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PROPHYLACTIC TREATMENT OF PIGLETS WITH LACTOBACILLUS STRAINS OF PORCINE ORIGIN

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SUMMARY

The studies presented here summarise our work directed at selecting a functional probiotic strain for oral administration to piglets. Historically the work was initiated in the mid eighties because Swedish authorities prohibited the use of low dose antibiotics in animal husbandry and alternative treatments were needed. From surveying the literature at that time, it was apparent that lactobacillus dosage could be useful, however, no single strain with conclusively proven results was reported. Our approach was to initially identify the major causative agent(s) responsible for economic losses in the pig industry. This was shown to be postweaning diarrhoea mediated by enterotoxigenic *Escherichia coli*, and especially those bearing K88 fimbriae. A strategy for *in vitro* selection was established for isolating strains of lactobacilli of host origin and which had the potential to reduce the susceptibility of the piglet to *E. coli* K88 induced diarrhoea. *In vitro* studies were directed both at identifying useful strains as well as to understand the mechanisms involved, thus allowing one to better predict the function of the strain *in vivo*. Field studies were then performed to evaluate the selected strains. It was concluded that the selection criteria used was satisfactory for isolating strains which could colonise the young piglet for extended periods, reduce the incidence of post-weaning diarrhoea and improve weight gain.

INTRODUCTION

The concept of orally administering lactic acid bacteria to piglets to improve health of the host was used as early as 1947 when *Møllgaard* (1947) dosed piglets with a lactic acid bacillus of host origin and reported improved health and improved skeletal formation. He proposed the phytic acid in ungerminated seeds in the diet interfered with absorption of calcium and phosphorus, an effect which he showed could be inhibited by lactic acid (*Møllgaard*, 1946), for example as produced by lactic acid bacteria.

The prophylactic use of lactic acid bacteria for piglets received little interest until the eighties when authorities and the consumers became concerned with the increasing need for the use of low dose antibiotics to facilitate good growth rates in intensive commercial units. Although numerous approaches for dealing with intestinal disturbances in piglets have been reported (reviewed

Table 1: Lactic acid bacteria utilised as probiotics for piglets (summarised from *Jonsson* and *Conway*, 1992).

Lactobacillus spp.	*Enterococcus faecalis*
L. acidophilus	*Enterococcus faecium*
L. lactis	
L. reuteri	*Bifidobacterium bifidus*
L. fermentum	*B. pseudolongum*
L. murinus	*B. thermophilus*

Mixed LABs prepared from the following:
L. plantarum, L. casei, L. fermentum, L. brevis, L. acidophilus, Enterococcus faecium, Streptococcus salivarius, L. delbrueckii

by *Jonsson* and *Conway*, 1992), there is increasing interest in the use of probiotics and in particular lactic acid bacteria for piglets. This is particularly evident from the number of reviews of the topic over the last decade e.g. (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982). As can be seen in Table 1, lactobacilli are the most commonly used lactic acid bacteria for probiotics for piglