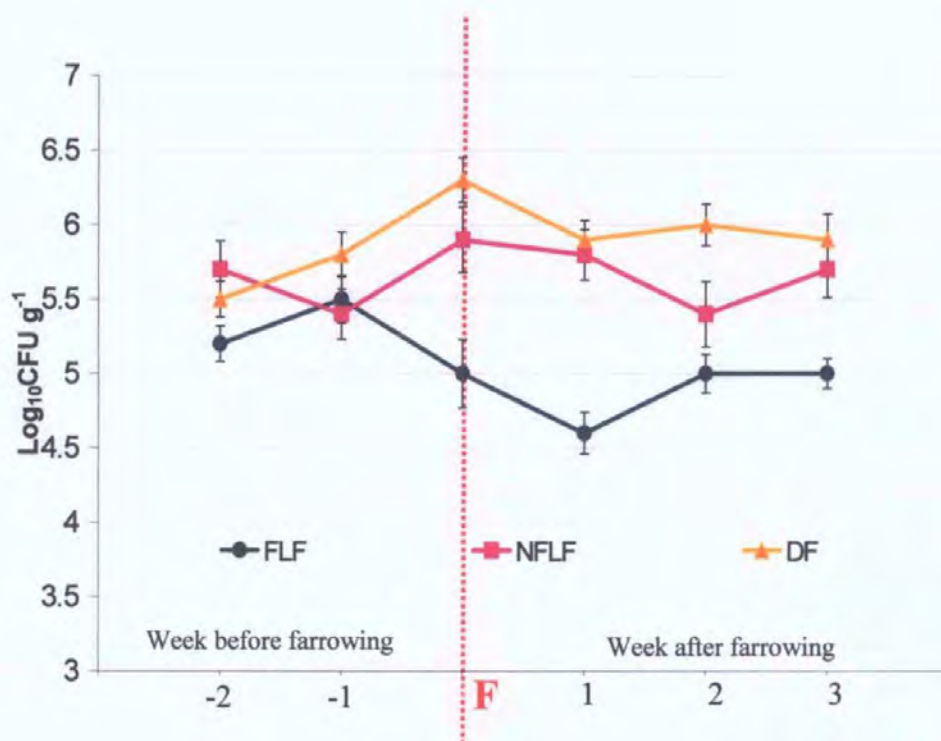


Table 4.4. LAB : coliform and LAB: *E. coli* ratio in the faeces of 2-week old piglets.

	FLF	NFLF	DF	P
LAB: coliforms	0.33 ^a	0.31 ^a	-0.19 ^b	**
LAB: <i>E. coli</i>	0.42 ^a	0.40 ^a	-0.17 ^b	***

FLF-fermented liquid feed; NFLF-non-fermented liquid feed; DF-dry feed.

^{a,b} within the rows, means with the same superscript are not significantly different.

Figure 4.3. Evaluation of species-specific PCR for the identification of *Lactobacillus salivarius* strain, used to ferment the liquid feed, in faecal samples of farrowing sows.

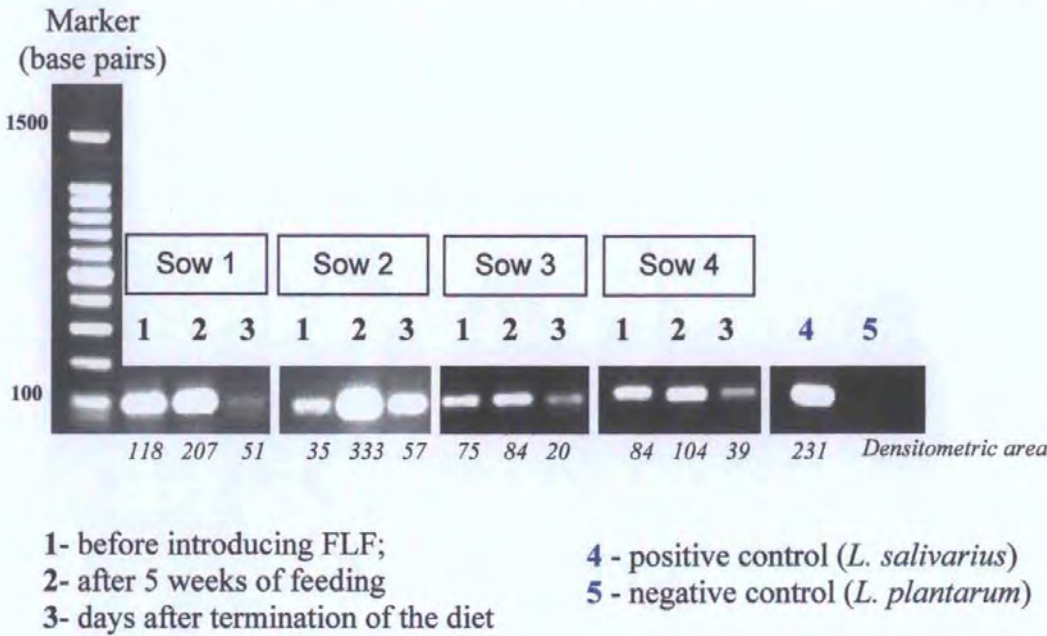
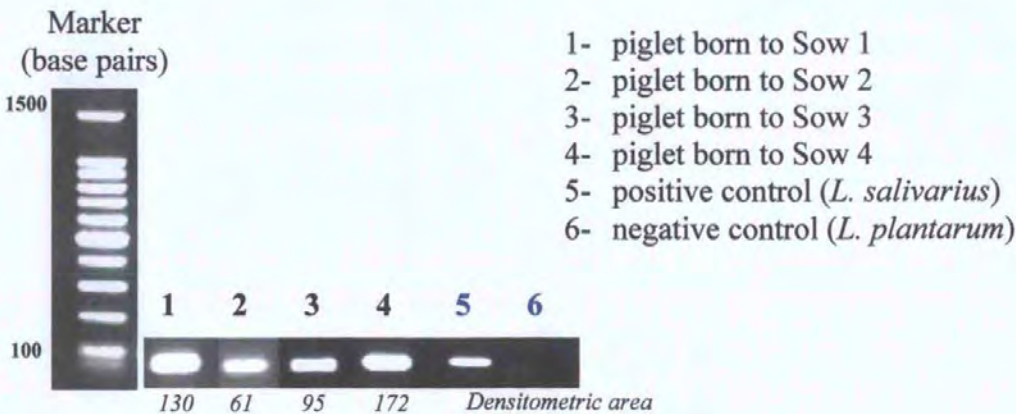


Figure 4.4. Evaluation of species-specific PCR for the identification of *Lactobacillus salivarius* strain, used to ferment the liquid feed, in faecal samples of 14-days old piglets.



The PCR method confirmed that the *L. salivarius* strain, used to ferment the feed, survived passage through the intestinal tract of sows (Figure 4.3) and that it was present also in piglets' faeces on the 14th day of suckling (Figure 4.4). As shown, each tested sow harbored *L. salivarius* naturally prior to administration of FLF (Figure 4.3). Feeding FLF for 5 weeks increased the numbers of *L. salivarius* in all tested faeces. However, the numbers decreased again 4 days after termination of feeding. Only in one case (sow 2) was the level of *L. salivarius* higher, four days after cessation of feeding, than at the starting point.

Dry matter and short-chain fatty acid (SCFA) concentration of the sows' faeces

At farrowing, the dry matter concentration of faeces from both FLF and NFLF fed sows were significantly lower than that of DF fed sows (Table 4.5). In addition sows fed FLF, but not sows fed NFLF, had faeces with lower DM content for 14 days postpartum.

Table 4.5. Dry matter content (g kg^{-1}) of faeces of gilts fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF), and dry feed (DF) during period 14, 7 days before farrowing, at farrowing and 7, 14 days post-farrowing.

	FLF	NFLF	DF	P
Day 14 BF	24.71±0.4	26.31±0.6	25.38±0.5	ns
Day 7 BF	27.04±0.7	28.34±0.5	26.37±0.6	ns
Farrowing	27.93±0.2 ^a	27.81±0.4 ^a	30.40±0.4 ^b	***
Day 7 AF	23.33±0.7 ^a	25.91±0.8 ^b	27.99±0.8 ^b	***
Day 14 AF	22.47±0.6 ^a	26.64±0.6 ^b	26.26±0.7 ^b	***

BF- before farrowing; AF-after farrowing; *** $P < 0.001$; ^{a,b} within the rows, means with the same superscript are not significantly different. Data are expressed as a mean \pm SEM

The mean values of SCFA concentrations in the sow faeces are presented in Table 4.6. Significant increases in the total faecal concentration of acetic acid ($P < 0.01$) and butyric

acid ($P<0.001$) were recorded in the FLF group after 5 weeks of intake compared with DF fed sows. There was also a significant increase ($P<0.01$) in propionic acid in NFLF-fed sows after 5 weeks of feeding compare to DF-fed sows. Lactic acid was not detectable in the faeces of any experimental group.

Table 4.6. Short-chain fatty acid (SCFA) concentration (mmol g^{-1}) of the sows' faeces at day 1, 14 and 35 of feeding.

SCFA		FLF	NFLF	DF	P
Acetate	Day 1	6.13 ± 1.29	6.36 ± 1.63	6.16 ± 1.56	ns
	Day 14	5.36 ± 1.25	5.01 ± 1.25	5.49 ± 0.72	ns
	Day 35	7.41 ± 2.31^a	$5.93 \pm 1.53^{a,b}$	4.80 ± 0.51^b	**
Propionate	Day 1	2.41 ± 0.83	2.53 ± 0.75	2.21 ± 0.71	ns
	Day 14	1.49 ± 0.76	1.45 ± 0.70	1.87 ± 0.54	ns
	Day 35	1.92 ± 0.75^a	2.48 ± 0.79^a	$1.24 \pm 0.63^{a,b}$	***
Butyrate	Day 1	1.33 ± 0.39	1.03 ± 0.25	1.12 ± 0.60	ns
	Day 14	0.96 ± 0.36	0.93 ± 0.41	1.11 ± 0.31	ns
	Day 35	2.18 ± 0.89^a	1.84 ± 0.49^a	1.02 ± 0.49^b	***

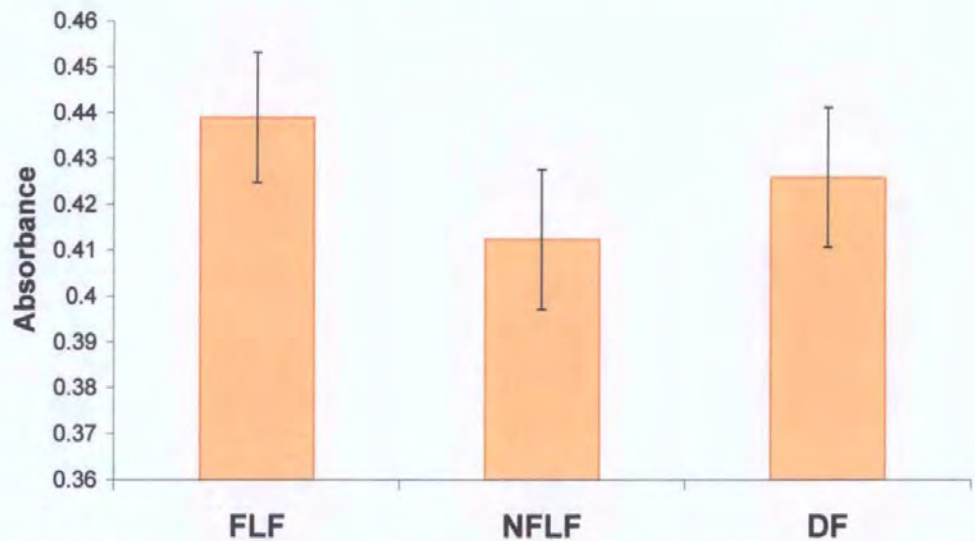
Data are expressed as a mean \pm SD; ns = not significant ** $P<0.01$; *** $P<0.001$

^{a, b} within rows, means with the same superscript are not significantly different

Mitogenic activity on intestinal epithelial cells (IEC-6) and pig lymphocytes

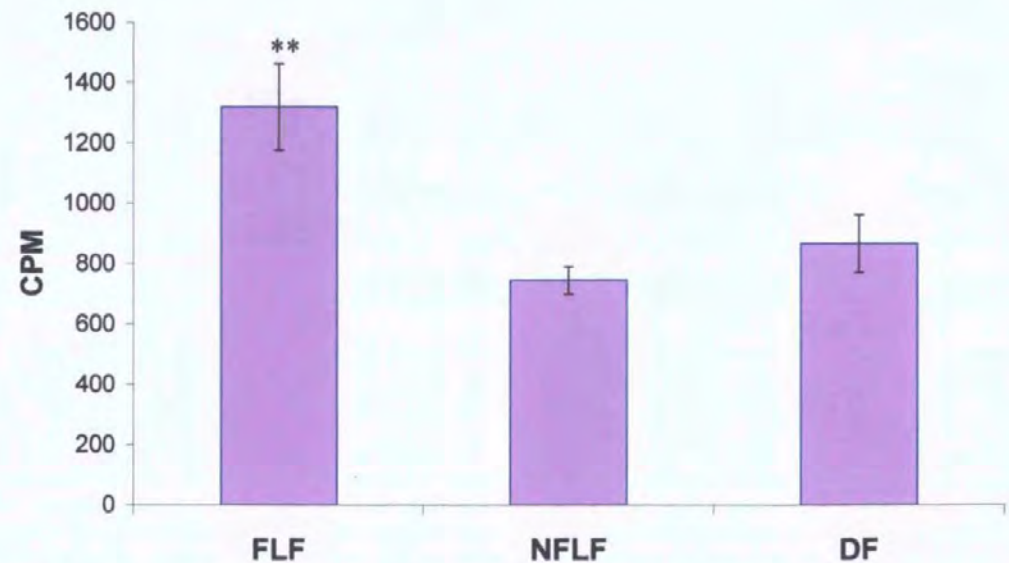
The mitogenic response of intestinal epithelial cells (IEC-6), exposed to the colostrum samples taken from the gilts on different diets, is presented in Figure 4.4. The results obtained by CellTiter 96[®] non-radioactive cell proliferation method showed that although all colostrum samples stimulated proliferation of IEC-6 compared with the negative control, no significant differences were observed between the treatments.

Figure 4.4. Mitogenic activity of gilts' colostrum on intestinal epithelial cells (IEC-6).



Data are expressed as a mean ± SEM

Figure 4.5. Mitogenic activity of gilts' colostrum on blood lymphocytes



Data are expressed as a mean counts per minute (CPM) ± SEM; **p<0.01

However, despite non significant results, colostrum from FLF-fed gilts showed numerically the best mitogenic potential. Lymphocyte proliferation was evaluated by the radioactive method, the same method that was used in the previous mitogenic experiments in Chapter 2. The results confirmed the significantly better proliferation activity ($P < 0.01$) of colostrum samples from the group of sows fed FLF compared with NFLF and DF respectively (Figure 4.5).

Immunoglobulin, protein and amino-acid content of colostrum samples

The mean concentrations of the total protein as well as the immunoglobulins A, G and M in the colostrum of gilts fed different diets are given in Table 4.7. The results showed that colostrum from FLF-fed sows contained 48.95 mg ml^{-1} of IgG which was significantly higher ($P < 0.05$ and $P < 0.01$) than colostrum of NFLF-fed (36.62 mg ml^{-1}) and DF-fed (30.81 mg ml^{-1}) gilts respectively. A significant difference ($P < 0.05$) was also observed in colostral IgA concentration of FLF-fed gilts (15.01 mg ml^{-1}) compared with NFLF-fed gilts (10.57 mg ml^{-1}). There were no significant differences in IgM concentration, protein (Table 4.7) or amino-acid (Table 4.8) content of the colostral samples.

Table 4.7. Concentration (mg ml^{-1}) of immunoglobulins G (IgG), A (IgA), M (IgM) and total proteins in colostrum of sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF).

	IgG	IgA	IgM	Total proteins
FLF	48.95 ± 3.27^a	15.01 ± 1.04^a	5.60 ± 0.62	215.8 ± 11.53
NFLF	$36.62 \pm 3.50^{b,c}$	10.57 ± 1.11^b	4.37 ± 0.66	175.4 ± 12.32
DF	30.81 ± 3.50^c	$11.96 \pm 1.11^{a,b}$	5.03 ± 0.66	198.1 ± 12.32

Data are expressed as a mean \pm SEM; ^{a,b,c} Within columns, means with a common superscript are not statistically different

Table 4.8. Amino-acid analysis of colostrum from gilts fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF).

Amino-acids (ppm)	FLF	NFLF	DF
<i>Arginin</i>	13183 ± 814.0	14966 ± 759.7	14118 ± 816.4
<i>Lysine</i>	8855 ± 564.3	9675 ± 526.6	9592 ± 565.9
<i>Alanin</i>	8025 ± 1366	5216 ± 1274	5586 ± 1373
<i>Threonine</i>	5731 ± 419.9	6410 ± 391.9	6061 ± 421.1
<i>Valine</i>	10003 ± 707.4	10324 ± 660.2	9877 ± 709.5
<i>Serine</i>	6435 ± 483.7	7148 ± 451.5	6981 ± 485.2
<i>Proline</i>	9647 ± 554.2	10482 ± 517.2	10552 ± 555.8
<i>Isoleucine</i>	6188 ± 461.3	6048 ± 430.5	6000 ± 462.6
<i>Leucine</i>	10573 ± 603.3	11425 ± 563.1	11205 ± 605.1
<i>Methionine</i>	1105 ± 91.12	1316 ± 85.04	1245 ± 91.39
<i>Phenylalanine</i>	4629 ± 262.0	4859 ± 244.6	4843 ± 262.8
<i>Tyrosine</i>	4703 ± 281.5	4855 ± 262.7	4980 ± 282.3

Data are expressed as a mean ± SD

Lysozyme analysis of colostrum samples

Colostrum from FLF-fed group of gilts had a numerically higher concentration of lysozyme however this was not significantly different from other two experimental groups (Table 4.9).

Table 4.9. Lysozyme concentrations ($\mu\text{g ml}^{-1}$) in colostrum of primiparous sows fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF) and dry feed (DF).

	FLF	NFLF	DF
<i>Lysozyme ($\mu\text{g ml}^{-1}$)</i>	13.26 ± 3.69	9.66 ± 2.69	10.09 ± 2.52

Data are expressed as a mean ± SD.

Animal performance

All gilts suckled their litters throughout the lactation period. Reproductive performance of sows and growth and survival traits of their piglets are presented in Table 4.11 and Table 4.12. Average daily feed intakes of sows fed liquid diets were significantly higher (15-20%) than those of DF sows during the second and third week of lactation (Table 4.10). However, because of the small number of replicates there was no significant relationship with piglet growth (Table 4.11).

Table 4.10. Average daily feed intake ADFI (DM kg/day) of gilts fed fermented liquid feed (FLF), non-fermented liquid feed (NFLF), and dry feed (DF) during 3-week lactation period.

	FLF (1)	NFLF (2)	DF (3)	<i>sed</i> (1-2)	<i>sed</i> (1-3)	<i>sed</i> (2-3)
Week 1	3.2	3.5	3.0	0.20	0.20	0.21
Week 2	5.6	6.0	4.8	0.21	0.22 ***	0.23 ***
Week 3	7.1	7.4	6.0	0.21	0.21 ***	0.23 ***

***P<0.001

Table 4.11. Average piglet weight (kg) after first and second week of suckling.

	FLF (1)	NFLF (2)	DF (3)	<i>sed</i> (1-2)	<i>sed</i> (1-3)	<i>sed</i> (2-3)
Week 1	2.71	2.89	2.72	0.10	0.10	0.12
Week 2	4.00	4.39	4.39	0.14 *	0.16	0.18

*P<0.05

Piglets from gilts fed FLF were 300 and 450g heavier than piglets from the gilts fed NFLF (P<0.01) and DF fed gilts (P<0.001) (Table 4.12). The number of piglets remained alive was numerically higher for sows fed on FLF.

Table 4.12. Effect of type and amount of feed on litter performance at birth.

Measurements (average)	FLF ₍₁₎	NFLF ₍₂₎	DF ₍₃₎	sed (1-2)	sed (1-3)	sed (2-3)
<i>Litter size</i>	11.37	11.27	10.15	0.319	0.351 **	0.341
<i>Piglets alive per litter</i>	11.13	9.72	9.79	0.742	0.699	0.343
<i>Birth-weight (kg) (BW)</i>	1.787	1.401	1.332	0.122 **	0.118 ***	0.056
<i>Feed Intake DM (kg) during 2 weeks period</i>	33.98	36.90	36.61	0.100 *****	0.110 ***	0.107 *

*P<0.05, ** P<0.01, *** P<0.001; litter size was used as a covariate in calculation of BW

The results from this study support the view that the provision of FLF to gilts at 14 days before farrowing, can improve the BW of piglets compared with the birth weight of piglets from gilts fed NFLF and DF, even though the sows on FLF consumed less DM (P<0.001) and had significantly larger litters (P<0.01) than gilts fed DF.

4.4. Discussion

Microbiology and analyses of the feed

Despite the finding in the previous experiment (Chapter 2), that under farm conditions the sanitation of pig feed with chlorine dioxide is not complete, it was decided to use the same sanitation procedure in this experiment. In an ideal situation it would be preferable to have a sterile feed. However, chlorine dioxide is able to eliminate coliforms to below detectable limits, reduce the level of natural occurring yeast and LAB and thus allow the starter culture to become the predominant strain in the feed (Chapter 2). The results obtained in this study were very similar to those reported in Chapter 2. In terms of coliforms, sanitation with chlorine dioxide was successful, as coliforms were eliminated below detectable limit in 24 hours in each feed sample during the whole experimental period. However, the way how the feed was prepared on the farm made total elimination of natural occurring LAB and yeasts impossible to achieve.

The loss of dry matter (7.8 %) in FLF in the current study was slightly higher than in the previous experiment (6%). However, no corrections were made in the dry matter calculations for the volatile components of the FLF diet, as the total organic acids and ethanol were not quantified. Therefore, the dry matter loss by fermentation does not necessarily mean the loss of dietary energy as some dietary substrates are only converted to different source of energy (e.g. carbohydrates to SCFA).

Microbiology of the sows' and piglets' faeces

Coliform results in this study were in agreement with the results reported in Chapter 2. Significant differences between dietary treatments were observed only 1 week postpartum. However, in terms of *E. coli* a significant reduction was observed already at farrowing. This supports the hypothesis that coliforms, and more importantly the *E. coli* challenge to the newborn piglet, can be reduced by feeding sows fermented liquid feed. The possible factors which may account for this beneficial antibacterial activity of FLF (pH of the feed, SCFA, lactic acid and high number of LAB) were thoroughly discussed in Chapter 2. In addition to these factors, Schiffrin *et al.*, (1995) demonstrated another possible means by which *E. coli* could be eliminated from the host GI-tract. Phagocytosis of *E. coli* by human blood leucocytes was enhanced after a 3-week consumption period of milk fermented with *L. acidophilus* or *Bifidobacterium bifidum*. This suggests that specific LAB strains can enhance the host's nonspecific defense mechanism. Similar results were reported by De Simone *et al.*, (1988), where it was concluded that beneficial modification of microflora with yogurt containing live lactobacilli influences the adherence of *Salmonella typhimurium* to intestinal mucosa, the natural antibacterial activity of the Peyer's patches lymphocytes, the accumulation of the macrophages in the liver, and the proliferative responses of the splenocytes. In addition, Romond *et al.*, (1997) showed that in the case of certain probiotic strains, abiotic compounds produced during fermentation might also be responsible for the enhancement of the antibacterial effect of fermented products. The cell-free whey from cow's milk fermented with *Bifidobacterium breve* and cleared from the presence of acetic and lactic acids, was able to decrease numbers of clostridia and bacilli and to increase the number of bifidobacteria in the faeces of human volunteers after 7 days of consumption. Detailed characterization of the whey compounds suggested that it is

possibly an enzyme produced during fermentation that might play a role in the induced microbial changes (Mullie *et al.*, 2002). The enzyme might reach the intestinal lumen and partly degrade substrates, such as mucins, usually used by the intestinal flora. The released molecules might then favor the development of a new microbial balance. Feeding FLF to sows did not seem to have any major effect on the indigenous lactobacilli population. However, it helped to balance the negative effect of stress associated with farrowing on the lactobacilli numbers as numbers of lactobacilli one week before farrowing were significantly higher in the faeces of FLF-fed sows compared with NFLF and DF-fed sows. Increased numbers of *L. salivarius* in the faeces of experimental sows after 5 weeks of feeding were observed in all sows, however with variable densities. Ohashi *et al.*, (2001) showed that milk fermented with *L. casei* strain *Shirota* appeared to affect the indigenous *Lactobacillus* population and constitution of the experimental pigs after 2 weeks of feeding. Although the number of bacteria of *L. casei* strain *Shirota* in the intestinal contents was much smaller than those of indigenous lactobacilli, (10^4 vs 10^8 CFU g⁻¹), the numbers of indigenous lactobacilli and bifidobacteria in the pig intestine appeared to increase with the fermented milk. The 16S rDNA method was also used to observe the increased faecal numbers of *L. acidophilus* after 2 week consumption period in the study of Sui *et al.*, (2002). Lactobacilli derived from the endogenous flora are being increasingly used as probiotics in functional foods. *L. salivarius* together with *L. delbrueckii* and *L. acidophilus* are typical obligately homofermentative lactobacilli isolated from the GI-tract of pigs (Tannock, 1992; Du Toit *et al.*, 2001). Indeed, each sow fed FLF harboured *L. salivarius* naturally before the experimental treatment as confirmed by the PCR method. Pascual *et al.*, (1999) reported a protective effect of *L. salivarius* strain against *Salmonella enteritidis* C-114 colonization in chickens. When the *L. salivarius* was dosed (10^5 CFU g⁻¹) by oral gavage together with *S. enteritidis* C-114 directly into the proventriculus of 1-day-

old chickens, the pathogen was completely removed from the birds after 21 days. The same results were obtained when the probiotic strain was administered through the feed and the drinking water. *L. salivarius* in this study expressed very good *in vitro* fermentative and probiotic features. However, as shown recently by Ibnou-Zekri *et al.*, (2003), distinct *Lactobacillus* strains colonized the intestinal lumen and translocated into mucosal lymphoid organs at different densities despite displaying similar growth, survival, and adherence properties *in vitro*. Therefore, there is a possibility that other lactobacilli that express similar *in vitro* characteristics to *L. salivarius* would perform better *in vivo* than the selected *L. salivarius*. PCR analyses of faecal samples from gilts taken four days after termination of the trial revealed that only in one case was the introduced *L. salivarius* sustained at higher levels compare with the numbers at the starting point. Surprisingly, the number of *L. salivarius* in the faeces excreted by the rest of the experimental sows dropped below the level detected at the beginning of the experiment. The reason for such a dramatic drop in *L. salivarius* is not known. One possible explanation may be the stress effect as the faecal samples were taken shortly after weaning and sows' movement into new accommodation as well as diet change (from liquid form to dry pellet). Stress is known to influence the GI microflora, and total *Lactobacillus* populations are among those that are shown to be affected (Tannock, 1997). On the other hand, several recent studies have indicated that it is not uncommon for introduced bacteria, particularly those under investigation for probiotic properties or preparations, to be undetectable 3 to 5 days after terminations of treatment (Jacobsen *et al.*, 1999; Netherwood *et al.*, 1999a; Netherwood *et al.*, 1999b; Simpson *et al.*, 2000; Walter *et al.*, 2000; Sui *et al.*, 2002). These results confirmed the already well established observation that colonization of the adult GI-tract by orally introduced strains is not a simple process. Certainly it needs to be realized that analysis of the composition of the faecal microflora does not show what happens in the

small intestine. But, taking in account the complexity of the intestine, individual variations of animals to probiotic inclusion may be the rule and not the exception. Probiotics used as feed additives do not act like essential nutrient in terms of a clear dose response until the requirements are met. Therefore, the range between no effect and significant effects seems to be reasonable (Simon *et al.*, 2003). However, more research is needed in order to be able to answer questions such as: what is the efficacy of probiotics in relation to the host age, physiological status and dietary intake, as well as how the introduced strains will interact within the microbial biofilm of the host GI-tract. However, despite this fact, we can conclude that liquid feed fermented with *L. salivarius* proved to have the ability to beneficially alter the composition of the intestinal microflora of farrowing gilts which was subsequently reflected in more 'friendly' bacterial flora in the neonate's GI-tract. Higher faecal LAB: coliform / *E. coli* ratios are usually associated with a bacterial flora that contributes to improved animal growth and performance (Muralidhara *et al.*, 1977). The possible factors which could be account for this beneficial microflora in piglets' faeces were discussed in Chapter 2. The fact that the mother animal represents a major source of piglets' lactic acid bacteria flora was also confirmed in this study as randomly selected piglets from FLF-fed dams excreted *L. salivarius* in high numbers at 2 weeks of age.

Dry matter and short-chain fatty acid (SCFA) concentration of the sows' faeces

Constipation of sows during late gestation or within a few days of farrowing is commonly encountered (Miller *et al.*, 1982). This reduction in feed passage can result in lowered feed intake and therefore reduced milk production which would have detrimental effect on the body weight gain of the piglets. Thus inclusion of a laxative in the sow diet around farrowing time is often recommended. The current study showed that FLF exerted mild

laxative effects on the experimental sows which represent another beneficial feature of this diet for the farrowing sows (Table 4.5). Ouwehand *et al.*, (2002) showed that supplementation of diet with certain probiotics can improve intestinal motility and reduce faecal enzyme activity. It has also been suggested that alleviation of constipation by feeding probiotics will only require high numbers of transient bacteria through the intestine and therefore this effect may not be strain specific (O'Sullivan, 2001).

At the end of the 5-week feeding period the faecal concentration of acetate and butyrate was significantly higher in the FLF-fed sows than DF-sows. The benefit of SCFA in terms of host health and pathogen elimination has been thoroughly reviewed in Chapter 2 and thus based on the previous discussion the increase of SCFA in the faeces of FLF-fed sows is considered as a beneficial outcome of this dietary treatment and most possibly represents one of the factors responsible for the significant reduction of coliforms and *E. coli* from the sow's GI-tract. A significant increase of faecal acetate and increasing tendency of butyrate after consumption of milk fermented with *L. casei* strain *Shirota*, was also reported by Ohashi *et al.*, (2001).

Mitogenic activity on intestinal epithelial cells (IEC-6) and pig lymphocytes

The results of the mitogenic studies confirmed the previous results only in the case of pig lymphocytes. This experiment was conducted according to the same protocol as the one used in Chapter 2. However, a different method was used to evaluate mitogenic activity of colostral samples on intestinal epithelial cells. The results of this experiment were not significant however there was a tendency for FLF-fed sows to produce colostrum with higher mitogenic activity. It would probably need more animals on the treatments to obtain

more significant results. The benefit of having colostrum of higher mitogenic activity and possible factors has been discussed in Chapter 2. In addition to colostrum with better mitogenic activity, the colonization of newborn gut with *L. salivarius* could itself be responsible for additive proliferation effect on the immune cells. In the study of Kirjavainen *et al.*, (1999a), oral administration of *L. rhamnosus* GG (10^9 viable bacteria / kg of body weight) appeared to enhance T and B-cell proliferation. However, it was shown that this immunostimulatory effect was dose- and duration-dependent. Higher bacterial numbers (10^{12} viable bacteria/kg of body weight) enhanced B-cell proliferation, but this was accompanied by a decrease in T-cell proliferation. This is another example of experiments that emphasize the importance of having information on dose response and time profile relationships of individual probiotic. Therefore, it can be speculated that different doses and consumption periods for *L. salivarius* may be needed to trigger sufficient amounts of the mediators responsible for proliferation of epithelial cells and lymphocytes. Strain specific effects on the proliferation of lymphocytes were demonstrated in the study of Kirjavainen *et al.*, (1999b). While oral consumption of *L. acidophilus* (10^9 viable bacteria / kg) by mice enhanced *ex vivo* basal proliferation of B cells, the opposite effect was obtained after consumption of *L. casei*, *L. gasseri* and *L. rhamnosus*. A similar beneficial immunomodulatory effect of different LAB on lymphocyte proliferation was described in other studies (Yasui and Ohwaki, 1991; Aattouri *et al.*, 2002; Easo *et al.*, 2002).

Immunoglobulin, protein and amino-acid content of colostrum samples

The trend for FLF to increase immunoglobulin levels in sow's colostrum, observed in Chapter 2, was also confirmed in this experiment. However, by choosing animals of the

same parity and by taking colostrum at a specific time from each sow (after the first piglet was born), significant differences were found in the concentration of IgG of colostrum from FLF-fed sows compared with NFLF and DF-fed sows. The concentrations of colostral IgA and IgM were also numerically higher in FLF-fed sows, with IgA being significantly higher than in the colostrum of NFLF-fed sows. This immunostimulatory effect of FLF may be due to the presence of high numbers of LAB, namely *L. salivarius*. However, it is difficult to conclude whether these significant differences were obtained just by changing inoculants from *L. plantarum* to *L. salivarius* or whether it was an effect of having a better experimental design in this study (in terms of animal age, health status and colostrum sampling time). The concentration of the immunoglobulins in colostrum of primiparous sows was much lower compared with those of multiparous sows in the first experiment, which is in agreement with the results reported in previous studies (Klobasa *et al.*, 1985; Klobasa *et al.*, 1986; Klobasa and Butler, 1987). The function and importance of colostral IgG and IgA for neonatal piglets as well as factors affecting the levels of immunoglobulins in sow colostrum and milk have been discussed in Chapter 2. It has been shown that not all lactobacilli are immunostimulatory and, for those that are, the size of the dose administered seems to be critical. Therefore, it could be that porcine *L. salivarius* might have greater immunoenhancing potential than *L. plantarum*. In studies with mice, Perdigon *et al.*, (1993) found that oral introduction of *L. casei*, but not *L. acidophilus* induced synthesis of IgA, in the gut, and long-term feeding of the bacterium increased proliferation of plasma cells, lymphocytes and macrophages. Similarly, another *in vitro* study by Easo *et al.*, (2002) showed that only yogurt starter-derived strains of *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* were able to induce high levels of antibody production (of IgM and IgG isotypes) by murine splenocytes, while two different strains, *L. delbrueckii* subsp. *bulgaricus* (ATCC 11977) and *L. acidophilus* (ATCC 521)

were not able to express this immunomodulatory property. Bonet *et al.*, (1999) demonstrated that all four lactobacilli strains (*L. casei*, *L. delbruecki* subsp. *bulgaricus* or *L. acidophilus* at a concentration of 1×10^9 cells/day/mouse) tested were able to increase the number of IgA secreting cells and mast cells associated with both gut and bronchus mucosa, with the effect being more marked for *L. delbruecki* subsp. *bulgaricus*. There are many other studies demonstrating that certain LAB may act as adjuvants of the humoral immune response. Saucier *et al.*, (1992) studied the effect of different diets (milk containing a washed suspension of eight LAB, milk fermented by the same strains in both live and heat-treated form) on the immune system (specific and nonspecific) before and after infection of mice with *Klebsiella pneumoniae* on the 13th day of feeding. Postinfection survival was 0.7 d longer for mice receiving fermented milk than for the saline control group. The percentage of phagocytosis by macrophages did not vary significantly, while serum immunoglobulin G levels differed between mice fed fermented milk and those fed bacterial suspensions in unfermented milk. Fermentation appears to be essential for the beneficial effects on the immune system and survival time as this effect no longer occurs after pasteurization of fermented milk. In another study, a group of volunteers consumed fermented milk containing *L. acidophilus* and bifidobacteria over a period of 3 week. During this period an attenuated *Salmonella typhi* was administered to mimic enteropathogenic infection. Subjects fed fermented milk had a significant increase in the titer of specific serum IgA in comparison to the control group (Link-Amster *et al.*, 1994). Oral administration of *Bifidobacterium bifidum* to mice significantly increased the number of Ig (IgM, IgG, and IgA) secreting cells in the culture of both mesenteric lymph nodes and spleen cells (Park *et al.*, 2002). It was shown that this immunostimulating activity of *B. bifidum* was due to its cellular components and not due to any component(s) actively secreted by the bacteria. Oral administration of live bifidobacteria increased faecal

IgA levels in children (Fukushima *et al.*, 1998) and faecal and milk IgA levels in lactating mice (Fukushima *et al.*, 1999). Oral administration of *Bifidobacterium breve* YIT4064 enhanced antigen-specific IgG (Yasui *et al.*, 1999) as well as IgA antibody in the mammary gland and in the intestine (Yasui *et al.*, 1995). Mouse pups born to and nursed by dams fed *B. breve* YIT4064 and immunized orally with rotavirus were more strongly protected against rotavirus-induced diarrhea than those born to and nursed by dams immunized with rotavirus only. Yasui *et al.*, (1999) also demonstrated that by oral administration of different bacteria (in this case *L. casei* strain Shirota (LcS)) different modulation of the immune system was observed compared with *B. breve*. *L. casei* stimulated type 1 helper T (Th1) cells, activated the cellular immune system and inhibited IgE production. This study provides clear evidence that different strains of lactic acid bacteria can modulate different parts of the immune systems and thus protect the host against various diseases (Yasui *et al.*, 1999). In addition, Paubert-Braquet *et al.*, (1995) demonstrated that even for one bacteria species, various strains exhibit different effects on the immune system. The study compared the effects of milks fermented with various *L. casei* strains on the survival rate of mice infected with *Salmonella typhimurium*. Although all fermented milks exhibited a protective effect against *S. typhimurium* infection, there were obvious differences in the level of protection between the treatments. Interestingly, the hierarchy established for survival rates did not entirely correlate with the effects noted on immune system, which indicates that stimulation of non-specific immunity by lactobacilli, especially macrophage activity, is not the only mechanism involved in protection against intestinal infections.

The ability of colostral sIgA and IgG to prevent bacterial translocation *in vitro* was studied by Albanese *et al.*, (1994). While both immunoglobulins bound *E. coli*, only sIgA

completely prevented passage of *E. coli* across a morphologically intact segment of viable intestinal tissue as compared with rats in the control group. The presence of IgG resulted in a significantly reduced number of bacteria that passed when compared with controls. In addition, it has been demonstrated that the microbicidal activity of colostrum phagocytes was dependent on previous pathogen opsonization with colostral IgA and IgG (Honorio-Franca *et al.*, 1997). It was suggested that this type of killing of enteropathogenic bacteria by colostral phagocytes may represent an additional mechanism of breast-feeding protection against intestinal infections during the first week of life. Therefore, significantly higher concentrations of these immunoglobulins in FLF-colostrum may also enhance the killing function of colostral phagocytes. Increased intestinal IgA production and thereby promotion of the gut immunological barrier during diarrhoea after oral introduction of lactobacilli was also observed in the human studies by Kaila *et al.*, (1992) as well as Malin *et al.*, (1996).

A study by Rychen and Nunes (1995) demonstrated that supplementation of the diet with lactobacilli (10^6 CFU g⁻¹ feed) did not influence piglet's blood concentration of glucose, galactose and lactic acid. However, the concentration of amino-N was significantly higher in the piglets consuming diet supplemented with these bacteria compared with those on the basal diet. The digestibility of bacterial protein has been shown to be high and similar to that of soybean meal. Recently, Canibe *et al.*, (2003) showed that fermentation of liquid feed resulted in increased concentration of total lysine, which could be explained by the transformation of dietary protein to bacterial protein, which contains higher levels of lysine compared with dietary proteins (Roth and Kirchgessner, 1977). Sows in the current study were consuming 10^9 CFU of *L. salivarius* / g feed for 2 weeks. However, it did not have any significant effect on colostrum protein or amino-acid composition.

Lysozyme analysis of colostrum samples

Lysozyme (1,4- β -N-acetylmuramidase) is a small basic protein (enzyme) with antibacterial activity. The concentration of lysozyme in colostrum of primiparous sows was not affected by dietary treatment and the levels obtained in this study (between 9.6 to 13.26 $\mu\text{g ml}^{-1}$) were similar to those estimated by Schulze and Müller (1980) (between 6.8 and 11.0 $\mu\text{g ml}^{-1}$). Day of lactation and parity both appear to have a marked influence on lysozyme concentration. Schulze and Müller (1980) reported that the concentration of lysozyme reached its highest point on the second day after parturition, followed by a decline to a much lower value ($5.2 \pm 2.0 \mu\text{g ml}^{-1}$) which then remained constant up to the 30th day of lactation. The colostrum samples in the current study were taken immediately after parturition thus the results presented in this study does not necessarily represent the highest possible amounts of colostral lysozyme. The concentration of lysozyme in colostrum of FLF-fed sows tended to be higher than those in the colostrum of NFLF and DF-fed sows. It would be interesting to see whether the increase in the lysozyme concentration would be the same for all the experimental group of sows or whether even greater differences between the dietary treatments would be found if colostrum samples were taken on the second day of lactation. Such high lysozyme levels in sow colostrum over the first two days of age suggest that this protein might affect the bacterial population of the gastrointestinal tract of suckling piglets. However, there is very little *in vivo* evidence for an involvement of milk lysozyme in the defence against infection in the newborn.

Besides its antibacterial activity lysozyme has many other functions including inactivation of certain viruses (Gluck, 1989), enhancing phagocytic activity of polymorphonuclear leukocytes and macrophages (Biggar and Sturgess, 1977), stimulation of monocytes and

lymphocyte proliferation (Lemarbre *et al.*, 1981; Rinehart *et al.*, 1982), antitumor activity (Lemarbre *et al.*, 1981; Warren *et al.*, 1981), and induction of fusion of phospholipid vesicles (Posse *et al.*, 1990; Vechetti *et al.*, 1997). Finally, lysozyme has been found to complex with lactoferrin (Ellison and Giehl, 1991) and IgA (Stephens *et al.*, 1980; Vaerman, 1984), resulting in bactericidal effects at concentrations at which lactoferrin and lysozyme alone are usually only bacteriostatic (Bernt and Walker, 2001).

Animal performance

The nutrition and management of the newborn pig begins prior to birth. One objective of feeding the pregnant sow during the last 3-4 weeks of gestation is to supply sufficient nutrients to the foetuses for optimal development and birth weight. The majority of foetal development occurs in the last trimester of gestation (Bazer *et al.*, 2001). The results from this study showed that the provision of FLF to gilts at 14 days before farrowing, can improve the BW of piglets compared with the birth weight of piglets from gilts fed NFLF and DF, even though the sows on FLF consumed less DM and had significantly larger litters than gilts fed DF.

The daily energy needs for lactation are 3 to 4 times greater than for pregnancy, ranging from 15-20 Mcal day⁻¹ depending on sow body weight (Burrin, 2001). The nutrient requirements of the lactating sow are a function of the sow's needs for growth and maintenance and the needs for milk production (Klopfenstein *et al.*, 1999). However, achieving adequate feed intakes by sows, and particularly gilts, in lactation can often be a problem under intensive farrowing-house conditions. It has been shown that sow feed intake during lactation can significantly affect subsequent reproductive efficiency such as

remating interval, conception rate and embryo survival (Einarson and Rojkittikhun, 1993). There are many nutritional, environmental and physiological factors that influence the sow's appetite during lactation. The physical form of the diet (wet or dry) has also been shown to affect feed intake as sows ate 12 % more feed given as a wet mash compare with dry form (O'Grady and Lynch, 1978; Genest and Dallaire, 1995). The growth of the piglets during the first 2 weeks of lactation depends entirely on the milk supply of the mother. As this has been shown to be influenced by the level of feed intake (Koketsu *et al.*, 1996), factors controlling feed intake during lactation should determine the rate and efficiency of piglet growth. Present results showed that average daily feed intakes of sows fed liquid diets were significantly higher (15-20%) than those of DF sows during the second and third week of lactation. However, no significant relationship between ADFI of sows during lactation and piglet growth was observed. Some previous studies have also concluded that energy intake during lactation has no or only very little effect on piglet growth (Genest and Dallaire, 1995). Even on wet diets, where the sow ate 12% more per day than those on dry feed, the difference in nutrient uptake were reflected in sow weight change and not in litter growth (O'Grady and Lynch, 1978). The study by Pluske (1998) also demonstrated that extra feed in primiparous sows (energy intake was 38% higher than that of *ad libitum*-fed sows) was apportioned to the sow's body rather than into milk production, since litter growth of overfed and *ad libitum*-fed sows was similar. This higher ADFI of FLF and NFLF-fed gilts may result in better subsequent reproductive efficiency as was shown in the recent study by Eissen (2000). Higher feed intake during lactation tends to reduce tissue loss by the sow and reduces the probability of a prolonged weaning-to-oestrus interval. In addition, sows with low feed intakes in lactation tend to produce fewer piglets in the subsequent litter (Hughes, 1993).

Finally, it needs to be pointed out that even though feeding the FLF to sow around farrowing and whole lactation period seemed to improve some of the sow's and piglet's performance compared with those fed the NFLF and DF, these result have to be considered carefully. It would take many more animals in a trial to demonstrate an effect of diet on animal performance.

4.5. Conclusion

This study strongly indicates that porcine *L. salivarius* can provide an inoculant for FLF with a potential double role: the role of the starter culture and the role of probiotic. This could help sows to overcome disorders due to the stress associated with farrowing. However, considering the complexity of the sow GI-tract, together with its large diversity depending on age, sex, breed and diet, it appears unlikely that a single probiotic bacterial strain will be capable of influencing the microbial ecology and of beneficially affecting different health parameters equally in pigs of different breed, sex and developmental stage. It is more likely that with a better understanding of these differences, future FLF inoculants / probiotic cultures may consist of multiple strains of the same species to account for this diversity and enable beneficial effects during different developmental stage of pig's life.

Chapter 5

Concluding Comments

The studies contained in this thesis were aimed at investigating the potential antimicrobial and immunostimulatory effect of fermented liquid feed fed to the sow at late gestation.

During the past 50 years, the pig industry has developed in several areas including nutrition, genetics, engineering, and management in order to maximize the efficiency of growth performance and meat yield. During this time, antibiotics have been used in animal agriculture to achieve these goals and protect animals from the adverse effects of pathogenic and non-pathogenic microorganism. However, nowadays antibiotics have come under increasing scrutiny because of the potential development of antibiotic resistant human pathogenic bacteria after long use. Consequently, animal agriculture must develop alternatives to antibiotic growth promoters, or at least, substantially reduced the amount of antibiotics used to maintain production efficiency. The three primary effects of antibiotic growth promoters are: 1) increased growth; 2) improved feed efficiency, and 3) a lower incidence of certain diseases. To be effective, alternatives to antibiotics should generate similar benefits.

Fermented liquid feeding represents a very promising feeding practice which may compensate, to some extent, for the reduction or elimination of antibiotic growth promoters in feeds. Previous studies in our laboratory have demonstrated that FLF satisfies the

criteria to replace antimicrobial growth promoters during the post-weaning period (Moran, 2001). However, the first critical stage of pig's life is the pre-weaning period. Recent UK data showed that an average of 10 % of potential growing pigs die before weaning (Meat and Livestock Commission, 2001) and thus greater individual attention from the producers at this point of the pig's life might pay off with more live pigs at birth and consequently larger litters at weaning. Therefore, this study examined the hypothesis that feeding the sow a diet fermented with lactic acid bacteria would significantly benefit her offspring during the neonatal period.

Fermented liquid feed is characterised as a diet which is high in lactic acid bacteria ($>9 \log \text{CFU ml}^{-1}$) and yeast ($\sim 6\text{--}7 \log \text{CFU ml}^{-1}$), high concentration of lactic acid (64.0–105.3 g kg^{-1}), and has a low pH ($\text{pH} < 4.0$). One of the proven benefits of FLF is its potential to improve bacterial ecology of the pig's GI-tract (Moran, 2001; van Winsen *et al.*, 2001b; van Winsen *et al.*, 2002). To control the pathogen load within the pigs' environment is a critical part of management. It is known that the stress occasioned by movement into the farrowing house and parturition may lead to a decreased excretion of lactobacilli and increased excretion of potential pathogens especially haemolytic *Escherichia coli* (Maclean and Thomas, 1974). As suckling piglets usually ingest a large quantity of sow's faeces (18–25 g day^{-1}) and bedding material (Sansom and Gleed, 1981), the risk of infection at this stage of their life is very high. Therefore, one possible way in which FLF could help to improve neonatal survival and performance would be to control the pathogen load within the piglet's environment by reducing the rapid *E. coli* multiplication in sows associated with farrowing. The relative proportion of different organisms in the sow's faeces and the extent to which the piglet comes into contact with the faeces may affect colonisation of its immature gut. This study demonstrated that FLF has great potential to

fulfill this antimicrobial role. The faeces excreted by sows fed FLF had significantly lower numbers of *E. coli* at parturition compared with sows fed NFLF and DF. More importantly, significantly lower numbers of both coliforms and *E. coli* were found in the faeces of FLF-fed sows throughout the whole lactation period. Consequently, the faecal LAB: *E. coli* ratio of piglets from FLF-fed mothers was significantly higher than for piglets born to DF-fed mothers. Higher LAB: *E. coli* ratio is usually associated with a bacterial flora that contributes to improved animal growth and performance.

Since the new-born piglet is immunologically naïve and the time lag before its immune system develops fully covers at least the period from birth until weaning, the piglet is dependent on the sow for immune protection during that period. Therefore, the pig industry is currently subject to pressures which may make the producer more reliant on successful suckling by the piglet to obtain adequate passive immune protection. Passive transfer of immunity via colostrum is important in pigs as the epitheliochorial nature of the placenta prevents the transfer of immunoglobulins across the placenta. Thus any beneficial dietary modulations of sow immune system which would be reflected in improved immunological quality of milk would help to enhance immunoglobulin uptake by the piglet. A review of the literature shows that there are considerable numbers of studies, which report the use of various *Lactobacillus* strains as probiotic agents. In addition to their nutritional and antimicrobial effects, many of them have immunomodulatory activity. It has been shown that they are able to interact with the immune system at many levels, including cytokine production, mononuclear cell proliferation, macrophage phagocytosis and killing, modulation of autoimmunity, and immunity to bacterial pathogens (Vitini *et al.*, 2000). The efficacy of immunomodulating LAB could be enhanced by using live cultures rather than killed bacteria (Vesely *et al.*, 1985; De Simone *et al.*, 1986) and by delivering them in

fermented rather than non-fermented products (Perdigon *et al.*, 1986b; Saucier *et al.*, 1992). Thus liquid feed, fermented with LAB inoculum, could in fact represent a form of probiotic diet with potential immunostimulatory activity.

Previous work at our laboratory included a large screening programme in order to find suitable LAB starter cultures for fermentation of liquid feed (Moran, 2001). The selection criteria were based purely on the fermentative ability of screened bacterial strains to ensure a rapid decrease of pH in the pig feed. As a result of this screening programme, *L. plantarum* (PC-81-1-06; Alltech Inc., Kentucky, USA) was selected and used to control the fermentation of liquid feed (Moran, 2001). The study reported in this thesis was aimed at selecting a new starter culture from LAB of porcine origin, which would have similar fermentative abilities to *L. plantarum*, but could exert possible 'probiotic' effects on the host (sow). Porcine homofermentative aggregating *L. salivarius* was selected for further *in vivo* studies, as in addition to having comparable fermentation properties to the *L. plantarum* it was able to resist bile and acid conditions, had good adherence abilities to all intestinal compartments (mucus, epithelial cells, ECM) and expressed potent IL-12 inducing potential. However, it would be interesting to perform *in vivo* experiments with some of the other lactobacilli strains with similar 'probiotic' attributes as they could exert very different *in vivo* effects. A recent study by Ibnou-Zekri *et al.*, (2003) clearly demonstrated that despite similar *in vitro* probiotic properties (similar growth, survival, and adherence properties), distinct *Lactobacillus* strains colonized the gut differently and generated divergent immune responses.

This study provides good evidence that porcine *L. salivarius* can provide an inoculant for FLF with a potential double role: the role of the starter culture and the role of probiotic.

Significant differences were found in the concentration of IgG of colostrum from FLF-fed sows compared with NFLF and DF-fed sows. The concentrations of colostral IgA and IgM were also higher in FLF-fed sows, with IgA being significantly higher than in the colostrum of NFLF-fed sows. It has been shown that the concentrations of IgG in the plasma of piglets shortly after birth are positively correlated with survival (Hendrix *et al.*, 1976) and that dead piglets had lower serum IgG concentrations than surviving piglets (Klobasa *et al.*, 1981; Drew and Owens, 1988).

In addition to increased immunoglobulin content, a beneficial effect on mitogenic activity of colostral samples from FLF-fed sows on both IEC and lymphocytes was also reported. Intestinal epithelium is a tissue with rapid cellular turnover. An increase in the cell proliferation rate in the crypts will result in an overall increase of the epithelial cell population and associated increases in villus height. Thus colostrum with higher mitogenic activity has the potential to speed up the maturation of the newborn's GI tract and provide the piglet with better protection by keeping the intestinal mucosa sealed and impermeable to toxins and bacteria.

Achieving adequate feed intakes by sows, and particularly gilts, in lactation can often be a problem under intensive farrowing-house conditions. There are many nutritional, environmental and physiological factors that influence the sow's appetite during lactation. The physical form of the diet (wet or dry) has also been shown to affect feed intake as sows ate 12 % more feed given as a wet mash compare with dry form (O'Grady and Lynch, 1978; Genest and Dallaire, 1995). Results obtained in the current experiment showed that the average daily feed intakes of sows fed liquid diets were significantly higher (15-20%) than those of DF sows during the second and third week of lactation. This higher ADFI of

FLF and NFLF-fed gilts may result in better subsequent reproductive efficiency as was shown in the recent study by Eissen *et al.*, (2000). Higher feed intake during lactation tends to reduce tissue loss by the sow and reduces the probability of a prolonged weaning-to-oestrus interval. In addition, sows with low feed intakes in lactation tend to produce fewer piglets in the subsequent litter (Hughes, 1993).

Future work

The effect of FLF on milk quality, especially mitogenic activity, is definitely one of the areas that warrant further investigation. It would be interesting to find out which factors are triggered and the exact mechanism of this stimulating effect of FLF. For example, is this effect due to the presence of the bacterial inoculant itself or some substances produced by fermentation?

The liquid form of FLF makes it very suitable for the inclusion and testing of additional alternative products currently available on the market (e.g. enzymes, metal chelates, prebiotics, probiotics, organic acids, herbs, and immunostimulators), which could enhance its protective effect. Probiotics used as feed additives do not act like essential nutrients in terms of a clear dose response until the requirements are met. Therefore, it would be interesting to test different *Lactobacillus* inoculants for their protective effect against different disease challenges induced *in vitro* with different pig pathogens or by adding some cytokines, whose expression profiles change *in vivo* during weaning or farrowing. Tight junction permeability, pathogen adherence and invasivity, and apoptosis of intestinal cells and lymphocytes could be possible protective parameters tested. Many other porcine

lactobacilli tested in Chapter 3 together with some non-commensal LAB could be tested for their interaction with intestinal epithelial cells and mucosal immune system.

Some of the questions mentioned above form part of the research proposal No. 506144 entitled '*Development of natural alternatives to antimicrobials for the control of pig health and promotion of performance*' which has been favourably evaluated under the current EU Framework 6 programme.

Finally, despite the significant progress has been made in the understanding of the impact of indigenous bacteria on the GI-tract, especially its immune part, there is still lack of clear answers on questions such as: what is the efficacy of LAB in relation to the host age, physiological status and dietary intake? Another very important issue is to define the effective dose for each strain as well as elucidate their exact mechanism of action. Moreover, as the majority of the immunostimulatory experiments have been carried out with pure strains and *in vitro* conditions, the next area in which knowledge has to be improved is the understanding of how the introduced strains will interact within the microbial biofilm of the host GI-tract. Considering the complexity of the sow GI-tract, together with its large diversity depending on age, sex, breed and diet, it appears unlikely that a single probiotic bacterial strain will be capable of influencing the microbial ecology and of beneficially affecting different health parameters equally in pigs of different breed, sex and developmental stage. It is more likely that with a better understanding of these diversities, future FLF inoculants / probiotic cultures may consist of multiple strains of the same species to account for this diversity and enable beneficial effects during different developmental stage of pig's life.

Summary of main conclusions

- This study demonstrated that by implementing appropriate nutritional regimes there is an opportunity to influence the sows' bacterial excretion around farrowing time. The faecal excretion of *E. coli* at parturition was significantly lower in FLF-fed sows compared with NFLF- and DF-fed sows.
- FLF showed potential to stimulate the immune system of the farrowing sow, which was reflected in higher concentration of colostral IgG.
- Feeding FLF had a beneficial effect on the mitogenic activity of sow colostrum. Cell proliferation and differentiation are key elements in the efficient function of the GI tract and immune system.

This combination of effective immunity and reduced level of environmental contamination with faecal pathogens can lead to improved management of sows for increased litter size and weight at weaning.

Bibliography

- Aattouri, N., Bouras, M., Tome, D., Marcos, A. and Lemonnier, D. (2002). Oral ingestion of lactic acid bacteria by rats increases lymphocyte proliferation and interferon-gamma production. *British Journal of Nutrition* **87**(4): 367-373.
- Abdel-Rahman, M. S., Couri, D. and Bull, R. J. (1982). Metabolism and pharmacokinetics of alternate drinking water disinfectants. *Environmental Health Perspectives* **46**: 19-23.
- Abe, F., Ishibashi, N. and Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *Journal of Dairy Science* **78**: 2838-2846.
- Abraham, R. and Ogra, P. L. (1994). Mucosal microenvironment and mucosal response. *American Journal of Tropical Medicine and Hygiene* **50**(5): 3-9.
- Acres, R. B., Lamb, J. R. and Feldman, M. (1985). Effects of platelet derived growth factor and epidermal growth factor on antigen induced proliferation of human T-cell lines. *Immunology* **54**(1): 9-16.
- Adachi, S. (1992). Lactic acid bacteria and the control of tumours. In: Wood, B. J. B. *The lactic acid bacteria. 1. The lactic acid bacteria in health and disease*: London, Elsevier Applied Science. 233 - 255.
- Adams, M. R. and Hall, C. J. (1988). Growth inhibition of foodborne pathogens by lactic and acetic acids and their mixtures. *International Journal of Food Science and Technology* **23**(3): 287-292.
- Adawi, D., Jeppsson, B., Ahrne, S. and Molin, G. (2001). Modulation of the colonic bacterial flora affects differently bacterial translocation and liver injury in an acute liver injury model. *American Journal of Clinical Nutrition* **73**(2): 493S-493S.
- Adlerberth, I. (1999). Establishment of a normal intestinal microflora in the newborn infant. In: Hanson, L. A. and Yolken, R. H. *Probiotics, other nutritional factors and intestinal microflora*. Philadelphia, Lippincott-Raven **42**: 63-79.
- Adlerberth, I., Ahrne, S., Johansson, M. L., Molin, G., Hanson, L. A. and Wold, A. E. (1996). A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT- 29. *Applied and Environmental Microbiology* **62**(7): 2244-2251.
- Agati, V., Guyot, J. P., Morlon-Guyot, J., Talamond, P. and Hounhouigan, D. J. (1998). Isolation and characterisation of new amylolytic strains of *Lactobacillus fermentum* from fermented maize doughs (mawe and ogi) from Benin. *Journal of Applied Microbiology* **85**(3): 512-520.
- Aherne, F. X., Foxcroft, G. R. and Pettigrew, J. E. (1999). Nutrition of the sow. In: Straw, B. E., D'Allaire, S., Mengeling, W. L. and Taylor, A. R. *Diseases of swine*. Ames, Iowa, Iowa State University Press: 1029-1045.
- Aherne, F. X., Hogberg, M. G., Kornegay, E. T. and Shurson, G. C. (1992). Management and nutrition of the newly weaned pig. *Pork Industry Handbook*. West Lafayette, Purdue University Cooperative Extension Service: 1 - 4.

- Aieta, E. M. and Berg, J. D. (1986). A review of chlorine dioxide in drinking water treatment. *Journal American Water Works Association* **78**(6): 62-72.
- Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T. and von Wright, A. (1999). Persistence of colonization of human colonic mucosa by probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. *Applied and Environmental Microbiology* **65**(1): 351-354.
- Albanese, C. T., Smith, S. D., Watkins, S., Kurkchubasche, A., Simmons, R. L. and Rowe, M. I. (1994). Effect of secretory IgA on transepithelial passage of bacteria across the intact ileum *in vitro*. *Journal of the American College of Surgeons* **179**(6): 679-688.
- Aleljung, P. (1994). *Collagen binding proteins of intestinal Lactobacillus reuteri: characterisation, purification and cloning*. Ph.D. Thesis, Lunds University, Stockholm, Sweden.
- Aleljung, P., Paulsson, M., Emody, L., Andersson, M., Naidu, A. S. and Wadstrom, T. (1991). Collagen binding by Lactobacilli. *Current Microbiology* **23**(1): 33-38.
- Aleljung, P., Shen, W., Rozalska, B., Hellman, U., Ljungh, A. and Wadström, T. (1994). Purification of collagen-binding proteins of *Lactobacillus reuteri* NCIB-11951. *Current Microbiology* **28**(4): 231-236.
- Alexopoulos, C., Karagiannidis, A., Kritas, S. K., Boscos, C., Georgoulakis, I. E. and Kyriakis, S. C. (2001). Field evaluation of a bioregulator containing live *Bacillus cereus* spores on health status and performance of sows and their litters. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine* **48**(3): 137-145.
- Allen, W. D. and Porter, P. (1973). The relative distribution of IgM and IgA cells in intestinal mucosa and lymphoid tissues of the young unweaned pig and their significance in ontogenesis of secretory immunity. *Immunology* **24**: 493-501.
- Alverdy, J. C. and Rocha, F. (1999). Surgical stress, bacteria, and mucosal immune function. *European Journal of Pediatric Surgery* **9**(4): 210-213.
- Alverdy, J. C., Spitz, J., Hecht, G. and Ghandi, S. (1994). Causes and consequences of bacterial adherence to mucosal epithelia during critical illness. *New Horizons* **2**: 264-272.
- Anderson, L. L. (2000). Pigs. In: Hafez, B. and Hafez, E. S. E. *Reproduction in farm animals*. Baltimore, Maryland, USA, Lippincott Williams & Wilkins: 182-192.
- Anderson, R. C., Stanker, L. H., Young, C. R., Buckley, S. A., Genovese, K. J., Harvey, R. B., DeLoach, J. R., Keith, N. K. and Nisbet, D. J. (1999). Effect of competitive exclusion treatment on colonization of early-weaned pigs by *Salmonella* serovar *choleraesuis*. *Swine Health and Production* **7**(4): 155-160.
- Apanius, V. (1998). Stress and immune defense. *Stress and Behavior* **27**: 133-153.
- Arbuckle, J. B. R. (1968). The distribution of certain *Escherichia coli* strains in pigs and their environment. *The British Veterinary Journal* **124**: 152-159.
- Atwood, C. S. and Hartmann, P. E. (1992). Collection of fore and hind milk from the sow and the changes in milk composition during suckling. *Journal of Dairy Research* **59**(3): 287-298.
- Auldish, D. E., Carlson, D., Morrish, L., Wakeford, C. M. and King, R. H. (2000). The influence of suckling interval on milk production of sows. *Journal of Animal Science* **78**(8): 2026-2031.

- Aumaitre, A. and Seve, B. (1978). Nutritional importance of colostrum in the piglet. *Annales de Recherches Veterinaires* 9(2): 181-192.
- Barber, J., Brooks, P. H. and Carpenter, J. L. (1991). The effect of four levels of food on the water intake and water to food ratio of growing pigs. *Animal Production* 52: 602 (Abstr.).
- Barcellos-Hoff, M. H. and Ewan, K. B. R. (2000). Transforming growth factor- β and breast cancer - Mammary gland development. *Breast Cancer Research* 2(2): 92-99.
- Barrow, P. A., Brooker, B. E., Fuller, R. and Newport, M. J. (1980). The attachment of bacteria to the gastric epithelium of the pig and its importance in the microecology of the intestine. *Journal of Applied Bacteriology* 48(1): 147-154.
- Bazer, F. W., Ford, J. J. and Kensinger, R. S. (2001). Reproductive physiology. In: Pond, W. G. and Mersmann, H. J. *Biology of the domestic pig*. New York, Cornell University Press: 150-225.
- Beal, J. D., Niven, S. J., Campbell, A. and Brooks, P. H. (2002). The effect of temperature on the growth and persistence of *Salmonella* in fermented liquid pig feed. *International Journal of Food Microbiology* 79(1-2): 99-104.
- Belay, T. and Sonnenfeld, G. (2002). Differential effects of catecholamines on *in vitro* growth of pathogenic bacteria. *Life Sciences* 71(4): 447-456.
- Ben Omar, N., Ampe, F., Raimbault, M., Guyot, J. P. and Tailliez, P. (2000). Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Systematic and Applied Microbiology* 23(2): 285-291.
- Berg, J. D., Hoff, J. C., Roberts, P. V. and Matin, A. (1988). Resistance of bacterial subpopulations to disinfection by chlorine dioxide. *Journal American Water Works Association* 80(9): 115-119.
- Berg, J. D., Matin, A. and Roberts, P. V. (1982). Effect of antecedent growth conditions on sensitivity of *Escherichia coli* to chlorine dioxide. *Applied and Environmental Microbiology* 44(4): 814-819.
- Berg, J. D., Roberts, P. V. and Matin, A. (1986). Effect of chlorine dioxide on selected membrane functions of *Escherichia coli*. *Journal of Applied Bacteriology* 60(3): 213-220.
- Berggren, A. M., Bjorck, I. M. E., Nyman, E. M. G. L. and Eggum, B. O. (1993). Short chain fatty acid content and pH in cecum of rats given various sources of carbohydrates. *Journal of the Science of Food and Agriculture* 63(4): 397-406.
- Bernt, K. and Walker, W. A. (2001). Human milk and the response of intestinal epithelium to infection. In: Newburg, D. S. *Bioactive components of human milk*. New York, Kluwer Academic / Plenum. 501: 11-30.
- Berrada, N., Lemeland, J. F., Laroche, G., Thouvenot, P. and Piaia, M. (1991). *Bifidobacterium* from fermented milks - survival during gastric transit. *Journal of Dairy Science* 74(2): 409-413.
- Berthier, F. and Ehrlich, S. D. (1998). Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. *FEMS Microbiology Letters* 161(1): 97-106.

- Bibiloni, R., Fernando, P. and De Antoni, G. L. (1999). Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia. *Anaerobe* 5(5): 519-524.
- Bichard, M., Stork, M. G., Rickatson, S. and Pease, A. H. R. (1976). The use of synchronized farrowing in large pig units. *Animal Production* 22: 138.
- Biggar, W. D. and Sturgess, J. M. (1977). Role of lysozyme in the microbicidal activity of rat alveolar macrophages. *Infection and Immunity* 16: 974-982.
- Billey, L. O., Erickson, A. K. and Francis, D. H. (1998). Multiple receptors on porcine intestinal epithelial cells for the three variants of *Escherichia coli* K88 fimbrial adhesin. *Veterinary Microbiology* 59(2-3): 203-212.
- Billiau, A. and Vandenbroeck, K. (2000). IFN- γ . In: Oppenheim, J. J. and Feldmann, M. *Cytokine reference*. London, UK, Academic Press. 1: 642-688.
- Blau, H., Passwell, J. H., Levanon, M., Davidson, J., Kohen, F. and Ramot, B. (1983). Studies on human milk macrophages - Effect of activation on phagocytosis and secretion of prostaglandin E2 and lysozyme. *Pediatric Research* 17(4): 241-245.
- Blecha, F. and Kelley, K. W. (1981). Cold stress reduces the acquisition of colostral immunoglobulin in piglets. *Journal of Animal Science* 52(3): 594-600.
- Bloksma, N., De Heer, E., Van Dijk, H. and Willers, J. M. (1979). Adjuvanticity of lactobacilli. *Clinical and Experimental Immunology* 37: 367-375.
- Blomberg, L., Cohen, P. S. and Conway, P. L. (1993a). A study of the adhesive capacity of *Escherichia coli* strain BD-1107/7508 (K88ac) in relation to growth phase. *Microbial Pathogenesis* 14(1): 67-74.
- Blomberg, L., Henriksson, A. and Conway, P. L. (1993a). Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. *Applied and Environmental Microbiology* 59(1): 34-39.
- Blomberg, L., Krivan, H. C., Cohen, P. S. and Conway, P. L. (1993b). Piglet ileal mucus contains protein and glycolipid (Galactosylceramide) receptors specific for *Escherichia coli* K88 fimbriae. *Infection and Immunity* 61(6): 2526-2531.
- Bomba, A., Gancarcikova, S., Nemcova, R., Herich, R., Kastel, R., Depta, A., Demeterova, M., Ledecsko, V. and Zitman, R. (1998). The effect of lactic acid bacteria on intestinal metabolism and metabolic profile in gnotobiotic pigs. *Deutsche Tierärztliche Wochenschrift* 105(10): 384-389.
- Bonet, M. E. B., De Petrino, S. F., Meson, O., De Budeguer, M. V. and Perdigon, G. (1999). Optimal effect of *Lactobacillus delbrueckii* subsp. *bulgaricus*, among other lactobacilli species, on the number of IgA and mast cells associated with the mucosa in immunosuppressed mice. *Food and Agricultural Immunology* 11(3): 259-267.
- Boris, S., Suarez, J. E. and Barbes, C. (1997). Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *Journal of Applied Microbiology* 83(4): 413-420.
- Boris, S., Suarez, J. E., Vazquez, F. and Barbes, C. (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infection and Immunity* 66(5): 1985-1989.

- Brandenburg, A. C. and Wilson, M. R. (1973). Immunity to *Escherichia coli* in pigs: IgG immunoglobulin in passive immunity to *E. coli* enteritis. *Immunology* **24**: 119-127.
- Bringel, F., Quenee, P. and Tailliez, P. (2001). Polyphasic investigation of the diversity within *Lactobacillus plantarum* related strains revealed two *L. plantarum* subgroups. *Systematic and Applied Microbiology* **24**(4): 561-571.
- Bromander, A. K., Kjerrulf, M., Holmgren, J. and Lycke, N. (1993). Cholera toxin enhances alloantigen presentation by cultured intestinal epithelial cells. *Scandinavian Journal of Immunology* **37**(4): 452-458.
- Brooks, P. H. (1999). The potential of liquid feeding systems. In: Lyons, T. P. and Cole, D. J. A. *Concepts in pig science*. Nottingham, Nottingham University Press: 81-98.
- Brooks, P. H., Moran, C. and Beal, J. D. (1999). Liquid feeding of pigs: potential for reducing environmental impact and for improving productivity and food safety. In: Lyons, T. P. and Jacques, K. A. *Biotechnology in the feed industry: Proceedings of Alltech's 15th annual symposium*. Nottingham, England, Nottingham University Press: 111-129.
- Brooks, P. H., Moran, C. A., Beal, J. D., Demecková, V. and Campbell, A. (2001). Liquid feeding for the young piglet. In: Varley, M. A. and Wiseman, J. *The weaner pig*. Wallingford, CAB International, UK: 153-178.
- Brown, H., Speer, J. C., Quinn, L. Y., Hays, V. M. and Catron, D. V. (1961). Studies on colostrum-acquired immunity and active antibody production in baby pigs. *American Journal of Veterinary Research* **20**: 323.
- Bryan, D. L., Hawkes, J. S. and Gibson, R. A. (1999). Interleukin-12 in human milk. *Pediatric Research* **45**(6): 858-859.
- Bückenhuskes, H. J. (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiology Reviews* **12**: 253-272.
- Bujnakova, D. and Kmet, V. (2002). Aggregation of animal lactobacilli with O157 enterohemorrhagic *Escherichia coli*. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **49**(3): 152-154.
- Burrin, D. G. (2001). Nutrient requirements and metabolism. In: Pond, W. G. and Mersmann, H. J. *Biology of the domestic pig*. London, Cornell University Press: 309-390.
- Burrin, D. G., Davis, T. A., Ebner, S., Schoknecht, P. A., Fiorotto, M. L. and Reeds, P. J. (1997). Colostrum enhances the nutritional stimulation of vital organ protein synthesis in neonatal pigs. *Journal of Nutrition* **127**: 1284-1289.
- Burrin, D. G., Wang, H., Heath, J. and Dudley, M. A. (1996). Orally administered lactoferrin increases hepatic protein synthesis in formula-fed newborn pigs. *Pediatric Research* **40**(1): 72-76.
- Busscher, H. J., Cowan, M. M. and Vandermei, H. C. (1992). On the relative importance of specific and nonspecific approaches to oral microbial adhesion. *FEMS Microbiology Reviews* **88**(3-4): 199-209.
- Butera, M., Smith, J. H., Morrison, W. D., Hacker, R. R., Kains, F. A. and Ogilvie, J. R. (1991). Concentration of respirable dust and bioaerosols and identification of certain microbial types in a hog growing facility. *Canadian Journal of Animal Science* **71**(2): 271-277.

- Butler, J. E. (1973). Synthesis and distribution of immunoglobulins. *Journal of American Veterinary Medicine Association* 163: 795-800.
- Butler, J. E. (1998). Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. *Revue Scientifique et Technique De L Office International Des Epizooties* 17(1): 43-70.
- Calderon, M., Loiseau, G. and Guyot, J. P. (2003). Fermentation by *Lactobacillus fermentum* Ogi E1 of different combinations of carbohydrates occurring naturally in cereals: consequences on growth energetics and α -amylase production. *International Journal of Food Microbiology* 80(2): 161-169.
- Canibe, N. and Jensen, B. B. (2000). Effect of non-fermented and fermented liquid feeding on the intestinal microbiota of growing pigs. *Reproduction, Nutrition, Development* 40: 203.
- Canibe, N., Peisker, M., Jensen, S. K. and Jensen, B. B. (2003). Effect of fermentation of liquid feed on lysine degradation. In: Ball, R. O. *Proceedings of the 9th International Symposium on Digestive Physiology in Pigs*. Banff, AB, Canada, University of Alberta. 2: 244-246.
- Carbonare, S. B., Silva, M. L. M., Palmeira, P. and Carneiro-Sampaio, M. M. S. (1997). Human colostrum IgA antibodies reacting to enteropathogenic *Escherichia coli* antigens and their persistence in the faeces of a breastfed infant. *Journal of Diarrhoeal Diseases Research* 15(2): 53-58.
- Carlton, B. D., Basaran, A. H., Mezza, L. E., George, E. L. and Smith, M. K. (1991). Reproductive effects in Long-Evans rats exposed to chlorine dioxide. *Environmental Research* 56(2): 170-177.
- Castro, G. A. and Arntzen, C. J. (1993). Immunophysiology of the gut - a research frontier for integrative studies of the common mucosal immune system. *American Journal of Physiology* 265(4): G599-G610.
- Cera, K., Mahan, D. C. and Simmen, F. A. (1987). *In vitro* growth-promoting activity of porcine mammary secretions: initial characterization and relationship to known peptide growth factors. *Journal of Animal Science* 65: 1149-1159.
- [CFR] Code of Federal Regulations (2000). Title 21, Part 173.325. Secondary direct food additives permitted in food for human consumption: Acidified sodium chlorite solutions. Available from <http://www.access.gpo.gov/nara/cfr/index.html> Accessed 2003, July 31.
- Chabaudie, N., Lejan, C., Olivier, M. and Salmon, H. (1993). Lymphocyte subsets in the mammary gland of sows. *Research in Veterinary Science* 55(3): 351-355.
- Chauviere, G., Aubel, D., Darfeuillemichaud, A., Kerneis, S., Joly, B. and Servin, A. L. (1990). Caco-2 and Ht-29 models for bacterial adhesion. *Gastroenterologie Clinique et Biologique* 14(5): 464-464.
- Chauviere, G., Coconnier, M. H., Kerneis, S., Darfeuillemichaud, A., Joly, B. and Servin, A. L. (1992). Competitive exclusion of diarrheogenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. *FEMS Microbiology Letters* 91(3): 213-218.
- Chen, A. C. and Donovan, S. M. (2000). Impact of insulin-like growth factor-I (IGF-I) on proliferation and lactase activity in Caco2-BBe cells. *Faseb Journal* 14(4): A362-A362.

- Chen, R. M., Wu, J. J., Lee, S. C., Huang, A. H. and Wu, H. M. (1999). Increase of intestinal *Bifidobacterium* and suppression of coliform bacteria with short-term yogurt ingestion. *Journal of Dairy Science* **82**: 2308-2314.
- Chen, W. J., Jin, W. W., Tian, H. S., Sicurello, P., Frank, M., Orenstein, J. M. and Wahl, S. M. (2001). Requirement for transforming growth factor beta 1 in controlling T cell apoptosis. *Journal of Experimental Medicine* **194**(4): 439-453.
- Cherrington, C. A., Hinton, M., Mead, G. C. and Chopra, I. (1991). Organic acids - chemistry, antibacterial activity and practical applications. *Advances in Microbial Physiology* **32**: 87-108.
- Chiang, J. Y. L. (2003). Bile acid regulation of hepatic physiology - III. Bile acids and nuclear receptors. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **284**(3): G349-G356.
- Chierici, R. (2001). Antimicrobial actions of lactoferrin. *Advances in Nutritional Research*, **10**: 247-269.
- Christensen, H. R., Frokiaer, H. and Pestka, J. J. (2002). Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *Journal of Immunology* **168**(1): 171-178.
- Clark, M. A., Reed, K. A., Lodge, J., Stephen, J., Hirst, B. H. and Jepson, M. A. (1996). Invasion of murine intestinal M cells by *Salmonella typhimurium* inv mutants severely deficient for invasion of cultured cells. *Infection and Immunity* **64**(10): 4363-4368.
- Clark, R., Strasser, J., McCabe, S., Robbins, K. and Jardieu, P. (1993). Insulin-like growth factor-I stimulation of lymphopoiesis. *Journal of Clinical Investigation* **92**(2): 540-548.
- Cleveland, J., Montville, T. J., Nes, I. F. and Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology* **71**(1): 1-20.
- Close, W. H. (1992). Thermoregulation in piglets: environmental and metabolic consequences. In: Varley, M. A., Williams, P. E. V. and Lawrence, T. L. J. *Neonatal survival and growth*. Edinburgh, BSAP. **15**: 25-33.
- Close, W. H. and Cole, D. J. A. (2000). *Nutrition of sows and boars*. Nottingham, Nottingham University Press, UK.
- Coconnier, M. H., Bernet, M. F., Kerneis, S., Chauviere, G., Fourniat, J. and Servin, A. L. (1993). Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. *FEMS Microbiology Letters* **110**(3): 299-305.
- Coconnier, M. H., Klaenhammer, T. R., Kerneis, S., Bernet, M. F. and Servin, A. L. (1992). Protein mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell-lines in culture. *Applied and Environmental Microbiology* **58**(6): 2034-2039.
- Cole, D. J. A., Beal, R. M. and Luscombe, J. R. (1968). The effect on performance and bacterial flora of lactic acid, propionic acid, calcium propionate and calcium acrylate in the drinking water of weaned pigs. *The Veterinary Record* **83**: 459-464.
- Coleman, M. E., Pan, Y. C. E. and Etherton, T. D. (1991). Identification and NH₂-terminal amino acid sequence of 3 insulin-like growth factor binding proteins in porcine serum. *Biochemical and Biophysical Research Communications* **181**(3): 1131-1136.

- Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinezmurcia, A., Phillips, B. A., Williams, A. M. and Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S ribosomal RNA. *FEMS Microbiology Letters* **77**(1): 5-12.
- Cone, R. A. (1999). Mucus. In: Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., Bienenstock, J. and McGhee, J. R. *Mucosal immunology*. London, Academic Press: 43-65.
- Conneely, O. M. (2001). Antiinflammatory activities of lactoferrin. *Journal of the American College of Nutrition* **20**(5): 389S-395S.
- Conway, B. and Ronald, A. (1988). An overview of some mechanisms of bacterial pathogenesis. *Canadian Journal of Microbiology* **34**: 281-286.
- Conway, P. L. (1997). Development of intestinal microbiota. In: Mackie, R. I., White, B. A. and Isaacson, R. E. *Gastrointestinal microbiology*. London, Chapman & Hall. **2**: 3-38.
- Cornberg, M., Enss, M. L. and Wagner, S. (2001). Differential mucin expression in the gastrointestinal tract. *Zeitschrift für Gastroenterologie* **39**(5): 369-376, 378.
- Cranwell, P. D. (1995). Development of the neonatal gut and enzyme systems. In: Varley, M. A. *The Neonatal Pig: Development and survival* Leeds, CAB International: 99-154.
- Craven, S. E. and Williams, D. D. (1997). Inhibition of *Salmonella typhimurium* attachment to chicken cecal mucus by intestinal isolates of *Enterobacteriaceae* and lactobacilli. *Avian Diseases* **41**(3): 548-558.
- Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerstrom, R., Matto, J., Saarela, M., Mattila-Sandholm, T. and Poutanen, K. (2002). *In vitro* fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. *Journal of the Science of Food and Agriculture* **82**(8): 781-789.
- Cummings, J. H., Jill, M. J., Bone, E. S., Branch, W. J. and Jenkins, D. J. A. (1979). The effect of meat protein and dietary fiber on colonic function and metabolism. II. Bacterial metabolites in feces and urine. *American Journal of Clinical Nutrition* **32**(10): 2094-2101.
- Curk, M. C., Hubert, J. C. and Bringel, F. (1996). *Lactobacillus paraplantarum*, a new species related to *Lactobacillus plantarum*. *International Journal of Systematic Bacteriology* **46**(2): 595-598.
- Curtis, J. and Bourne, F. J. (1971). Immunoglobulin quantitation in sow serum, colostrum and milk and the serum of young pigs. *Biochimica et biophysica acta* **236**(1): 319-332.
- Curtis, J. and Bourne, F. J. (1973). Half-life of immunoglobulins IgG, IgA and IgM in the serum of new-born pigs. *Immunology* **24**(147-155).
- Cutler, R. S., Fahy, V. A., Spicer, E. M. and Cronin, G. M. (1999). Prewaning mortality. In: Straw, B. E., D'Allaire, S., Mengeling, W. L. and Taylor, A. R. *Diseases of swine*. Ames, Iowa, Iowa State University Press: 985-1003.
- CVB (1999). Veevoedertabel. Centraal Veevoederbureau in Nederland. Lelystad, The Netherlands.
- Davidkova, G., Popova, P., Guencheva, G., Bogdanov, A., Pacelli, E., Auteri, A. and Mincheva, V. (1992). Endogenous production of tumor necrosis factor in normal mice orally treated with

- deodan - a preparation from *Lactobacillus bulgaricus* Lb51. *International Journal of Immunopharmacology* **14**(8): 1355-1362.
- Davidson, L. A. and Lonnerdal, B. (1988). Specific binding of lactoferrin to brush border membrane - ontogeny and effect of glycan chain. *American Journal of Physiology* **254**(4): G580-G585.
- De Araujo, A. N. and Giugliano, L. G. (2000). Human milk fractions inhibit the adherence of diffusely adherent *Escherichia coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells. *FEMS Microbiology Letters* **184**(1): 91-94.
- De Roos, N. M. and Katan, M. B. (2000). Effects of probiotics bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *The American Journal of Clinical Nutrition* **71**(2): 405-411.
- De Simone, C., Salvadori, B. B., Negri, R., Ferrazzi, M., Baldinelli, L. and Vesely, R. (1986). The adjuvant effect of yogurt on production of γ -interferon by Con-A-stimulated human peripheral blood lymphocytes. *Nutrition Reports International* **33**(3): 419-433.
- De Simone, C., Tzantzoglou, S., Baldinelli, L., Di Fabio, S., Bianchi-Salvadori, B., Jirillo, E. and Vesely, R. (1988). Enhancement of host resistance against *Salmonella typhimurium* infection by a diet supplemented with yogurt. *Immunopharmacology and Immunotoxicology* **10**(3): 399-415.
- Del Re, B., Busetto, A., Vignola, G., Sgorbati, B. and Palenzona, D. L. (1998). Autoaggregation and adhesion ability in a *Bifidobacterium suis* strain. *Letters in Applied Microbiology* **27**(5): 307-310.
- Del Re, B., Sgorbati, B., Miglioli, M. and Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in Applied Microbiology* **31**(6): 438-442.
- Delgado, A., Brito, D., Fevereiro, P., Peres, C. and Marques, J. F. (2001). Antimicrobial activity of *L. plantarum*, isolated from a traditional lactic acid fermentation of table olives. *Lait* **81**(1-2): 203-215.
- Dellaglio, F., Bottazzi, V. and Vescovo, M. (1975). Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium*. *International Journal of Systematic Bacteriology* **25**: 160-172.
- Delneri, M. T., Carbonare, S. B., Silva, M. L. M., Palmeira, P. and Carneiro-Sampaio, M. M. S. (1997). Inhibition of enteropathogenic *Escherichia coli* adhesion to HEp-2 cells by colostrum and milk from mothers delivering low- birth-weight neonates. *European Journal of Pediatrics* **156**(6): 493-498.
- Demeckova, V., Moran, C. A., Caveney, C., Campbell, A., Kuri, V. and Brooks, P. H. (2001). The effect of fermentation and/or sanitization of liquid diets on the feeding preferences of newly weaned pigs. In: Lindberg, J. E. and Ogle, B. *Digestive physiology of pigs*. Wallingford, UK, CAB International: 291-293.
- Deplancke, B. and Gaskins, H. R. (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *American Journal of Clinical Nutrition* **73**(6): 1131S-1141S.
- Dignass, A. U. (2001). Mechanisms and modulation of intestinal epithelial repair. *Inflammatory Bowel Diseases* **7**(1): 68-77.

- Donham, K. J. (1991). Association of Environmental Air Contaminants with Disease and Productivity in Swine. *American Journal of Veterinary Research* **52**(10): 1723-1730.
- Donnet-Hughes, A., Rochat, F., Serrant, P., Aeschlimann, J. M. and Schiffrin, E. J. (1999). Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *Journal of Dairy Science* **82**: 863-869.
- Donovan, S. M., McNeil, L. K., Jimenezflores, R. and Odle, J. (1994). Insulin-like growth factors and insulin like growth factor binding proteins in porcine serum and milk throughout lactation. *Pediatric Research* **36**(2): 159-168.
- Drago, L., Gismondo, M. R., Lombardi, A., de Haen, C. and Gozzini, L. (1997). Inhibition of *in vitro* growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. *FEMS Microbiology Letters* **153**(2).
- Drescher, K., Roos, N., Pfeuffer, M., Seyfert, H. M., Schrezenmeir, J. and Hagemester, H. (1999). Recovery of N-15-lactoferrin is higher than that of N-15-casein in the small intestine of suckling, but not adult miniature pigs. *Journal of Nutrition* **129**(5): 1026-1030.
- Drew, M. D. and Owens, B. D. (1988). The provision of passive the neonatal pig and lamb. *Canadian Journal of Animal Science* **68**: 1277-1284.
- Du, J. H., Han, Y. and Linton, R. H. (2002). Inactivation by chlorine dioxide gas (ClO₂) of *Listeria monocytogenes* spotted onto different apple surfaces. *Food Microbiology* **19**(5): 481-490.
- Du Toit, M., Dicks, L. M. T. and Holzapfel, W. H. (2001). Taxonomy of obligately homofermentative and facultatively heterofermentative lactobacilli in pig faeces. *Letters in Applied Microbiology* **32**(3): 199-204.
- Du Toit, M., Franz, C. M. A. P., Dicks, L. M. T., Schillinger, U., Haberer, P., Warlies, B., Ahrens, F. and Holzapfel, W. H. (1998). Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. *International Journal of Food Microbiology* **40**(1-2): 93-104.
- Dulin, A. M., Paape, M. J. and Nickerson, S. C. (1988). Comparison of phagocytosis and chemiluminescence by blood and mammary-gland neutrophils from multiparous and nulliparous cows. *American Journal of Veterinary Research* **49**(2): 172-177.
- Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F. and Collins, J. K. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition* **73**(2): 386S-392S.
- Durum, S. K. (2000). Cytokines and the immune system. In: Oppenheim, J. J. and Feldmann, M. *Cytokine reference*. London, UK, Academic Press. 1: 95-97.
- Dvorak, B., Halpern, M. D., Holubec, H., Williams, C. S., McWilliam, D. L., Dominguez, J. A., Stepankova, R., Payne, C. M. and McCuskey, R. S. (2002). Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **282**(1): G156-G164.
- Dziarski, R. (1991). Demonstration of peptidoglycan-binding sites on lymphocytes and macrophages by photoaffinity cross-linking. *Journal of Biological Chemistry* **266**(8): 4713-4718.

- Easo, J. G., Measham, J. D., Munroe, J. and Green-Johnson, J. M. (2002). Immunostimulatory actions of lactobacilli: Mitogenic induction of antibody production and spleen cell proliferation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*. *Food and Agricultural Immunology* **14**(1): 73-83.
- Eddie, D. S., Schulkind, M. L. and Robbins, J. B. (1971). The isolation and biologic activities of purified secretory IgA and IgG anti-*Salmonella typhimurium* "O" antibodies from rabbit intestinal fluid and colostrum. *Journal of Immunology* **106**: 181-190.
- Edelman, S., Westerlund-Wikstrom, B., Leskela, S., Kettunen, H., Rautonen, N., Apajalahti, J. and Korhonen, T. K. (2002). *In vitro* adhesion specificity of indigenous lactobacilli within the avian intestinal tract. *Applied and Environmental Microbiology* **68**(10): 5155-5159.
- Einarson, S. and Rojkittikhun, T. (1993). Effects of nutrition on pregnant and lactating sows. *Journal of Reproduction and Fertility* (Suppl. **48**): 229-239.
- Eissen, J. J., Merks, J. W. M., Verstegen, M. V. A. and de Greef, K. H. (2000). The importance of a high feed intake during lactation of primiparous sows nursing larger litters. *Journal of Animal Science* **78** (Suppl. 1): 147.
- Ellendorff, F. and Poulain, D. (1984). A means to assess nursing efficiency in the pig - the study of the milk ejection reflex. *Annales de Recherches Veterinaires* **15**(2): 271-274.
- Elliot, J. I., Senft, B., Erhard, G. and Fraser, D. (1984). Isolation of lactoferrin and its concentration in sows' colostrum and milk during a 21-day lactation. *Journal of Animal Science* **59**(4): 1080-1084.
- Ellison, R. T. and Giehl, T. J. (1987). Human lactoferrin releases lipopolysaccharide from *Escherichia coli*. *Clinical Research* **35**(3): A473-A473.
- Ellison, R. T., Giehl, T. J. and Laforce, F. M. (1988). Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity* **56**(11): 2774-2781.
- Ellison, R. T., Laforce, F. M., Giehl, T. J., Boose, D. S. and Dunn, B. E. (1990). Lactoferrin and transferrin damage of the Gram-negative outer membrane is modulated by Ca^{2+} and Mg^{2+} . *Journal of General Microbiology* **136**: 1437-1446.
- Ellison, R. T. and Giehl, T. J. (1991). Killing of Gram-negative bacteria by lactoferrin and lysozyme. *Journal of Clinical Investigation* **88**(4): 1080-1091.
- Erickson, A. K., Baker, D. R., Bosworth, B. T., Casey, T. A., Benfield, D. A. and Francis, D. H. (1994). Characterization of porcine intestinal receptors for the K88ac fimbrial adhesin of *Escherichia coli* as mucin-type sialoglycoproteins. *Infection and Immunity* **62**(12): 5404-5410.
- Erickson, K. L. and Hubbard, N. E. (2000). Probiotic immunomodulation in health and disease. *Journal of Nutrition* **130**: 403S-409S.
- Esche, C., Shurin, M. R. and Lotze, M. T. (2000). IL-12. In: Oppenheim, J. J. and Feldmann, M. *Cytokine reference*. London, UK, Academic Press. **1**: 187-201.
- Evans, P. A., Newby, T. J., Stokes, C. R. and Bourne, F. J. (1982). A study of cells in the mammary secretions of sows. *Veterinary Immunology and Immunopathology* **3**(5): 515-527.

- Everts, H., Salden, N., Lemmens, A. G., Wijers, J. and Beynen, A. C. (2000). High intakes of L⁺ and D⁻ lactic acid are efficiently metabolized by pigs and rats. *Journal of Animal Physiology and Animal Nutrition* **83**: 224-230.
- Ewing, W. N. and Cole, D. J. A. (1994). *The living gut*. Dungannon, N. Ireland, Context.
- Falk, P. G., Hooper, L. V., Midtvedt, T. and Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. *Microbiology and Molecular Biology Reviews* **62**(4): 1157-1170.
- Fedorka-Cray, P. J., Bailey, J. S., Stern, N. J., Cox, N. A., Ladely, S. R. and Musgrove, M. (1999). Mucosal competitive exclusion to reduce *Salmonella* in swine. *Journal of Food Protection* **62**(12): 1376-1380.
- Ferket, P. R. (2003). *Managing gut health in a world without antibiotics*. Alltech's 17th European, Middle Eastern and African Lecture Tour, Birmingham, UK.
- Fernandes, R. M., Carbonare, S. B., Carneiro-Sampaio, M. M. S. and Trabulsi, L. R. (2001). Inhibition of enteroaggregative *Escherichia coli* adhesion to HEp-2 cells by secretory immunoglobulin A from human colostrum. *Pediatric Infectious Disease Journal* **20**(7): 672-678.
- Fetherston, C. M., Lee, C. S. and Hartmann, P. E. (2001). Mammary gland defense: the role of colostrum, milk, and involution secretion. *Advances in Nutritional Research* **10**: 167-198.
- Fiessinger, F., Richard, Y., Montiel, A. and Musquere, P. (1981). Advantages and disadvantages of chemical oxidation and disinfection by ozone and chlorine dioxide. *Science of the Total Environment* **18**(4): 245-261.
- Finlay, B. B. and Caparon, M. (2000). Bacterial adherence to cell surfaces and extracellular matrix. In: Cossart, P., Boquet, P., Normark, S. and Rappuoli, R. *Cellular Microbiology*. Washington, D.C., ASM Press: 67-80.
- Finlay, B. B. and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews* **61**(2): 136-169.
- Fiocchi, C. (1997). Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* **273**: G769-G775.
- Flanders, K. C. and Roberts, A. B. (2000). TGF β . In: Oppenheim, J. J. and Feldmann, M. *Cytokine reference*. London, UK, Academic Press. 1: 719-747.
- Floch, M. H., Binder, H. J., Filburn, B. and Gershengoren, W. (1972). The effect of bile acids on intestinal microflora. *American Journal of Clinical Nutrition* **25**: 1418-1426.
- Flock, J.-I. (1999). Extracellular-matrix-binding proteins as targets for the prevention of *Staphylococcus aureus* infections. *Molecular Medicine Today* **5**: 532-537.
- Foegeding, P. M., Hemstapat, V. and Giesbrecht, F. G. (1986). Chlorine dioxide inactivation of *Bacillus* and *Clostridium* spores. *Journal of Food Science* **51**(1): 197-201.
- Foschino, R., Nervegna, I., Motta, A. and Galli, A. (1998). Bactericidal activity of chlorine dioxide against *Escherichia coli* in water and on hard surfaces. *Journal of Food Protection* **61**(6): 668-672.

- Francis, D. H., Erickson, A. K. and Grange, P. A. (1999). K88 adhesins of enterotoxigenic *Escherichia coli* and their porcine enterocyte receptors. In: Paul, P. S. *Mechanisms in the Pathogenesis of Enteric Diseases 2*. New York, Kluwer Academic / Plenum Publ. **473**: 147-154.
- Freter, R. (1992). Factors affecting the microecology of the gut. In: Fuller, R. *Probiotics: The scientific basis*. London, Chapman & Hall: 111 - 144.
- Fujiwara, S., Seto, Y., Kimura, A. and Hashiba, H. (2001a). Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *Journal of Applied Microbiology* **90**(3): 343-352.
- Fujiwara, S., Seto, Y., Kimura, A. and Hashiba, H. (2001b). Intestinal transit of an orally administered streptomycin-rifampicin resistant variant of *Bifidobacterium longum* SBT2928: its long-term survival and effect on the intestinal microflora and metabolism. *Journal of Applied Microbiology* **90**(1): 43-52.
- Fukayama, M. Y., Tan, H., Wheeler, W. B. and Wei, C. I. (1986). Reactions of aqueous chlorine and chlorine dioxide with model food compounds. *Environmental Health Perspectives* **69**: 267-274.
- Fukushima, Y., Kawata, Y., Hara, H., Terada, A. and Mitsuoka, T. (1998). Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. *International Journal of Food Microbiology* **42**(1-2): 39-44.
- Fukushima, Y., Kawata, Y., Mizumachi, K., Kurisaki, J. and Mitsuoka, T. (1999). Effect of bifidobacteria feeding on fecal flora and production of immunoglobulins in lactating mouse. *International Journal of Food Microbiology* **46**(3): 193-197.
- Fuller, R. (1973). Ecological studies on the *Lactobacillus* flora associated with the crop epithelium of the fowl. *Journal of Applied Bacteriology* **36**: 131-139.
- Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology* **66**: 365-378.
- Garofalo, R., Chheda, S., Mei, F., Palkowetz, K. H., Rudloff, H. E., Schmalstieg, F. C., Rassin, D. K. and Goldman, A. S. (1995). Interleukin-10 in Human-Milk. *Pediatric Research* **37**(4): 444-449.
- Gartner, L. P. and Hiatt, J. L. (2001). *Color textbook of histology*. Philadelphia, Pennsylvania, W.B. Saunders Company.
- Gaskins, H. R. and Kelley, K. W. (1995). Immunology and Neonatal Mortality. In: Varley, M. A. *The Neonatal Pig: Development and survival*. Oxon, CAB International: 39-55.
- Geary, T. M. (1997). *Improving the performance of weaner pigs through developments in liquid feeding*. Ph.D. thesis, University of Plymouth, UK.
- Geary, T. M. and Brooks, P. H. (1998). The effect of weaning weight and age on the post-weaning growth performance of piglets fed fermented liquid diets. *The Pig Journal* **42**: 10-23.
- Geary, T. M., Brooks, P. H., Morgan, D. T., Campbell, A. and Russell, P. J. (1996). Performance of weaner pigs fed *ad libitum* with liquid feed at different dry matter concentrations. *Journal of Science of Food and Agriculture* **72**: 17-24.

- Genest, M. and Dallaire, S. (1995). Feeding strategies during the lactation period for first-parity sows. *Canadian Journal of Animal Science* **75**(3): 461-467.
- Genovese, K. J., Anderson, R. C., Harvey, R. B. and Nisbet, D. J. (2000). Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* **64**(4): 204-207.
- Gill, H. S. and Rutherford, K. J. (2001). Immune enhancement conferred by oral delivery of *Lactobacillus rhamnosus* HN001 in different milk-based substrates. *Journal of Dairy Research* **68**(4): 611-616.
- Gislason, J., Douglas, G. C., Hutchens, T. W. and Lonnerdal, B. (1995). Receptor mediated binding of milk lactoferrin to nursing piglet enterocytes-a model for studies on absorption of lactoferrin bound iron. *Journal of Pediatric Gastroenterology and Nutrition* **21**(1): 37-43.
- Gislason, J., Iyer, S., Hutchens, T. W. and Lonnerdal, B. (1993). Lactoferrin receptors in piglet small intestine-Lactoferrin binding properties, ontogeny, and regional distribution in the gastrointestinal tract. *Journal of Nutritional Biochemistry* **4**(9): 528-533.
- Gluck, U. (1989). *In vivo* tests of lysozyme of the nasal mucosa. *HNO* **37**(5): 207-210.
- Goepfert, J. M. and Hicks, R. (1969). Effect of volatile fatty acids on *Salmonella typhimurium*. *Journal of Bacteriology* **97**: 956-958.
- Goldman, A. S., Chheda, S. and Garofalo, R. (1997). Spectrum of immunomodulating agents in human milk. *International Journal of Pediatric Hematology/Oncology* **4**(5): 491-497.
- Goldsby, R. A., Kindt, T. J. and Osborne, B. A. (2000). *Kuby Immunology*. New York, W.H. Freeman and Company.
- Goodlad, R. A. and Wright, N. A. (1996). Epidermal growth factor (EGF). *Baillieres Clinical Gastroenterology* **10**(1): 33-47.
- Gopal, P. K., Prasad, J., Smart, J. and Gill, H. S. (2001). *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal of Food Microbiology* **67**(3): 207-216.
- Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A. and Brassart, D. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* LA1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* **65**(3): 1071-1077.
- Grange, P. A., Erickson, A. K., Anderson, T. J. and Francis, D. H. (1998). Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of *Escherichia coli*. *Infection and Immunity* **66**(4): 1613-1621.
- Greene, J. D. and Klaenhammer, T. R. (1994). Factors involved in adherence of Lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology* **60**(12): 4487-4494.
- Grun, D., Reiner, G. and Dzapo, V. (1993). Investigations on breed differences in milk yield of swine. 2. Commitment - milk composition and its relation towards piglet development during suckling period. *Reproduction in Domestic Animals* **28**(1): 22-27.

- Gusils, C., Chaia, A. P., Gonzalez, S. and Oliver, G. (1999). Lactobacilli isolated from chicken intestines: Potential use as probiotics. *Journal of Food Protection* **62**(3): 252-256.
- Gusils, C., Cuozzo, S., Sesma, F. and Gonzalez, S. (2002). Examination of adhesive determinants in three species of *Lactobacillus* isolated from chicken. *Canadian Journal of Microbiology* **48**(1): 34-42.
- Guttenberg, T. J., Dalaker, K. and Vorland, L. H. (1990). Early response in neonatal septicemia. The effect of *Escherichia coli*, *Streptococcus agalactiae* and tumor necrosis factor on the generation of lactoferrin. *Apmis* **98**: 1027-1032.
- Hagiwara, K., Kataoka, S., Yamanaka, H., Kirisawa, R. and Iwai, H. (2000). Detection of cytokines in bovine colostrum. *Veterinary Immunology and Immunopathology* **76**: 183-190.
- Hagiwara, T., Shinoda, I., Fukuwatari, Y. and Shimamura, S. (1995). Effects of lactoferrin and its peptides on proliferation of rat intestinal cell line, IEC-18, in the presence of epidermal growth factor. *Bioscience Biotechnology and Biochemistry* **59**: 1875-1881.
- Haller, D., Blum, S., Bode, C., Hammes, W. P. and Schiffrin, E. J. (2000). Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: Evidence of NK cells as primary targets. *Infection and Immunity* **68**(2): 752-759.
- Haller, D., Bode, C., Hammes, W. P., Pfeifer, A. M. A., Schiffrin, E. J. and Blum, S. (2000b). Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* **47**(1): 79-87.
- Han, Y., Linton, R. H., Nielsen, S. S. and Nelson, P. E. (2000). Inactivation of *Escherichia coli* O157:H7 on surface-uninjured and injured green pepper (*Capsicum annuum* L.) by chlorine dioxide gases demonstrated by confocal laser scanning microscopy. *Food Microbiology* **17**(6): 643-655.
- Han, Y., Linton, R. H., Nielsen, S. S. and Nelson, P. E. (2001). Reduction of *Listeria monocytogenes* on green peppers (*Capsicum annuum* L.) by gaseous and aqueous chlorine dioxide and water washing and its growth at 7°C. *Journal of Food Protection* **64**(11): 1730-1738.
- Hansen, L. L., Mikkelsen, L. L., Agerhem, H., Laue, A., Jensen, M. T. and Jensen, B. B. (2000). Effect of fermented liquid food and zinc bacitracin on microbial metabolism in the gut and sensoric profile of *M. longissimus dorsi* from entire male and female pigs. *Animal Science* **71**: 65-80.
- Hara, T., Irie, K., Saito, S., Ichijo, M., Yamada, M., Yanai, N. and Miyazaki, S. (1995). Identification of macrophage colony stimulating factor in human milk and mammary gland epithelial cells. *Pediatric Research* **37**(4): 437-443.
- Hardin, J. A., Buret, A., Meddings, J. B. and Gall, D. G. (1993). Effect of epidermal growth factor on enterocyte brush border surface area. *American Journal of Physiology* **264**(2): G312-G318.
- Harkins, M., Boyd, R. D. and Bauman, D. E. (1989). Effect of recombinant porcine somatotropin on lactational performance and metabolite patterns in sows and growth of nursing pigs. *Journal of Animal Science* **67**(8): 1997-2008.
- Harvey, R. B., Droleskey, R. E., Hume, M. E., Anderson, R. C., Genovese, K. J., Andrews, K. and Nisbet, D. J. (2002). *In vitro* inhibition of *Salmonella enterica* serovars *choleraesuis* and

- typhimurium*, *Escherichia coli* F-18, and *Escherichia coli* O157:H7 by a porcine continuous-flow competitive exclusion culture. *Current Microbiology* **45**(3): 226-229.
- Hashira, S., Okitsu-Negishi, S. and Yoshino, K. (2002). Interleukin 8 in the human colostrum. *Biology of the Neonate* **82**(1): 34-38.
- Hashizume, S., Kuroda, K. and Murakami, H. (1983). Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum free medium. *Biochimica et Biophysica Acta* **763**(4): 377-382.
- Havenaar, R., Brink, B. T. and Huis in't veld, J. H. J. (1992). Selection of strains for probiotic use. In: Fuller, R. *Probiotics: The Scientific Basis*. London, Chapman & Hall: 209-224.
- Havenaar, R. and Huis in't Veld, J. H. J. (1991). Probiotics: health claims and selection criteria. In: Novel, G. and le Querler, J-F. *Lactic acid bacteria: research and industrial applications in the agro-food industries*. University of Caen, France: 235-243.
- Hawkes, J. S., Bryan, D. L., James, M. J. and Gibson, R. A. (1999). Cytokines (IL-1 beta, IL-6, TNF-alpha, TGF-beta 1, and TGF-beta 2) and prostaglandin E-2 in human milk during the first three months postpartum. *Pediatric Research* **46**(2): 194-199.
- Heilig, H. G. H. J., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermans, A. D. L. and de Vos, W. M. (2002). Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Applied and Environmental Microbiology* **68**(1): 114-123.
- Helander, I. M., von Wright, A. and Mattila-Sandholm, T. M. (1997). Potential of lactic acid bacteria and novel antimicrobials against gram-negative bacteria. *Trends in Food Science & Technology* **8**(5): 146-150.
- Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *American Journal of Clinical Nutrition* **73**(2): 374S-379S.
- Henderson, R. (1814). *A treatise on the breeding of swine and curing bacon*. Leith, Constable and Co.
- Hendrix, W. F., Kelley, K. W., Gaskins, C. T. and Hinrich, D. J. (1976). Porcine neonatal survival and serum gamma globulins. *Journal of Animal Science* **47**: 1281-1286.
- Henriksson, A. and Conway, P. L. (1992). Adhesion to porcine squamous epithelium of saccharide and protein moieties of *Lactobacillus fermentum* strain 104-S. *Journal of General Microbiology* **138**(12): 2657 - 2661.
- Henriksson, A. and Conway, P. L. (1996). Adhesion of *Lactobacillus fermentum* 104-S to porcine stomach mucus. *Current Microbiology* **33**(1): 31-34.
- Herias, M. V., Hessle, C., Telemo, E., Midtvedt, T., Hanson, L. A. and Wold, A. E. (1999). Immunomodulatory effects of *Lactobacillus plantarum* colonizing the intestine of gnotobiotic rats. *Clinical and Experimental Immunology* **116**(2): 283-290.
- Hessle, C., Andersson, B. and Wold, A. E. (2000). Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. *Infection and Immunity* **68**(6): 3581-3586.

- Hessle, C., Hanson, L. A. and Wold, A. E. (1999). Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. *Clinical and Experimental Immunology* **116**(2): 276-282.
- Heumann, D., Barras, C., Severin, A., Glauser, M. P. and Tomasz, A. (1994). Gram-positive cell walls stimulate synthesis of tumor necrosis factor- α and interleukin-6 by human monocytes. *Infection and Immunity* **62**(7): 2715-2721.
- Hill, I. R. and Porter, P. (1974). Studies of bactericidal activity to *Escherichia coli* of porcine serum and colostral immunoglobulins and the role of lysozyme with secretory IgA. *Immunology* **26**: 1239-1250.
- Hillman, K., Murdoch, T. A., Spencer, R. J. and Stewart, C. S. (1994). Inhibition of enterotoxigenic *Escherichia coli* by the microflora of the porcine ileum, in an *in vitro* semicontinuous culture system. *Journal of Applied Bacteriology* **76**(3): 294-300.
- Hillman, K., Spencer, R. J., Murdoch, T. A. and Stewart, C. S. (1995). The effect of mixtures of *Lactobacillus* spp on the survival of enterotoxigenic *Escherichia coli* in *in vitro* continuous culture of porcine intestinal bacteria. *Letters in Applied Microbiology* **20**(2): 130-133.
- Hinton, A., Buhr, R. J. and Ingram, K. D. (2000). Reduction of *Salmonella* in the crop of broiler chickens subjected to feed withdrawal. *Poultry Science* **79**(11): 1566-1570.
- Hofacre, C. L., Johnson, A. C., Kelly, B. J. and Froyman, R. (2002). Effect of a commercial competitive exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic *Escherichia coli* in day-old broiler chickens. *Avian Diseases* **46**(1): 198-202.
- Holderbaum, D., Spech, T., Ehrhart, L. A., Keys, T. and Hall, G. S. (1987). Collagen binding in clinical isolates of *Staphylococcus aureus*. *Journal of Clinical Microbiology* **25**(12): 2258-2261.
- Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J. H. J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology* **41**(2): 85-101.
- Hong, S. I., Kim, Y. J. and Pyun, Y. R. (1999). Acid tolerance of *Lactobacillus plantarum* from kimchi. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie* **32**(3): 142-148.
- Honorio-Franca, A. C., Carvalho, M. P., Isaac, L., Trabulsi, L. R. and Carneiro-Sampaio, M. M. S. (1997). Colostral mononuclear phagocytes are able to kill enteropathogenic *Escherichia coli* opsonized with colostral IgA. *Scandinavian Journal of Immunology* **46**(1): 59-66.
- Hood, S. K. and Zottola, E. A. (1988). Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *Journal of Food Science* **53**(5): 1514-1516.
- Hori, T., Kiyoshima, J., Shida, K. and Yasui, H. (2002). Augmentation of cellular immunity and reduction of influenza virus titer in aged mice fed *Lactobacillus casei* strain *Shirota*. *Clinical and Diagnostic Laboratory Immunology* **9**(1): 105-108.
- Hosseini, S., Insera, P., Araghi-Niknam, M. and Watson, R. R. (2001). Colostrum and milk in the treatment of disease. In: Woodward, B. and Draper, H. H. *Advances in Nutritional Research*. New York, Kluwer Academic / Plenum. **10**: 201-213.

- Houle, V. M., Dudley, M. A. and Donovan, S. M. (1997). Mechanisms of oral IGF-I induced intestinal growth and lactase specific activity in the neonatal piglet. *Faseb Journal* 11(3): 842-842.
- Houle, V. M., Schroeder, E. A., Park, Y. K., Odle, J. and Donovan, S. M. (1995). Orally administered insulin like growth factor-I (IGF-I) stimulates neonatal piglet intestinal development. *Faseb Journal* 9(3): A580-A580.
- Howard, J. C., Heinemann, C., Thatcher, B. J., Martin, B., Gan, B. S. and Reid, G. (2000). Identification of collagen-binding proteins in *Lactobacillus* spp. with surface-enhanced laser desorption/ionization-time of flight ProteinChip technology. *Applied and Environmental Microbiology* 66(10): 4396-4400.
- Hu, W. L., Mazurier, J., Sawatzki, G., Montreuil, J. and Spik, G. (1988). Lactotransferrin receptor of mouse small intestinal brush border - binding characteristics of membrane bound and triton X-100-solubilized forms. *Biochemical Journal* 249(2): 435-441.
- Huang, L. Y., Krieg, A. M., Eller, N. and Scott, D. E. (1999). Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. *Infection and Immunity* 67(12): 6257-6263.
- Hughes, P. E. (1993). The effects of food level during lactation and early gestation on the reproductive performance of mature sows. *Animal Production* 57: 437-445.
- Huis in't Veld, J. H. J. and Havenaar, R. (1993). Selection criteria for microorganisms for probiotic use. In: Jensen, J. F. Hinton, M. H. and Mulder, R. W. A. W. *Prevention and control of potentially pathogenic microorganisms in poultry and poultry meat processing*, Vedbaek, Denmark, DLO Spelderholt Centre for Poultry Research and Information Services 6:11-17.
- Huis in't Veld, J. H. J. and Shortt, C. (1996). Selection criteria for probiotic micro-organisms. In: Leeds, A.R. and Rowland, I.R. *Gut flora and health-past, present and future*. London, UK, The Royal Society of Medicine Press Ltd.: 27-36.
- Hurley, W. L. (2001). Mammary gland growth in the lactating sow. *Livestock Production Science* 70: 149-157.
- Hurley, W. L. and Grieve, R. C. J. (1988). Total and differential cell counts and N-acetyl-beta-D-glucosaminidase activity in sow milk during lactation. *Veterinary Research Communications* 12(2-3): 149-153.
- Iba, A. M. and Berchieri, A. (1995). Studies on the use of a formic acid propionic acid mixture (Bio-add™) to control experimental *Salmonella* infection in broiler chickens. *Avian Pathology* 24(2): 303-311.
- Ibnou-Zekri, N., Blum, S., Schiffrin, E. J. and von der Weid, T. (2003). Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*. *Infection and Immunity* 71(1): 428-436.
- Ibrahim, S. A. and O'Sullivan, D. J. (1998). Acid and bile resistance of *Bifidobacterium* spp., *Lactobacillus bulgaricus* and *Streptococcus salivarius* subspecies *thermophilus* and their β -galactosidase activities. *Journal of Dairy Science* 81(Suppl.): Abstract 21.
- Iijima, H., Takahashi, I. and Kiyono, H. (2001). Mucosal immune network in the gut for the control of infectious diseases. *Reviews in Medical Virology* 11(2): 117-133.

- Iizasa, H. and Matsushima, K. (2000). IL-8. In: Oppenheim, J. J. and Feldmann, *Cytokine reference*. M. London, UK, Academic Press. 1: 1061-1068.
- Inoue, T. (1981). Possible factors influencing immunoglobulin A concentration in swine colostrum. *American Journal of Veterinary Research* 42: 533-536.
- Inoue, T., Kitano, K. and Inoue, K. (1980). Possible factors influencing the immunoglobulin G concentration in swine colostrum. *American Journal of Veterinary Research* 41: 1134-1136.
- Isaacs, C. E. (2001). The antimicrobial function of milk lipids. *Advances in Nutritional Research* 10: 271-285.
- Ismail, M. and Brock, J. H. (1993). Binding of lactoferrin and transferrin to the human promonocytic cell line U937 - effect on iron uptake and release. *Journal of Biological Chemistry* 268(29): 21618-21625.
- Isolaure, E. (2001). Probiotics in human disease. *American Journal of Clinical Nutrition* 73(6): 1142S-1146S.
- Isolaure, E., Kirjavainen, P. V. and Salminen, S. (2002). Probiotics: a role in the treatment of intestinal infection and inflammation? *Gut* 50: 54-59.
- Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Moller, P. L., Michaelsen, K. F., Paerregaard, A., Sandstrom, B., Tvede, M. and Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology* 65(11): 4949-4956.
- Jaeger, L. A. and Lamar, C. H. (1992). Immunolocalization of epidermal growth factor (EGF) and EGF receptors in the porcine upper gastrointestinal tract. *American Journal of Veterinary Research* 53(9): 1685-1692.
- Jaeger, L. A., Lamar, C. H., Bottoms, G. D. and Cline, T. R. (1987). Growth-stimulating substances in porcine milk. *American Journal of Veterinary Research* 48(10): 1531-1533.
- Jaeger, L. A., Lamar, C. H., Cline, T. R. and Cardona, C. J. (1990). Effect of orally administered epidermal growth factor on the jejunal mucosa of weaned pigs. *American Journal of Veterinary Research* 51(3): 471-474.
- Jensen, B. B. and Mikkelsen, L. L. (1998). Feeding liquid diets to pigs. In: Gamsworthy, P. C. and Wiseman, J. *Recent Advances in Animal Nutrition*. Nottingham, Nottingham University Press: 107-126.
- Jepson, M. A. and Clark, M. A. (2001). The role of M cells in *Salmonella* infection. *Microbes and Infection* 3(14-15): 1183-1190.
- Jepson, M. A. a. C., M. A. (1998). Studying M cells and their role in infection. *Trends in Microbiology* 6(9): 359-365.
- Jin, L. Z., Marquardt, R. R. and Baidoo, S. K. (2000). Inhibition of enterotoxigenic *Escherichia coli* K88, K99 and 987P by the *Lactobacillus* isolates from porcine intestine. *Journal of the Science of Food and Agriculture* 80(5): 619-624.

- Johannson, M. L., Sanni, A., Lonner, C. and Mollin, G. (1995). Phenotypically based taxonomy using API 50CH of lactobacilli from Nigerian ogi, and the occurrence of starch fermenting strains. *International Journal of Food Microbiology* **25**: 159-168.
- Johansson, M. L., Nobaek, S., Berggren, A., Nyman, M., Bjorck, I., Ahrne, S., Jeppsson, B. and Molin, G. (1998). Survival of *Lactobacillus plantarum* DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. *International Journal of Food Microbiology* **42**(1-2): 29-38.
- Jonsson, H., Strom, E. and Roos, S. (2001). Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* *in vitro*. *FEMS Microbiology Letters* **204**(1): 19-22.
- Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J. and Isolauri, E. (2001). Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. *Clinical and Diagnostic Laboratory Immunology* **8**(2): 293-296.
- Juto, P. (1985). Human milk stimulates B-cell function. *Archives of Disease in Childhood* **60**(7): 610-613.
- Kagnoff, M. F. and Eckmann, L. (1997). Epithelial cells as sensors for microbial infection. *Journal of Clinical Investigation* **100**(12): S51-S55.
- Kaila, M., Isolauri, E., Saxelin, M., Arvilommi, H. and Vesikari, T. (1995). Viable versus inactivated *Lactobacillus* strain GG in acute rotavirus diarrhea. *Archives of Disease in Childhood* **72**(1): 51-53.
- Kaila, M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S. and Arvilommi, H. (1992). Enhancement of the circulating antibody secreting cell response in human diarrhoea by a human *Lactobacillus* strain. *Pediatric Research* **32**(2): 141-144.
- Kapczynski, D. R., Meinersmann, R. J. and Lee, M. D. (2000). Adherence of *Lactobacillus* to intestinal 407 cells in culture correlates with fibronectin binding. *Current Microbiology* **41**(2): 136-141.
- Karlsson, N. G., Nordman, H., Karlsson, H., Carlstedt, I. and Hansson, G. C. (1997). Glycosylation differences between pig gastric mucin populations: A comparative study of the neutral oligosaccharides using mass spectrometry. *Biochemical Journal* **326**: 911-917.
- Kato, I., Tanaka, K. and Yokokura, T. (1999). Lactic acid bacterium potently induces the production of interleukin-12 and interferon- γ by mouse splenocytes. *International Journal of Immunopharmacology* **21**(2): 121-131.
- Kato, I., Yokokura, T. and Mutai, M. (1983). Macrophage activation by *Lactobacillus casei* in mice. *Microbiology and Immunology* **27**(7): 611-618.
- Kato, I., Yokokura, T. and Mutai, M. (1984). Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. *Microbiology and Immunology* **28**(2): 209-217.
- Katouli, M., Lund, A., Wallgren, P., Kühn, I., Söderlind, O. and Möllby, R. (1995). Phenotypic characterization of intestinal *Escherichia coli* of pigs during suckling, postweaning, and fattening periods. *Applied and Environmental Microbiology* **61**(2): 778-783.

- Katouli, M., Lund, A., Wallgren, P., Kühn, I., Söderlind, O. and Möllby, R. (1997). Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre-and post-weaning periods. *Journal of Applied Microbiology* **83**: 147-154.
- Kawakami, H. and Lonnerdal, B. (1991). Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush border membranes. *American Journal of Physiology* **261**(5): G841-G846.
- Keller, R., Keist, R. and Joller, P. (1995). Macrophage response to microbial pathogens - modulation of the expression of adhesion, CD14, and MHC class-II molecules by viruses, bacteria, protozoa and fungi. *Scandinavian Journal of Immunology* **42**(3): 337-344.
- Kelly, D., Begbie, R. and King, T. P. (1992a). Postnatal intestinal development. In: Varley, M. A., Williams, P. E. V. and Lawrence, T. L. J. *Neonatal survival and growth*. Edinburgh, BSAP Occasional Publication, BSAS **15**: 63-79.
- Kelly, D. and King, T. P. (1991). The influence of lactation products on the temporal expression of histo-blood group antigens in the intestines of suckling pigs: lectin histochemical and immunohistochemical analysis. *Histochemical Journal* **23**: 55-60.
- Kelly, D. and King, T. P. (2001a). Digestive physiology and development in pigs. *The weaner pig: Nutrition and management*. Varley, M. A. and Wiseman, J., CAB International: 179-206.
- Kelly, D. and King, T. P. (2001b). Luminal bacteria: regulation of gut function and immunity. In: Piva, A., Bach Knudsen, K. E. and Lindberg, J. E. *Gut environment of pigs*. Nottingham, The Nottingham University Press: 113-131.
- Kelly, D., King, T. P., Brown, D. S. and McFadyen, M. (1991). Polyamide profiles of porcine milk and of intestinal tissue of pigs during suckling. *Reproduction Nutrition Development* **31**(1): 73-80.
- Kelly, D., McFadyen, M., King, T. P. and Morgan, P. J. (1992b). Characterization and autoradiographic localization of the epidermal growth factor receptor in the jejunum of neonatal and weaned pigs. *Reproduction Fertility and Development* **4**(2): 183-191.
- Kendrew, J. and Lawrence, E. (1994). *The encyclopedia of molecular biology*, Blackwell Science (UK).
- Khalil, N. (1999). TGF-beta: from latent to active. *Microbes and Infection* **1**(15): 1255-1263.
- Kidder, D. E. (1982). Nutrition of the early weaned pig compared with the sow-reared pig. *Pig News and Information* **3**(1): 25-28.
- Kidder, D. E. and Manners, M. J. (1978). *Digestion in the pig*. Bristol, Sciencetechnica.
- Kim, S. W., Hurley, W. L. and Easter, R. A. (1999). Changes in tissue composition associated with mammary gland growth during lactation in sows. *Journal of Animal Science* **77**(9): 2510.
- King, R. H. (2000). Factors that influence milk production in well-fed sows. *Journal of Animal Science* **78** (Suppl. 3): 19-25.
- Kingamkono, R., Sjogren, E. and Svanberg, U. (1999). Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels. *Epidemiology and Infection* **122**(1): 23-32.

- Kirjavainen, P. V., El-Nezami, H. S., Salminen, S. J., Ahokas, J. T. and Wright, P. F. A. (1999a). Effects of orally administered viable *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* subsp *shermanii* JS on mouse lymphocyte proliferation. *Clinical and Diagnostic Laboratory Immunology* 6(6): 799-802.
- Kirjavainen, P. V., El-Nezami, H. S., Salminen, S. J., Ahokas, J. T. and Wright, P. F. A. (1999b). The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation. *FEMS Immunology and Medical Microbiology* 26(2): 131-135.
- Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E. and Salminen, S. J. (1998). The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters* 167(2): 185-189.
- Kirkwood, R. N., Baidoo, S. K. and Aherne, F. X. (1990). The influence of feeding level during lactation and gestation on the endocrine status and reproductive performance of 2nd parity sows. *Canadian Journal of Animal Science* 70(4): 1119-1126.
- Klaenhammer, T. R. (1982). Microbiological considerations in selection and preparation of *Lactobacillus* strains for use as dietary adjuncts. *Journal of Dairy Science* 65(7): 1339-1349.
- Klobasa, F. and Butler, J. E. (1987). Absolute and relative concentrations of immunoglobulin G, immunoglobulin M, and immunoglobulin A, and albumin in the lacteal secretion of sows of different lactation numbers. *American Journal of Veterinary Research* 48(2): 176-182.
- Klobasa, F., Butler, J. E., Werhahn, E. and Habe, F. (1986). Maternal neonatal immunoregulation in swine 2. Influence of multiparity on de novo immunoglobulin synthesis by piglets. *Veterinary Immunology and Immunopathology* 11(2): 149-159.
- Klobasa, F., Habe, F., Werhahn, E. and Butler, J. E. (1985). The influence of age and breed on the concentrations of serum IgG, IgA and IgM in sows throughout the reproductive cycle. *Veterinary Immunology and Immunopathology* 10(4): 355-366.
- Klopfenstein, C., Farmer, C. and Martineau, G. P. (1999). Diseases of the mammary glands and lactation problems. In: Straw, B. E., D'Allaire, S., Mengeling, W. L. and Taylor, A. R. *Diseases of swine*. Ames, Iowa, Iowa State University Press: 833-861.
- Kmet, V., Callegari, M. L., Bottazzi, V. and Morelli, L. (1995). Aggregation promoting factor in pig intestinal *Lactobacillus* strains. *Letters in Applied Microbiology* 21(6): 351-353.
- Kmet, V. and Lucchini, F. (1999). Aggregation of sow lactobacilli with diarrhoeagenic *Escherichia coli*. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* 46(10): 683-687.
- Kmet, V. and Lucchini, R. (1997). Aggregation-promoting factor in human vaginal *Lactobacillus* strains. *FEMS Immunology and Medical Microbiology* 19(2): 111-114.
- Ko, T. C., Beauchamp, R. D., Townsend, C. M., Thompson, J. C. and Souba, W. W. (1993). Glutamine is essential for epidermal growth factor stimulated intestinal cell proliferation. *Surgery* 114(2): 147-154.
- Koketsu, Y., Dial, G. D., Pettigrew, J. E. and King, V. L. (1996). Feed intake pattern during lactation and subsequent reproductive performance of sows. *Journal of Animal Science* 74(12): 2875-2884.

- Kolenbrander, P. E., Andersen, R. N. and Moore, L. V. H. (1990). Intrageneric coaggregation among strains of human oral bacteria - potential role in primary colonization of the tooth surface. *Applied and Environmental Microbiology* **56**(12): 3890-3894.
- Kolenbrander, P. E., Ganeshkumar, N., Cassels, F. J. and Hughes, C. V. (1993). Coaggregation - specific adherence among human oral plaque bacteria. *Faseb Journal* **7**(5): 406-413.
- Kumprecht, I. and Zobac, P. (1998). Study of the effect of a combined preparation containing *Enterococcus faecium* M-74 and mannan-oligosaccharides in diets for weanling piglets. *Czech Journal of Animal Science* **43**(10): 477-481.
- Kumura, H., Sone, T., Shimazaki, K. and Kobayashi, E. (2000). Sequence analysis of porcine polymeric immunoglobulin receptor from mammary epithelial cells present in colostrum. *Journal of Dairy Research* **67**(4): 631-636.
- Kyriakis, S. C., Tsiloyiannis, V. K., Vlemmas, J., Sarris, K., Tsinas, A. C., Alexopoulos, C. and Jansegers, L. (1999). The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. *Research in Veterinary Science* **67**(3): 223-228.
- La Via, M. F. and Workman, E. A. (1991). Psychoneuroimmunology: where are we, where are we going? *Recenti Progressi in Medicina* **82**(12): 637-641.
- Laffineur, E., Genetet, N. and Leonil, J. (1996). Immunomodulatory activity of β -casein permeate medium fermented by lactic acid bacteria. *Journal of Dairy Science* **79**(12): 2112-2120.
- Lamm, M. E. (1998). Current concepts in mucosal immunity IV. How epithelial transport of IgA antibodies relates to host defense. *American Journal of Physiology (Gastrointestinal and Liver Physiology 37)* **274**: G614-G617.
- Lawrence, D. A. (1996). Transforming growth factor-beta: A general review. *European Cytokine Network* **7**(3): 363-374.
- Lawton, J. W. M., Shortridge, K. F., Wong, R. C. L. and Ng, M. H. (1979). Interferon synthesis by human colostrum leucocytes. *Archives of Disease in Childhood* **54**: 127-130.
- Le Dividich, J. (2002). Preface. *Livestock Production Science* **78**: 1.
- Le Dividich, J. and Noblet, J. (1981). Colostrum intake and thermoregulation in the neonatal pig in relation to environmental temperature. *Biology of the Neonate* **40**: 167-174.
- Le Jan, C. (1996). Cellular components of mammary secretions and neonatal immunity: a review. *Veterinary Research* **27**: 403-417.
- Lecce, J. G. and Morgan, D. O. (1962). Effect of dietary regime on cessation of intestinal absorption of large molecules (closure) in neonatal pigs and lambs. *Journal of Nutrition* **78**: 265-270.
- Ledeist, F., Desaintbasile, G., Angelescano, E. and Griscelli, C. (1986). Prostaglandin-E2 and plasminogen activators in human milk and their secretion by milk macrophages. *American Journal of Reproductive Immunology and Microbiology* **11**(1): 6-10.
- Lee, C. S., McCauley, I. and Hartmann, P. E. (1983). Light and electron microscopy of cells in pig colostrum, milk and involution secretion. *Acta anatomica* **116**: 126-135.
- Lejan, C. (1994). A study by flow-cytometry of lymphocytes in sow colostrum. *Research in Veterinary Science* **57**(3): 300-304.

- Lemarbre, P., Rinehart, J. J., Kay, N. E., Vesella, R. and Jacob, H. S. (1981). Lysozyme enhances monocyte mediated tumoricidal activity - a potential amplifying mechanism of tumor killing. *Blood* **58**(5): 994-999.
- Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M. and Moller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology* **68**(2): 673-690.
- Lessard, M. and Brisson, G. J. (1987). Effect of a *Lactobacillus* fermentation product on growth, immune response and fecal enzyme activity in weaned pigs. *Canadian Journal of Animal Science* **67**: 509-516.
- Liebler-Tenorio, E. M., Pohlenz, J. F. and Whipp, S. C. (1999). Diseases of the digestive system. In: Straw, B. E., D'Allaire, S., Mengeling, W. L. and Taylor, D. J. *Disease of swine*. Ames, Iowa, Iowa State University Press: 821-833.
- Lindgren, S. E. and Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentation. *FEMS Microbiology Reviews* **87**: 149-164.
- Link-Amster, H., Rochat, F., Saudan, K. Y., Mignot, O. and Aeschlimann, J. M. (1994). Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunology and Medical Microbiology* **10**(1): 55-63.
- Lipkin, M. (1985). Growth and development of gastrointestinal cells. *Annual Review of Physiology* **47**: 175-197.
- Ljungh, A., Emody, L., Steinruck, H., Sullivan, P., West, B., Zetterberg, E. and Wadstrom, T. (1990). Fibronectin, vitronectin, and collagen binding to *Escherichia coli* of intestinal and extraintestinal origin. *Zentralblatt für Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **274**(1): 126-134.
- Ljungh, A., Moran, A. P. and Wadström, T. (1996). Interactions of bacterial adhesins with extracellular matrix and plasma proteins: Pathogenic implications and therapeutic possibilities. *FEMS Immunology and Medical Microbiology* **16**(2): 117-126.
- Longman, R. J., Thomas, M. G. and Poulsom, R. (1999). Trefoil peptides and surgical disease. *British Journal of Surgery* **86**(6): 740-748.
- Lonnerdal, B. and Iyer, S. (1995). Lactoferrin - molecular structure and biological function. *Annual Review of Nutrition* **15**: 93-110.
- Loser, C. (2000). Polyamines in human and animal milk. *British Journal of Nutrition* **84**: S55-S58.
- Louveau, I., Dauncey, M. J. and Le Dividich, J. (2000). Regulation of development by nutrition and by the somatotrophic and thyroid axes in the neonatal pig. *Livestock Production Science* **66**: 121-131.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265-275.
- Lucchini, F., Kmet, V., Cesena, C., Coppi, L., Bottazzi, V. and Morelli, L. (1998). Specific detection of a probiotic *Lactobacillus* strain in faecal samples by using multiplex PCR. *FEMS Microbiology Letters* **158**(2): 273-278.

- Maassen, C. B. M., van Holten, J. C. A. M., Balk, F., Heijne den Bac-Glashouwer, M. J., Leer, R., Laman, J. D., Boersma, W. J. A. and Claassen, E. (1998). Orally administered *Lactobacillus* strains differentially affect the direction and efficacy of the immune response. *The Veterinary Quarterly* **20**(3): S81-S83.
- Macfarlane, G. T., Gibson, G. R. and Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. *Journal of Applied Bacteriology* **72**: 57-64.
- Mackie, R. I., Sghir, A. and Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *American Journal of Clinical Nutrition* **69**(5): 1035S-1045S.
- Maclean, C. W. and Thomas, N. D. (1974). Faecal and vaginal bacteriology of sows during the reproductive cycle. *The British Veterinary Journal* **130**: 230-237.
- Macpherson, A. J., Hunziker, L., McCoy, K. and Lamarre, A. (2001). IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes and Infection* **3**(12): 1021-1035.
- Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L. and De Simone, C. (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* **121**(3): 580-591.
- Magnusson, J., Strom, K., Roos, S., Sjogren, J. and Schnurer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters* **219**(1): 129-135.
- Magnusson, U., Rodriguez-Martinez, H. and Einarsson, S. (1991). A simple, rapid method for differential cell counts in porcine mammary secretions. *The Veterinary Record* **129**(22): 485-490.
- Mahida, Y. R. and Johal, S. (2001). NF-kappa B may determine whether epithelial cell-microbial interactions in the intestine are hostile or friendly. *Clinical and Experimental Immunology* **123**(3): 347-349.
- Makarov, S. S. (2000). NF- κ B as a therapeutic target in chronic inflammation: recent advances. *Molecular Medicine Today* **6**: 441-448.
- Makkink, C. A. and Schrama, J. W. (1998). Thermal requirements of the lactating sow. In: Verstegen, M. W. A., Moughan, P. J. and Schrama, J. W. *The lactating sow*. Wageningen, Netherlands, Wageningen Pers.: 271-285.
- Malin, M., Suomalainen, H., Saxelin, M. and Isolauri, E. (1996). Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. *Annals of Nutrition & Metabolism* **40**: 137-145.
- Mallard, B. A., Dekkers, J. C., Ireland, M. J., Leslie, K. E., Sharif, S., Vankampen, C. L., Wagter, L. and Wilkie, B. N. (1998). Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. *Journal of Dairy Science* **81**(2): 585-595.
- Manteuffel, G. (2001). Stress as a modulator of the immune system. *Tierarztliche Umschau* **56**(4): 206-206.
- Mao, Y., Yu, J. L., Ljungh, A., Molin, G. and Jeppsson, B. (1996). Intestinal immune response to oral administration of *Lactobacillus reuteri* R2LC, *Lactobacillus plantarum* DSM 9843, pectin

- and oatbase on methotrexate-induced enterocolitis in rats. *Microbial Ecology in Health and Disease* 9(6): 261-269.
- Maribo, H., Jensen, B. B. and Hedemann, M. S. (2000). Different doses of organic acids to piglets, Danish Bacon and Meat Council, Report No: 469.
- Martin, W. T., Zhang, Y. H., Willson, P., Archer, T. P., Kinahan, C. and Barber, E. M. (1996). Bacterial and fungal flora of dust deposits in a pig building. *Occupational and Environmental Medicine* 53(7): 484-487.
- Masson, P. L., Heremans, J. F. and Dive, C. (1966). An iron-binding protein common to many external secretions. *Clinica Chimica Acta* 14: 735-739.
- Matar, C., Valdez, J. C., Medina, M., Rachid, M. and Perdigon, G. (2001). Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *Journal of Dairy Research* 68(4): 601-609.
- Mathew, A. G., Sutton, A. L., Scheidt, A. B., Patterson, J. A., Kelly, D. T. and Meyerholtz, K. A. (1993). Effect of galactan on selected microbial populations and pH and volatile fatty acids in the ileum of the weanling pig. *Journal of Animal Science* 71: 1503-1509.
- Mayer, L. (1998). Current concepts in mucosal immunity I. Antigen presentation in the intestine: new rules and regulations. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* 274: G7-G9.
- Mäyrä-Mäkinen, A., Manninen, M. and Gyllenberg, H. (1983). The adherence of lactic acid bacteria to the columnar epithelial cells of pigs and calves. *Journal of Applied Bacteriology* 55(2): 241-245.
- Mayrhuber, E., Holzer, M., Danner, H., Madzingaidzo, L. and Braun, R. (1999). Comparison of homofermentative and heterofermentative *Lactobacillus* strains as silage inoculum, to improve aerobic stability. *The XII International Silage conference*, Uppsala, Sweden: 276-277.
- McCracken, V. J. and Lorenz, R. G. (2001). The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cellular Microbiology* 3(1): 1-11.
- McDonald, L. C., Fleming, H. P. and Hassan, H. M. (1990). Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 56(7): 2120-2124.
- McNeil, N. I. (1982). Human large-intestinal absorption of short-chain fatty acids. In: Kasper, H. and Goebell, H. *Colon and Nutrition*. Lancaster, Boston, The Hague, MTP Press Limited: 37-45.
- Meat and Livestock Commission (2001). *Pig Yearbook 2001*. Milton Keynes, UK, Meat and Livestock Commission.
- Mehrazar, K., Gilmansachs, A. and Kim, Y. B. (1993). Intestinal absorption of immunologically intact macromolecules in germ-free colostrum deprived piglets maintained on total parenteral nutrition. *Journal of Parenteral and Enteral Nutrition* 17(1): 8-15.
- Mercenier, A., Pavan, S. and Pot, B. (2003). Probiotics as biotherapeutic agents: Present knowledge and future prospects. *Current Pharmaceutical Design* 9(2): 175-191.

- Michail, S. and Abernathy, F. (2002). *Lactobacillus plantarum* reduces the *in vitro* secretory response of intestinal epithelial cells to enteropathogenic *Escherichia coli* infection. *Journal of Pediatric Gastroenterology and Nutrition* 35(3): 350-355.
- Midtvedt, T. (1985). Microflora-associated characteristics (MACs) and germfree animal characteristics (GACs) in man and animal. *Microecology and Therapy* 15: 295-302.
- Miettinen, A., Westerlund, B., Tarkkanen, A. M., Tornroth, T., Ljungberg, P., Renkonen, O. V. and Korhonen, T. K. (1993). Binding of bacterial adhesins to rat glomerular mesangium *in vivo*. *Kidney International* 43(3): 592-600.
- Miettinen, M., Lehtonen, A., Julkunen, I. and Matikainen, S. (2000). Lactobacilli and streptococci activate NF-kappa B and STAT signalling pathways in human macrophages. *Journal of Immunology* 164(7): 3733-3740.
- Miettinen, M., VuopioVarkila, J. and Varkila, K. (1996). Production of human tumour necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infection and Immunity* 64(12): 5403-5405.
- Mikkelsen, L. L. and Jensen, B. B. (1997). Effect of fermented liquid feed (FLF) on growth performance and microbial activity in the gastrointestinal tract of weaned piglets. In: Laplace, J. P., Fevrier, C. and Barbeau, A. *Digestive Physiology in Pigs*. Paris, EAAP Publication No.88, INRA: 639-642.
- Mikkelsen, L. L. and Jensen, B. B. (1998). Performance and microbial activity in the gastrointestinal tract of piglets fed fermented liquid feed at weaning. *Journal of Animal and Feed Sciences* 7(Suppl.1): 211-215.
- Miller, E. R., Hogberg, M. G. and Ku, P. K. (1982). Dietary laxatives for sows during gestation and lactation. *Michigan Agricultural Experiment Station Research Report* (437): 13-15.
- Minekus, M., Marteau, P., Havenaar, R. and Huisintveld, J. H. J. (1995). A multicompartamental dynamic computer controlled model simulating the stomach and small intestine. *Atla-Alternatives to Laboratory Animals* 23(2): 197-209.
- Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G. and Veld, J. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Applied Microbiology and Biotechnology* 53(1): 108.
- MLC (1999). *The stotfold feeding strategy*. Milton Keynes, UK, Meat & Livestock Commission.
- Moberg, G. P. (2000). Biological response to stress: Implications for animal welfare. In: Moberg, G. P. and Mench, J. A. *The biology of animal stress. Basic principles and implications for animal welfare*. Wallingford, Oxon, CABI Publishing: 1-23.
- Modlin, I. M. and Poulson, R. (1997). Trefoil peptides: Mitogens, motogens, or mirages? *Journal of Clinical Gastroenterology* 25: S94-S100.
- Monaco, M. H., Chen, A. C., Correa, N., Ganessunker, D., Gaskins, H. R., Lynch, J. K., McCarthy, M. C., Pelletier, S. E. and Donovan, S. M. (2000). Effect of oral insulin-like growth factor-I (IGF-I) during the suckling period on intestinal structure and immune cell composition during weaning in pigs. *Faseb Journal* 14(4): A506-A506.

- Monaco, M. H. and Donovan, S. M. (1997). Insulin-like growth factor-I infusion increases *in vivo* skeletal muscle and mammary alpha-amino[1-C-14]isobutyric acid accumulation in food restricted lactating rats. *Nutrition Research* 17(7): 1143-1154.
- Moran, C. A. (2001). *Developments and benefits of liquid feeding for the post-weaned pig*. Ph.D. Thesis, University of Plymouth, UK.
- Morita, H., He, F., Fuse, T., Ouwehand, A. C., Hashimoto, H., Hosoda, M., Mizumachi, K. and Kurisaki, J. I. (2002). Adhesion of lactic acid bacteria to Caco-2 cells and their effect on cytokine secretion. *Microbiology and Immunology* 46(4): 293-297.
- Morrowtesch, J. and McGlone, J. J. (1990). Sources of maternal odors and the development of odor preferences in baby pigs. *Journal of Animal Science* 68(11): 3563-3571.
- Motyl, T., Ploszaj, T., Wojtasik, A., Kukulska, W. and Podgurniak, M. (1995). Polyamines in cows and sows milk. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 111(3): 427-433.
- Mubiru, J. N. and Xu, R. J. (1997). Growth and development of the exocrine pancreas in newborn pigs: The effect of colostrum feeding. *Biology of the Neonate* 71(5): 317-326.
- Muller, T. (1990). Comparison of methods for differentiation between homofermentative and heterofermentative lactic acid bacteria. *Zentralblatt für Mikrobiologie* 145(5): 363-366.
- Mullie, C., Yazourh, A., Singer, E., Lecroix, F., Blareau, J. P., Romond, M. B. and Romond, C. (2002). Partial characterization of *Bifidobacterium breve* C50 cell-free whey compounds inducing modifications to the intestinal microflora. *Journal of Dairy Science* 85(6): 1383-1389.
- Muralidhara, K. S., Sheggeby, G. G., Elliker, P. R., England, D. C. and Sandine, W. E. (1977). Effect of feeding *Lactobacillus* on the coliform and *Lactobacillus* flora of intestinal tissue and faeces from piglets. *Journal of Food Protection* 40(5): 288-295.
- Murphy, L. C., Dunne, C., Kiely, B., Shanahan, F., O'Sullivan, G. and Collins, K. (1999). *In vivo* assessment of potential probiotic *Lactobacillus salivarius* strains: evaluation of their establishment, persistence, and localisation in the murine gastrointestinal tract. *Microbial Ecology of Health and Disease* 11: 149-157.
- Naidu, A. S., Bidlack, W. R. and Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition* 39(1): 13-126.
- Naito, S., Hayashidani, H., Kaneko, K., Ogawa, M. and Benno, Y. (1995). Development of intestinal lactobacilli in normal piglets. *Journal of Applied Bacteriology* 79: 230-236.
- Nakao, K., Imoto, I., Ikemura, N., Shibata, T., Takaji, S., Taguchi, Y., Misaki, M., Yamauchi, K. and Yamazaki, N. (1997). Relation of lactoferrin levels in gastric mucosa with *Helicobacter pylori* infection and with the degree of gastric inflammation. *American Journal of Gastroenterology* 92: 1005-1011.
- Nallapareddy, S. R., Qin, X., Weinstock, G. M., Höök, M. and Murray, B. E. (2000). *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infection and Immunity* 68(9): 5218-5224.

- Nallapareddy, S. R., Weinstock, G. M. and Murray, B. E. (2003). Clinical isolates of *Enterococcus faecium* exhibit strain specific collagen binding mediated by Acm, a new member of the MSCRAMM family. *Molecular Microbiology* **47**(6): 1733-1747.
- Neeser, J. R., Granato, D., Rouvet, M., Servin, A., Teneberg, S. and Karlsson, E. A. (2000). *Lactobacillus johnsonii* LA1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology* **10**(11): 1193-1199.
- Nemcova, R., Bomba, A., Herich, R. and Gancarcikova, S. (1998). Colonization capability of orally administered *Lactobacillus* strains in the gut of gnotobiotic piglets. *Deutsche Tierärztliche Wochenschrift* **105**(5): 199-200.
- Netherwood, T., Bowden, R., Harrison, P., O'Donnell, A. G., Parker, D. S. and Gilbert, H. J. (1999a). Gene transfer in the gastrointestinal tract. *Applied and Environmental Microbiology* **65**(11): 5139-5141.
- Netherwood, T., Gilbert, H. J., Parker, D. S. and O'Donnell, A. G. (1999b). Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Applied and Environmental Microbiology* **65**(11): 5134-5138.
- Nigatu, A. (2000). Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum*, *Lact. fermentum*, *Lact. rhamnosus*, *Lact. sake*, *Lact. parabuchneri*, *Lact. gallinarum*, *Lact. casei*, *Weissella minor* and related taxa isolated from kocho and tef. *Journal of Applied Microbiology* **89**(6): 969-978.
- Neutra, M. R. (1998). Current Concepts in Mucosal Immunity - V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **37**(5): G785-G791.
- Neutra, M. R., Mantis, N. J., Frey, A. and Giannasca, P. J. (1999). The composition and function of M cell apical membranes: Implications for microbial pathogenesis. *Seminars in Immunology* **11**: 171-181.
- Neutra, M. R., Pringault, E. and Kraehenbuhl, J. P. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology* **14**: 275-300.
- Nietfeld, J. C., Yeary, T. J., Basaraba, R. J. and Schauenstein, K. (1999). Norepinephrine stimulates *in vitro* growth but does not increase pathogenicity of *Salmonella choleraesuis* in an *in vivo* model. *Mechanisms in the Pathogenesis of Enteric Diseases* **2**, **473**: 249-260.
- Nigatu, A. (2000). Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum*, *Lact. fermentum*, *Lact. rhamnosus*, *Lact. sake*, *Lact. parabuchneri*, *Lact. gallinarum*, *Lact. casei*, *Weissella minor* and related taxa isolated from kocho and tef. *Journal of Applied Microbiology* **89**(6): 969-978.
- Nigatu, A., Ahrne, S. and Molin, G. (2001). Randomly amplified polymorphic DNA (RAPD) profiles for the distinction of *Lactobacillus* species. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **79**(1): 1-6.
- Nisbet, D. (2002). Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**(1-4): 481-486.

- Nissley, S. P., Haskell, J. F., Sasaki, N., Devroede, M. A. and Rechler, M. M. (1985). Insulin-like growth factor receptors. *Journal of Cell Science* **3**: 39-51.
- Noblet, J., Dourmad, J. Y. and Etienne, M. (1990). Energy utilization in pregnant and lactating sows: modeling of energy requirements. *Journal of Animal Science* **68**: 562-572.
- Noblet, J. and Le Dividich, J. (1981). Energy metabolism of the newborn pig during the first 24 hours of life. *Biology of the Neonate* **40**: 175-182.
- Norcross, N. L. (1982). Secretion and composition of colostrum and milk. *Journal of the American Veterinary Medical Association* **181**(10): 1057-1060.
- Nowak, D. (1998). Health effects of airborne pollutants particularly in swine confinement houses - a review focusing on aspects of occupational medicine. *Deutsche Tierärztliche Wochenschrift* **105**(6): 225-234.
- Nunes, I., Munger, J. S., Harpel, J. G., Nagano, Y., Shapiro, R. L., Gleizes, P. E. and Rifkin, D. B. (1996). Structure and activation of the large latent transforming growth factor-beta complex. *International Journal of Obesity* **20**: S4-S8.
- Nusrat, A., Turner, J. R. and Madara, J. L. (2000). Molecular physiology and pathophysiology of tight junctions IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* **279**: G851-G857.
- O'Brien, J., Crittenden, R., Ouwehand, A. C. and Salminen, S. (1999). Safety evaluation of probiotics. *Trends in Food Science & Technology* **10**(12): 418-424.
- Oda, Y., Ito, M., Kikuta, Y., Park, B. S. and Tonomura, K. (2000). Comparison and characterization of *Lactobacillus amylovorus* strains for lactic acid fermentation of food by-products. *Food Microbiology* **17**(3): 341-347.
- Odle, J., Zijlstra, R. T. and Donovan, S. M. (1996). Intestinal effects of milkborne growth factors in neonates of agricultural importance. *Journal of Animal Science* **74**(10): 2509-2522.
- Official Journal of the European Communities (2002). Council Directive 70/524/EEC.
- O'Grady, J. F. and Lynch, P. B. (1978). Voluntary feed intake by lactating sows: Influence of system of feeding and nutrient density of the diet. *Irish Journal of Agricultural Research* **17**: 1-5.
- Oguchi, S., Walker, W. A. and Sanderson, I. R. (1995). Iron saturation alters the effect of lactoferrin on the proliferation and differentiation of human enterocytes (Caco-2 cells). *Biology of the Neonate* **67**(5): 330-339.
- Ohashi, Y., Inoue, R., Tanaka, K., Matsuki, T., Umesaki, Y. and Ushida, K. (2001). *Lactobacillus casei* strain shirota-fermented milk stimulates indigenous lactobacilli in the pig intestine. *Journal of Nutritional Science and Vitaminology* **47**(2): 172-176.
- Ohlsson, B. G., Westrom, B. R. and Karlsson, B. W. (1986). Proteinase-inhibitors in the gastrointestinal tract, pancreas and liver during fetal and postnatal development of the pig. *Biology of the Neonate* **49**(5): 292-300.

- Olsen, A., Halm, M. and Jakobsen, M. (1995). The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interactions during fermentation. *Journal of Applied Bacteriology* **79**: 506-512.
- Olusanya, O., Steinruck, H., Aleljang, P., Ascencio, F., Curman, B., Jarmerot, G., Wadstrom, T. and Ljungh, A. (1992). Surface properties, connective tissue protein binding and shiga like toxin production of *Escherichia coli* isolated from patients with ulcerative colitis. *Zentralblatt für Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **276**(2): 254-263.
- Opletamadsen, K., Meddings, J. B. and Gall, D. G. (1991). Epidermal growth factor and postnatal development of intestinal transport and membrane structure. *Pediatric Research* **30**(4): 342-350.
- Osterlundh, I., Holst, H. and Magnusson, U. (2001). Effect of mammary secretions on functions of polymorphonuclear leukocytes in pigs. *American Journal of Veterinary Research* **62**(8): 1250-1254.
- O'Sullivan, D. J. (2001). Screening of intestinal microflora for effective probiotic bacteria. *Journal of Agricultural and Food Chemistry* **49**(4): 1751-1760.
- Ouwehand, A. C. and Conway, P. L. (1996). Specificity of spent culture fluids of *Lactobacillus* spp. to inhibit adhesion of enteropathogenic fimbriated *Escherichia coli* cells. *Microbial Ecology in Health and Disease* **9**(6): 239-246.
- Ouwehand, A. C., Kirjavainen, P., Isolauri, E. and Salminen, S. (2001). Adhesion of probiotic strains to human intestinal mucus. *American Journal of Clinical Nutrition* **73**(2): 490S-490S.
- Ouwehand, A. C., Lagstrom, H., Suomalainen, T. and Salminen, S. (2002). Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Annals of Nutrition and Metabolism* **46**(3-4): 159-162.
- Park, J. H., Um, J. I., Lee, B. J., Goh, J. S., Park, S. Y., Kim, W. S. and Kim, P. H. (2002a). Encapsulated *Bifidobacterium bifidum* potentiates intestinal IgA production. *Cellular Immunology* **219**(1): 22-27.
- Park, Y. S., Lee, J. Y., Kim, Y. S. and Shin, D. H. (2002b). Isolation and characterization of lactic acid bacteria from feces of newborn baby and from dongchimi. *Journal of Agricultural and Food Chemistry* **50**(9): 2531-2536.
- Partridge, G. and Gill, B. P. (1993). New approaches with pig weaner diets. In: Garnsworthy, P. C. and Cole, D. J. A. *Recent advances in animal nutrition - 1993*. Nottingham, Nottingham University Press. 13: 221-248.
- Pascual, M., Hugas, M., Badiola, J. I., Monfort, J. M. and Garriga, M. (1999). *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. *Applied and Environmental Microbiology* **65**(11): 4981-4986.
- Patti, J. M. and Höök, M. (1994). Microbial adhesins recognizing extracellular matrix macromolecules. *Current Opinion in Cell Biology* **6**(5): 752-758.
- Paubert-Braquet, M., Gan, X. H., Gaudichon, C., Hedef, N., Serikoff, A., Bouley, C., Bonavida, B. and Braquet, P. (1995). Enhancement of host resistance against *Salmonella typhimurium* in mice fed a diet supplemented with yogurt or milks fermented with various *Lactobacillus casei* strains. *International Journal of Immunotherapy* **11**(4): 153-161.

- Paul, D. and Hoskins, L. C. (1972). Effect of oral *Lactobacillus* feedings on fecal *Lactobacillus* counts. *American Journal of Clinical Nutrition* **25**: 763-765.
- Pedersen, K., Christensen, G. W., Steffensen, M., Schyum, P. and Johansen, A. K. (1992). Transfer of lactic acid bacterial strains from the feed to the sow, the environment, and the piglets. *Acta Veterinaria Scandinavica* **33**(4): 297-303.
- Pedersen, K. and Jørgensen, M. (1992). Lactic acid bacteria for mink. *Acta Veterinaria Scandinavica* **33**: 95-103.
- Pedersen, K. and Tannock, G. W. (1989). Colonization of the porcine gastrointestinal tract by lactobacilli. *Applied and Environmental Microbiology* **55**(2): 279-283.
- Penco, S., Pastorino, S., Bianchiscarra, G. and Garre, C. (1995). Lactoferrin down-modulates the activity of the granulocyte- macrophage colony stimulating factor promoter in interleukin-1 β -stimulated cells. *Journal of Biological Chemistry* **270**(20): 12263-12268.
- Percival, M. (1997). Choosing a probiotic supplement. *Clinical Nutrition Insights* **6**(1): 1-4.
- Perdigon, G., Alvarez, S., Demacias, M. E. N., Roux, M. E. and Holgado, A. P. D. (1990a). The oral administration of lactic acid bacteria increase the mucosal intestinal immunity in response to enteropathogens. *Journal of Food Protection* **53**(5): 404-410.
- Perdigon, G., Alvarez, S. and Holgado, A. P. D. (1991). Immunoadjuvant activity of oral *Lactobacillus casei* - influence of dose on the secretory immune response and protective capacity in intestinal infections. *Journal of Dairy Research* **58**(4): 485-496.
- Perdigon, G., Alvarez, S., Medina, M., Vintini, E. and Roux, E. (1999). Influence of the oral administration of lactic acid bacteria on IgA producing cells associated to bronchus. *International Journal of Immunopathology and Pharmacology* **12**(2): 97-102.
- Perdigon, G., Demacias, M. E. N., Alvarez, S., Medici, M., Oliver, G. and Holgado, A. A. P. D. (1986a). Effect of a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* administered orally on the immune system in mice. *Journal of Food Protection* **49**(12): 986
- Perdigon, G., Demacias, M. E. N., Alvarez, S., Oliver, G. and Holgado, A. A. P. D. (1986b). Effect of perorally administered lactobacilli on macrophage activation in mice. *Infection and Immunity* **53**(2): 404-410.
- Perdigon, G., Demacias, M. E. N., Alvarez, S., Oliver, G. and Holgado, A. A. P. D. (1990b). Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. *Journal of Dairy Research* **57**(2): 255-264.
- Perdigon, G., Medici, M., Dejorrat, M. E. B. B., Debudeguer, M. V. and Deruiz, A. H. P. (1993). Immunomodulating effects of lactic acid bacteria on mucosal and tumoral immunity. *International Journal of Immunotherapy* **9**(1): 29-52.
- Perdigon, G., Medina, M., Vintini, E. and Valdez, J. C. (2000). Intestinal pathway of internalisation of lactic acid bacteria and gut mucosal immunostimulation. *International Journal of Immunopathology and Pharmacology* **13**(3): 141-150.
- Perdue, M. H. (1999). Mucosal immunity and inflammation III. The mucosal antigen barrier: cross talk with mucosal cytokines. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* **40**: 277: G1-G5.

- Perrin, D. R. (1955). The chemical composition of the colostrum and milk of the sow. *Journal of Dairy Research* **22**: 103 - 107.
- Petschow, B. W., Carter, D. L. and Hutton, G. D. (1993). Influence of orally-administered epidermal growth factor on normal and damaged intestinal mucosa in rats. *Journal of Pediatric Gastroenterology and Nutrition* **17**(1): 49-58.
- Pinto, M., Robineleon, S., Appay, M. D., Keding, M., Triadou, N., Dussaulx, E., Lacroix, B., Simonassmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology of the Cell* **47**(3): 323-330.
- Pluske, J. R., Williams, I. H., Zak, L. J., Clowes, E. J., Cegielski, A. C. and Aherne, F. X. (1998). Feeding lactating primiparous sows to establish three divergent metabolic states: III. Milk production and pig growth. *Journal of Animal Science* **76**(4): 1165-1171.
- Podolsky, D. K. (1999). Mucosal immunity and inflammation V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* **40**: 277: G495-G499.
- Pond, W. G. and Houpt, K. A. (1978). *The Biology of the Pig*. London, Cornell University Press Ltd.
- Porter, P. (1969). Transfer of immunoglobulins IgG, IgA and IgM to lacteal secretions in the parturient sow and their absorption by the neonatal piglet. *Biochimica et biophysica acta* **181**: 381-392.
- Porter, P. (1973). Studies of porcine secretory IgA and its component chains in relation to intestinal absorption of colostral immunoglobulins by the neonatal pig. *Immunology* **24**: 163-176.
- Porter, P. (1975). The immune system. In: Dunne, H. W. and Leman, A. D. *Diseases of swine*. Ames, The Iowa State University Press: 122-138.
- Portier, A., Boyaka, N. P., Bougoudogo, F., Dubarry, M., Huneau, J. F., Tome, D., Dodin, A. and Coste, M. (1993). Fermented milks and increased antibody responses against cholera in mice. *International Journal of Immunotherapy* **9**(4): 217-224.
- Posse, E., Vinals, A. L., Dearcuri, B. F., Farias, R. N. and Morero, R. D. (1990). Lysozyme induced fusion of negatively charged phospholipid vesicles. *Biochimica et Biophysica Acta* **1024**(2): 390-394.
- Preissner, K. T. and Chhatwal, G. S. (2000). Extracellular matrix and host cell surfaces: potential sites of pathogen interaction. In: Cossart, P., Boquet, P., Normark, S. and Rappuoli, R. *Cellular Microbiology*. Washington, D.C., ASM Press: 49-66.
- Prohászka, L. and Baron, F. (1982). Antibacterial effect of volatile fatty acids on enterobacteriaceae in the large intestine. *Acta Veterinaria Academiae Scientiarum Hungaricae* **30**: 9-16.
- Prohászka, L., Jayarao, B. M., Fabian, A. and Kovacs, S. (1990). The role of intestinal volatile fatty acids in the *Salmonella* shedding of pigs. *Journal of Veterinary Medicine Series B-Zentralblatt für Veterinärmedizin Reihe B-Infectious Diseases and Veterinary Public Health* **37**(8): 570-574.

- Prohászka, L. and Lukács, K. (1984). Influence of the diet on the antibacterial effect of volatile fatty acids and on the development of swine dysentery. *Zentralblatt für Veterinärmedizin B* **31**: 779-785.
- Pryde, S. E., Richardson, A. J., Stewart, C. S. and Flint, H. J. (1999). Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. *Applied and Environmental Microbiology* **65**(12): 5372-5377.
- Quellette, A. J. (1997). Paneth cells and innate immunity in the crypt microenvironment. *Gastroenterology* **113**: 1779-1784.
- Quere, F., Deschamps, A. and Urdaci, M. C. (1997). DNA probe and PCR-specific reaction for *Lactobacillus plantarum*. *Journal of Applied Microbiology* **82**(6): 783-790.
- Quinn, P. J. (1990). Mechanisms of action of some immunomodulators used in veterinary medicine. In: Blecha, F. and Charley, B. *Advances in Veterinary Science and Comparative Medicine: Immunomodulation in domestic food animals*. San Diego, California, Academic Press, Inc. **35**: 43-99.
- Rada, V. a. R., I. (1995). The effect of *Lactobacillus salivarius* administration on coliforms and enterococci in the crop and caeca of chicken broilers. *Veterinary Medicine-Czech* **40**(10): 311-315.
- Rechler, M. M. and Nissley, S. P. (1985). The nature and regulation of the receptors for insulin-like growth factors. *Annual Review of Physiology* **47**: 425-442.
- Reeds, P. J., Burrin, D. G., Davis, T. A., Fiorotto, M. L., Stoll, B. and van Goudoever, J. B. (2000). Protein nutrition of the neonate. *Proceedings of the Nutrition Society* **59**(1): 87-97.
- Reid, G., Howard, J. and Gan, B. S. (2001). Can bacterial interference prevent infection? *Trends in Microbiology* **9**(9): 424-428.
- Reid, G., McGroarty, J., Angotti, R. and Cook, R. (1988). *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Canadian Journal of Microbiology* **34**: 344-351.
- Reiss, K., Valentini, B., Tu, X., Xu, S. Q. and Baserga, R. (1998). Molecular markers of IGF-I-mediated mitogenesis. *Experimental Cell Research* **242**(1): 361-372.
- Reniero, R., Cocconcelli, P., Bottazzi, V. and Morelli, L. (1992). High frequency of conjugation in *Lactobacillus* mediated by an aggregation promoting factor. *Journal of General Microbiology* **138**: 763-768.
- Rice, D. W., Soderlund, S. D., Phillip, I. E. and Harrison, J. H. (1990). Effect of microbial inoculation on digestibility of legume silages. *Journal of Dairy Science* **73** (Suppl. 1): 195.
- Rickard, A. H., Gilbert, P., High, N. J., Kolenbrander, P. E. and Handley, P. S. (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology* **11**(2): 94-100.
- Riedel-Caspari, G. (2001). The antimicrobial and immunomodulating actions of milk leukocytes. *Advances in Nutritional Research*, **10**: 287-307.

- Rinehart, J. J., Cerilli, J. G., Jacob, H. S. and Osserman, E. F. (1982). Lysozyme stimulates lymphocyte proliferation in monocyte depleted mixed lymphocyte cultures. *Journal of Laboratory and Clinical Medicine* **99**(3): 370-381.
- Roberts, A. B. (1998). Molecular and cell biology of TGF-beta. *Mineral and Electrolyte Metabolism* **24**(2-3): 111-119.
- Roe, J. M., Patel, D. and Morgan, K. L. (1993). Isolation of porcine IgE, and preparation of polyclonal antisera. *Veterinary Immunology and Immunopathology* **37**(2): 83-97.
- Rojas, M., Ascencio, F. and Conway, P. L. (2002). Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin. *Applied and Environmental Microbiology* **68**(5): 2330-2336.
- Rojas, M. and Conway, P. L. (1996). Colonization by lactobacilli of piglet small intestinal mucus. *Journal of Applied Bacteriology* **81**(5): 474-480.
- Rolfe, R. D. (1997). Colonization resistance. In: Mackie, R. I., White, B. A. and Isaacson, R. E. *Gastrointestinal Microbiology* London, UK, Chapman & Hall. **2**: 501-536.
- Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition* **130**: 396S-402S.
- Romond, M. B., Ais, A., Yazourh, A. and Romond, C. (1997). Cell-free wheys from bifidobacteria fermented milks exert a regulatory effect on the intestinal microflora of mice and humans. *Anaerobe* **3**(2-3): 137-143.
- Rooke, J. A. and Bland, I. M. (2002). The acquisition of passive immunity in the new-born piglet. *Livestock Production Science* **78**(1): 13-23.
- Roos, S. and Jonsson, H. (2002). A high-molecular mass cell surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology-Sgm* **148**: 433-442.
- Roos, S., Karner, F., Axelsson, L. and Jonsson, H. (2000). *Lactobacillus mucosae* - a new species with *in vitro* mucus-binding activity isolated from pig intestine. *International Journal of Systematic and Evolutionary Microbiology* **50**: 251-258.
- Rostand, K. S. and Esko, J. D. (1997). Microbial adherence to and invasion through proteoglycans. *Infection and Immunity* **65**(1): 1-8.
- Roth, F. X. and Kirchgessner, M. (1977). N-utilization and N-evaluation of increasing amounts of bacterial and soya protein fed to growing pigs. *Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde* **39**(3): 156-170.
- Roth, F. X. and Kirchgessner, M. (1998). Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. *Journal of Animal and Feed Sciences* **7**: 25-33.
- Roth, F. X., Kirchgessner, M. and Eidelsburger, U. (1993). Nutritive efficiency of lactic acid in the rearing of piglets. *Agribiological Research* **46**(3): 229-239.
- Rubin, H. E., Nerad, T. and Vaughan, F. (1982). Lactate acid inhibition of *Salmonella typhimurium* in yogurt. *Journal of Dairy Science* **65**(2): 197-203.
- Rudloff, H. E., Schmalstieg, F. C., Mushtaha, A. A., Palkowetz, K. H., Liu, S. K. and Goldman, A. S. (1992). Tumor necrosis factor-alpha in human milk. *Pediatric Research* **31**(1): 29-33.

- Rudloff, H. E., Schmalstieg, F. C., Palkowetz, K. H., Paszkiewicz, E. J. and Goldman, A. S. (1993). Interleukin-6 in human milk. *Journal of Reproductive Immunology* **23**(1): 13-20.
- Russell, P. J., Geary, T. M., Brooks, P. H. and Campbell, A. (1996). Performance, water use and effluent output of weaner pigs fed *ad libitum* with either dry pellets or liquid feed and the role of activity in the liquid feed. *Journal of Science of Food and Agriculture* **72**: 8-16.
- Rychen, G. and Nunes, C. S. (1995). Effects of 3 microbial probiotics on postprandial porto-arterial concentration differences of glucose, galactose and amino-nitrogen in the young-pig. *British Journal of Nutrition* **74**(1): 19-26.
- Sainz, B., Loutsch, J. M., Marquart, M. E. and Hill, J. M. (2001). Stress-associated immunomodulation and *Herpes simplex* virus infections. *Medical Hypotheses* **56**(3): 348-356.
- Salminen, S., Bouley, C., Boutron-Ruault, M.-C., Cummings, J. H., Franck, A., Gibson, G. R., Isolauri, E., Moreau, M.-C., Roberfroid, M. and Rowland, I. (1998). Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* **80**: S147-S171.
- Sanbrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbour, NY, Cold Spring Harbour Laboratory Press.
- Sanderson, I. R. and Walker, W. A. (1999). Mucosal barrier: An overview. In: Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., Bienenstock, J. and McGhee, J. R. *Mucosal immunology*. London, Academic Press: 5-18.
- Sangild, P. T. (2001). Transitions in the life of the gut at birth. In: Lindberg, J. E. and Ogle, B. *Digestive physiology of pigs*. Wallingford, Oxon, CABI Publishing: 3-17.
- Sangild, P. T., Fowden, A. L. and Trahair, J. F. (2000). How does the foetal gastrointestinal tract develop in preparation for enteral nutrition after birth? *Livestock Production Science* **66**(2): 141-150.
- Sansom, B. F. and Gleed, P. T. (1981). The ingestion of sow's faeces by suckling piglets. *British Journal of Nutrition* **46**: 451 - 456.
- Sansonetti, P. J. and Phalipon, A. (1999). M cells as ports of entry for enteroinvasive pathogens: Mechanisms of interaction, consequences for the disease process. *Seminars in Immunology* **11**(3): 193-203.
- Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* **21**(1): 55-89.
- Saucier, L., Julien, M., Cheour, F., Letarte, R. and Goulet, J. (1992). Effect of feeding lactic acid bacteria and fermented milk on specific and nonspecific immune responses of mice infected with *Klebsiella pneumoniae* Ad-1. *Journal of Food Protection* **55**(8): 595-600.
- SCANN (2000). Report of the Scientific Committee on Animal Nutrition on the assessment under directive 87 / 153 / EEC of the efficacy of micro-organisms used as feed additives. Brussels, European Commission: 9.
- Schiffrin, E. J., Brassart, D., Servin, A. L., Rochat, F. and Donnet-Hughes, A. (1997). Immune modulation of blood leukocytes in humans by lactic acid bacteria: Criteria for strain selection. *American Journal of Clinical Nutrition* **66**(2): S515-S520.

- Schiffrin, E. J., Rochat, F., Linkamster, H., Aeschlimann, J. M. and Donnethughes, A. (1995). Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *Journal of Dairy Science* **78**(3): 491-497.
- Scholten, R. H. J., Peet-Schwering, C. M. C. v. d., Verstegen, M. W. A., Hartog, L. A. d., Schrama, J. W. and Vesseur, P. C. (1999). Fermented co-products and fermented compound diets for pigs: a review. *Animal Feed Science and Technology* **82**: 1-19.
- Scholten, R. H. J., Peet-Schwering, V. D., den Hartog, L. A., Vesseur, P. C. and Verstegen, M. W. A. (1998). *Effect of liquid by-products on performance and health of pigs*. 49th Annual Meeting of the European Association for Animal Production, Warsaw, Poland.
- Schulze, F. and Müller, G. (1980). Lysozyme in sow's milk and its importance to bacterial population of the gastrointestinal tract in suckling piglets. *Archiv für Experimentelle Veterinarmedizin* **34**: 317-324.
- Seydel, K. B., Li, E., Swanson, P. E. and Stanley, S. L. (1997). Human intestinal epithelial cells produce proinflammatory cytokines in response to infection in a SCID mouse-human intestinal xenograft model of amebiasis. *Infection and Immunity* **65**(5): 1631-1639.
- Shanahan, F. (2000). Nutrient tasting and signaling mechanisms in the gut V. Mechanisms of immunologic sensation of intestinal contents. *American Journal of Physiology (Gastrointestinal and Liver Physiology)* **278**: G191-G196.
- Shen, W. H. and Xu, R. J. (1998). Stability and distribution of orally administered epidermal growth factor in neonatal pigs. *Life Sciences* **63**(10): 809-820.
- Shen, W. H. and Xu, R. J. (2000a). Gastrointestinal stability and absorption of insulin in suckling pigs. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology* **125**(3): 389-401.
- Shen, W. H. and Xu, R. J. (2000b). Stability of insulin-like growth factor I in the gastrointestinal lumen in neonatal pigs. *Journal of Pediatric Gastroenterology and Nutrition* **30**(3): 299-304.
- Sheridan, J. F., Dobbs, C., Brown, D. and Zwilling, B. (1994). Psychoneuroimmunology: stress effects on pathogenesis and immunity during infection. *Clinical Microbiology Review* **7**(2): 200-212.
- Shortt, C. (1999). The probiotic century: historical and current perspectives. *Trends in Food Science & Technology* **10**(12): 411-417.
- Shu, Q. and Gill, H. S. (2001). A dietary probiotic (*Bifidobacterium lactis* HN019) reduces the severity of *Escherichia coli* O157:H7 infection in mice. *Medical Microbiology and Immunology* **189**(3): 147-152.
- Shu, Q., Qu, F. and Gill, H. S. (2001). Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model. *Journal of Pediatric Gastroenterology and Nutrition* **33**(2): 171-177.
- Simmen, F. A., Cera, K. R. and Mahan, D. C. (1990). Stimulation by colostrum or mature milk of gastrointestinal tissue development in newborn pigs. *Journal of Animal Science* **68**: 3596-3603.
- Simmons, C. P., Clare, S. and Dougan, G. (2001). Understanding mucosal responsiveness: lessons from enteric bacterial pathogens. *Seminars in Immunology* **13**(3): 201-209.

- Simon, O., Vahjen, W. and Scharek, L. (2003). Micro-organisms as feed additives-probiotics. In: Ball, R. O. *Proceedings of the 9th international symposium on digestive physiology in pigs*. Banff, AB, Canada, University of Alberta. 1: 295-318.
- Simpson, J. M., McCracken, V. J., Gaskins, H. R. and Mackie, R. I. (2000). Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Applied and Environmental Microbiology* 66(11): 4705-4714.
- Sirotkin, A. V., Dukesova, J., Makarevich, A. V., Kubek, A. and Bulla, J. (2000). Evidence that growth factors IGF-I, IGF-II and EGF can stimulate nuclear maturation of porcine oocytes via intracellular protein kinase A. *Reproduction Nutrition Development* 40(6): 559-569.
- Smith, H. W. and Crab, W. E. (1961). The faecal bacterial flora of animals and man: its development in the young. *Journal of Pathological Bacteriology* 82: 53-66.
- Smith, P. (1976). A comparison of dry, wet and soaked meal for fattening bacon pigs. *Experimental Husbandry* 30: 87-94.
- Soderholm, J. D. and Perdue, M. H. (2001). Stress and the gastrointestinal tract II. Stress and intestinal barrier function. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 280(1): G7-G13.
- Sompayrac, L. (1999). *How the immune system works*, Blackwell Science, Inc.
- Spanhaak, S., Havenaar, R. and Schaafsma, G. (1998). The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *European Journal of Clinical Nutrition* 52(12): 899-907.
- Speer, V. C. (1990). Partitioning nitrogen and amino-acids for pregnancy and lactation in swine - a review. *Journal of Animal Science* 68(2): 553-561.
- Spencer, R. J. and Chesson, A. (1994). The effect of *Lactobacillus* spp. on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes. *Journal of Applied Bacteriology* 77(2): 215-220.
- Spinka, M., Illmann, G., Algers, B. and Stetkova, Z. (1997). The role of nursing frequency in milk production in domestic pigs. *Journal of Animal Science* 75(5): 1223-1228.
- Stavric, S., Gleeson, T. M., Blanchfield, B. and Pivnick, H. (1987). Role of adhering microflora in competitive exclusion of from young chicks. *Journal of Food Protection* 50(11): 928-932.
- Stephens, S., Brenner, M. K., Duffy, S. W., Lakhani, P. K., Kennedy, C. R. and Farrant, J. (1986). The effect of breast feeding on proliferation by infant lymphocytes *in vitro*. *Pediatric Research* 20(3): 227-231.
- Stephens, S., Dolby, J. M., Montreuil, J. and Spik, G. (1980). Differences in inhibition of the growth of comensal and enteropathogenic strains of *Escherichia coli* by lactotransferrin and secretory immunoglobulin A isolated from human milk. *Immunology* 41: 597-603.
- Štyriak, I., Demečková, V., Žatkovič, B. and Kmeť, V. (2001). Collagen binding by vaginal aggregative lactobacilli. *Veterinary Medicine-Czech* 46: 89-94.
- Štyriak, I., Demečková, V. and Nemcová, R. (1999a). Collagen (Cn-I) binding by gut lactobacilli. *Berliner und Münchener Tierärztliche Wochenschrift* 112(8): 301-304.

- Štyriak, I., Lauková, A., Fallgren, C. and Wadstrom, T. (1999b). Binding of extracellular matrix proteins by animal strains of staphylococcal species. *Veterinary Microbiology* **67**(2): 99-112.
- Sui, J., Leighton, S., Busta, F. and Brady, L. (2002). 16S ribosomal DNA analysis of the faecal lactobacilli composition of human subjects consuming a probiotic strain *Lactobacillus acidophilus* NCFM (R). *Journal of Applied Microbiology* **93**(5): 907-912.
- Svendsen, J., Bengtsson, A. C. and Svendsen, L. S. (1986). Occurrence and causes of traumatic injuries in neonatal pigs. *Pig News and Information* **7**: 159-170.
- Takahashi, M., Iwata, S., Yamazaki, N. and Fujiwara, H. (1991). Phagocytosis of the lactic acid bacteria by M cells in the rabbit Peyer's patches. *Journal of Clinical Electron Microscopy* **24**: 5-6.
- Takahata, Y., Takada, H., Nomura, A., Ohshima, K., Nakayama, H., Tsuda, T., Nakano, H. and Hara, T. (2001). Interleukin-18 in human milk. *Pediatric Research* **50**(2): 268-272.
- Tannock, G. W. (1997). Modification of the normal microbiota by diet, stress, antimicrobial agents and probiotics. In: Mackie, R. I., White, B. A. and Isaacson, R. E. *Gastrointestinal microbiology*. New York, Chapman and Hall **2**: 434-465.
- Tannock, G. W. (1992). The lactic microflora of pigs, mice and rats. In: Wood, B. J. B. *The lactic acid bacteria*. London, Elsevier Applied Science. 1. The lactic acid bacteria in health and disease: 21 - 48.
- Tannock, G. W. (2001). Molecular assessment of intestinal microflora. *American Journal of Clinical Nutrition* **73**: 410S-414S.
- Tannock, G. W., Fuller, R. and Pedersen, K. (1990). *Lactobacillus* succession in the piglet digestive tract demonstrated by plasmid profiling. *Applied and Environmental Microbiology* **56**(5): 1310-1316.
- Taormina, P. J. and Beuchat, L. R. (1999). Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. *Journal of Food Protection* **62**(4): 318-324.
- Targowski, S. P. and Niemialtowski, M. (1986). Inhibition of lacteal leukocyte phagocytosis by colostrum, nonlactating secretion, and mastitic milk. *American Journal of Veterinary Research* **47**(9): 1940-1945.
- Taylor, B. C., Keefe, R. G., Dellinger, J. D., Nakamura, Y., Cullor, J. S. and Stott, J. L. (1997). T cell populations and cytokine expression in milk derived from normal and bacteria-infected bovine mammary glands. *Cellular Immunology* **182**(1): 68-76.
- Tejada-Simon, M. V., Lee, J. H., Ustunol, Z. and Pestka, J. J. (1999a). Ingestion of yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium* to potentiate immunoglobulin A responses to cholera toxin in mice. *Journal of Dairy Science* **82**(4): 649-660.
- Tejada-Simon, M. V., Ustunol, Z. and Pestka, J. J. (1999b). *Ex vivo* effects of lactobacilli, streptococci, and bifidobacteria ingestion on cytokine and nitric oxide production in a murine model. *Journal of Food Protection* **62**(2): 162-169.
- Thim, L. (1997). Trefoil peptides: from structure to function. *Cellular and Molecular Life Sciences* **53**(11-12): 888-903.

- Thompson, J. L. and Hinton, M. (1996). Effect of short-chain fatty acids on the size of enteric bacteria. *Letters in Applied Microbiology* 22(6): 408-412.
- Thompson, J. L. and Hinton, M. (1997). Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. *British Poultry Science* 38(1): 59-65.
- Tielen, M. J. M., van Schie, F. W., van der Wolf, P. J., Elbers, A. R. W., Koppens, J. M. C. C. and Wolbers, W. B. (1997). Risk factors and control measures for subclinical *Salmonella* infection in pig herds. In: Bech-Nielsen, S. and Nielsen, J. P. *Proceedings of the second international symposium on epidemiology and control of Salmonella in pork. Copenhagen, Denmark, August 20-22*. Copenhagen, Federation of Danish Pig Producers and Slaughterhouses: 32-35.
- Tizard, I. (2001). The protective properties of milk and colostrum in non-human species. In: Woodward, B. and Draper, H. H. *Advances in Nutritional Research*. New York, Kluwer Academic / Plenum. 10: 139-167.
- Toba, T., Virkola, R., Westerlund, B., Bjorkman, Y., Sillanpaa, J., Vartio, T., Kalkkinen, N. and Korhonen, T. K. (1995). A collagen binding S-layer protein in *Lactobacillus crispatus*. *Applied and Environmental Microbiology* 61(7): 2467-2471.
- Toner, M. S., King, R. H., Dunshea, F. R., Dove, H. and Atwood, C. S. (1996). The effect of exogenous somatotropin on lactation performance of first-litter sows. *Journal of Animal Science* 74(1): 167-172.
- Torriani, S., Felis, G. E. and Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Applied and Environmental Microbiology* 67(8): 3450-3454.
- Trottier, N. L. and Johnston, L. J. (2001). Feeding gilts during development and sows during gestation and lactation. In: Lewis, A. J. and Southern, L. L. *Swine nutrition*. New York, CRC Press: 725-771.
- Tuboly, S., Bernath, S., Glavits, R. and Medveczky, I. (1988). Intestinal absorption of colostral lymphoid cells in newborn piglets. *Veterinary Immunology and Immunopathology* 20(1): 75-85.
- Tuchscherer, M., Kanitz, E., Otten, W. and Tuchscherer, A. (2002). Effects of prenatal stress on cellular and humoral immune responses in neonatal pigs. *Veterinary Immunology and Immunopathology* 86(3-4): 195-203.
- Tuomola, E. M., Ouwehand, A. C. and Salminen, S. J. (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunology and Medical Microbiology* 26(2): 137-142.
- Urlings, H. A. P., Bijker, P. G. H. and van Logtestijn, J. G. (1993). Fermentation of raw poultry byproducts for animal nutrition. *Journal of Animal Science* 71: 2420-2426.
- Vaerman, J. P. (1984). IgA effector mechanisms. *Annales De Biologie Clinique* 42(1): 61-70.
- van der Strate, B. W. A., Beljaars, L., Molema, G., Harmsen, M. C. and Meijer, D. K. F. (2001). Antiviral activities of lactoferrin. *Antiviral Research* 52(3): 225-239.
- van der Waaij, L. A., Limburg, P. C., Mesander, G. and vanderWaaij, D. (1996). *In vivo* IgA coating of anaerobic bacteria in human faeces. *Gut* 38(3): 348-354.

- van der Wolf, P. J., Bongers, J. H., Elbers, A. R. W., Franssen, F. M. M. C., Hunneman, W. A., van Exsel, A. C. A. and Tielen, M. J. M. (1999). *Salmonella* infections in finishing pigs in the Netherlands: bacteriological herd prevalence, serogroup and antibiotic resistance of isolates and risk factors for infection. *Veterinary Microbiology* **67**(4): 263-275.
- van Hooijdonk, A. C. M., Kussendrager, K. D. and Steijns, J. M. (2000). *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *British Journal of Nutrition* **84**(Suppl. 1): S127-S134.
- van Winsen, R. L., Keuzenkamp, D., Urlings, B. A. P., Lipman, L. J. A., Snijders, J. A. M., Verheijden, J. H. M. and van Knapen, F. (2002). Effect of fermented feed on shedding of *Enterobacteriaceae* by fattening pigs. *Veterinary Microbiology* **87**(3): 267-276.
- van Winsen, R. L., Lipman, L. J. A., Biesterveld, S., Urlings, B. A. P., Snijders, J. M. A. and van Knapen, F. (2001). Mechanism of *Salmonella* reduction in fermented pig feed. *Journal of the Science of Food and Agriculture* **81**(3): 342-346.
- van Winsen, R. L., Urlings, B. A. P., Lipman, L. J. A., Snijders, J. M. A., Keuzenkamp, D., Verheijden, J. H. M. and van Knapen, F. (2001b). Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. *Applied and Environmental Microbiology* **67**(7): 3071-3076.
- Vandevoorde, L., Christiaens, H. and Verstraete, W. (1992). Prevalence of coaggregation reactions among chicken *Lactobacilli*. *Journal of Applied Bacteriology* **72**(3): 214-219.
- Vaughan, E. E., Schut, F., Heilig, H. G. H. J., Zoetendal, E. G., de Vos, W. M. and Akkermans, A. D. L. (2000). A molecular view of the intestinal ecosystem. *Current Issues in Intestinal Microbiology* **1**(1): 1-12.
- Vechetti, G. F., deArcuri, B. F., Posse, E., Arrondo, J. L. R. and Morero, R. D. (1997). Aggregation, fusion and aqueous content release from liposomes induced by lysozyme derivatives: effect on the lytic activity. *Molecular Membrane Biology* **14**(3): 137-142.
- Ventura, M., Jankovic, I., Walker, D. C., Pridmore, R. D. and Zink, R. (2002). Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Applied and Environmental Microbiology* **68**(12): 6172-6181.
- Verstegen, M. W. A., Verhagen, J. M. F. and Denhartog, L. A. (1987). Energy requirements of pigs during pregnancy - a review. *Livestock Production Science* **16**(1): 75-89.
- Vesely, R., Negri, R., Salvadori, B. B., Lavezzari, D. and Desimone, C. (1985). Influence of a diet supplemented with yogurt on the mouse immune system. *Eos-Rivista Di Immunologia ed Immunofarmacologia* **5**(1): 30-35.
- Vitini, E., Alvarez, S., Medina, M., Medici, M., de Budeguer, M. V. and Perdigon, G. (2000). Gut mucosal immunostimulation by lactic acid bacteria. *Biocell* **24**(3): 223-232.
- von der Weid, T., Bulliard, C. and Schiffrin, E. J. (2001). Induction by a lactic acid bacterium of a population of CD4⁺ T cells with low proliferative capacity that produce transforming growth factor beta and interleukin-10. *Clinical and Diagnostic Laboratory Immunology* **8**(4): 695-701.
- von Moll, L. K. and Cantey, J. R. (1997). Peyer's patch adherence of enteropathogenic *Escherichia coli* strains in rabbits. *Infection and Immunity* **65**(9): 3788-3793.

- Wadström, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S. and Gullmar, B. (1987). Surface properties of lactobacilli isolated from the small intestine of pigs. *Journal of Applied Bacteriology* **62**(6): 513-520.
- Wagner, R. D., Holland, M. and Cerniglia, C. E. (2002). An *in vitro* assay to evaluate competitive exclusion products for poultry. *Journal of Food Protection* **65**(5): 746-751.
- Wagstrom, E. A., Yoon, K. J. and Zimmerman, J. J. (2000). Immune components in porcine mammary secretion. *Viral Immunology* **13**(3): 383-397.
- Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K. and Alatossava, T. (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Applied and Environmental Microbiology* **66**(1): 297-303.
- Walton, J. (1991). Poor production in growing pigs. In: Boden, E. *Swine practise*. London, UK, Baillière Tindall: 13-28.
- Wang, T. and Xu, R. J. (1996). Effects of colostrum feeding on intestinal development in newborn pigs. *Biology of the Neonate* **70**(6): 339-348.
- Ward, P. P., Uribe-Luna, S. and Conneely, O. M. (2002). Lactoferrin and host defense. *Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire* **80**(1): 95-102.
- Warren, J. S., Rinehart, J. J., Zwilling, B. S. and Neidhart, J. A. (1981). Lysozyme enhancement of tumor - cell immunoprotection in a murine fibro-sarcoma. *Cancer Research* **41**(5): 1642-1645.
- Watson, W. A. (1980). Veterinary and legislative aspects relating to the feeding of by-products and waste. In: Orskov, E. R. *By-products and wastes in animal feeding: Occasional publication No 3*. Edinburgh, British Society of Animal Production. **3**: 19-24.
- Wei, C. I., Huang, T. S., Kim, J. M., Lin, W. F., Tamplin, M. L. and Bartz, J. A. (1995). Growth and survival of *Salmonella montevideo* on tomatoes and disinfection with chlorinated water. *Journal of Food Protection* **58**(8): 829-836.
- Weldon, W. C., Thulin, A. J., Macdougald, O. A., Johnston, L. J., Miller, E. R. and Tucker, H. A. (1991). Effects of increased dietary energy and protein during late gestation on mammary development in gilts. *Journal of Animal Science* **69**(1): 194-200.
- Westerlund, B. and Korhonen, T. K. (1993). Bacterial proteins binding to the mammalian extracellular matrix. *Molecular Microbiology* **9**(4): 687-694.
- Williams, P. P. (1993). Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* **57**(1): 1-8.
- Wilson, K. H. and Blitchington, R. B. (1996). Human colonic biota studied by ribosomal DNA sequence analysis. *Applied and Environmental Microbiology* **62**(7): 2273-2278.
- Winniczuk, P. P. and Parish, M. E. (1997). Minimum inhibitory concentrations of antimicrobials against micro-organisms related to citrus juice. *Food Microbiology* **14**(4): 373-381.

- Wiseman, J., Pickard, J. and Zarkadas, L. (2001). Starch digestion in piglets. In: Varley, M. A. and Wiseman, J. *The weaner pig. Nutrition and management*. Wallingford, UK, CABI Publishing: 65-80.
- Wisniewsky, M. A., Glatz, B. A., Gleason, M. L. and Reitmeier, C. A. (2000). Reduction of *Escherichia coli* O157:H7 counts on whole fresh apples by treatment with sanitizers. *Journal of Food Protection* **63**(6): 703-708.
- Wolinski, J., Switonska, M., Jakubowski, T., Babelowska, M., Nowak, K. W. and Zabielski, R. (2003). Leptin in sow colostrum and milk during the first 7 days of lactation. In: Ball, R. O. *Proceedings of the 9th International Symposium on the Digestive Physiology in Pigs*. Banff, AB, Canada, University of Alberta. **2**: 235-237.
- Wonnacott, K. M. and Bonneau, R. H. (2002). The effects of stress on memory cytotoxic T lymphocyte-mediated protection against *Herpes simplex* virus infection at mucosal sites. *Brain, Behaviour and Immunity* **16**: 104-117.
- Woolford, M. K. (1984). The microbiology of silage. In: Woolford, M. K. *The Silage Fermentation*. New York, Marcel Dekker, Inc.: 35-42.
- Wu, G. and Knabe, D. A. (1994). Free and protein bound amino acids in sow's colostrum and milk. *Journal of Nutrition* **124**: 415-424.
- Xanthopoulos, V., Hatzikamari, M., Adamidis, T., Tsakalidou, E., Tzanetakis, N. and Litopoulou-Tzanetaki, E. (2000). Heterogeneity of *Lactobacillus plantarum* isolates from Feta cheese throughout ripening. *Journal of Applied Microbiology* **88**(6): 1056-1064.
- Xu, R. J., Doan, Q. C. and Regester, G. O. (1999). Detection and characterisation of transforming growth factor- beta in porcine colostrum. *Biology of the Neonate* **75**(1): 59-64.
- Xu, R. J., Mellor, D. J., Birtles, M. J., Breier, B. H. and Gluckman, P. D. (1994). Effects of oral IGF-I or IGF-II on digestive organ growth in newborn piglets. *Biology of the Neonate* **66**: 280-287.
- Xu, R. J., Mellor, D. J., Birtles, M. J., Reynolds, G. W., Simpson, H. V., Breier, B. H. and Gluckman, P. D. (1996). Morphological changes in the oesophagus of newborn pigs: Effects of age, diet and oral insulin-like growth factor I (IGF-I) or IGF-II. *Reproduction Fertility and Development* **8**(5): 903-909.
- Xu, R. J., Wang, F. and Zhang, S. H. (2000). Postnatal adaptation of the gastrointestinal tract in neonatal pigs: a possible role of milk-borne growth factors. *Livestock Production Science* **66**(2): 95-107.
- Yamauchi, K., Tomita, M., Giehl, T. J. and Ellison, R. T. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infection and Immunity* **61**(2): 719-728.
- Yang, E. V. and Glaser, R. (2002). Stress-induced immunomodulation and the implications for health. *International Immunopharmacology* **2**(2-3): 315-324.
- Yang, T. S., Wu, S. C. and Wang, S. R. (2000). Serum and milk lactoferrin concentration and the correlation with some blood components in lactating sows. *Research in Veterinary Science* **69**: 95-97.

- Yasui, H., Kiyoshima, J. and Ushijima, H. (1995). Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed *Bifidobacterium breve* YIT 4064. *Journal of Infectious Diseases* 172(2): 403-409.
- Yasui, H. and Ohwaki, M. (1991). Enhancement of immune-response in Peyer patch cells cultured with *Bifidobacterium breve*. *Journal of Dairy Science* 74(4): 1187-1195.
- Yasui, H., Shida, K., Matsuzaki, T. and Yokokura, T. (1999). Immunomodulatory function of lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 76(1): 383-389.
- Yen, J. T. (2001a). Anatomy of the digestive system and nutritional physiology. In: Lewis, A. J. and Southern, L. L. *Swine nutrition*. CRC Press: 31-65.
- Yen, J. T. (2001b). Digestive system. In: Pond, W. G. and Mersmann, H. J. *Biology of the domestic pig*. Ithaca and London, Cornell University Press: 390-454.
- Youatt, W. (1847). *The Pig: A treatise on the breeds, management, feeding, and medical treatment, of swine*. London, Craddock and Co.
- Zabielski, R., Wolinski, J., Biernat, M., Westrom, B. and Guilloteau, P. (2003). The role of milk-borne leptin in gut development in neonatal pigs. In: Ball, R. O. *Proceedings of the 9th International symposium on Digestive Physiology in Pigs*. Banff, AB, Canada, University of Alberta. 2: 226-227.
- Zar, J. H. (1999). *Biostatistical analysis*. London, U.K., Prentice Hall (UK).
- Zhang, G. L., Wu, H., Shi, J. S., Ganz, T., Ross, C. R. and Blecha, F. (1998). Molecular cloning and tissue expression of porcine beta- defensin-1. *Febs Letters* 424(1-2): 37-40.
- Zhang, H., Malo, C. and Buddington, R. K. (1997). Suckling induces rapid intestinal growth and changes in brush border digestive functions of newborn pigs. *Journal of Nutrition* 127: 418-426.
- Zhou, J. S., Gopal, P. K. and Gill, H. S. (2001). Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*. *International Journal of Food Microbiology* 63(1-2): 81-90.
- Zijlstra, R. T., Odle, J., Hall, W. F., Petschow, B. W., Gelberg, H. B. and Litov, R. E. (1994). Effect of orally administered epidermal growth factor on intestinal recovery of neonatal pigs infected with rotavirus. *Journal of Pediatric Gastroenterology and Nutrition* 19(4): 382-390.
- Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D. L. and de Vos, W. M. (2002). Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and Environmental Microbiology* 68(7): 3401-3407.
- Zucker, B. A. and Muller, W. (2002). Investigations on airborne microorganisms in animal stables - 4. Report: Airborne gram-negative bacteria and airborne endotoxin in pig houses. *Berliner und Munchener Tierarztliche Wochenschrift* 115(1-2): 30-36.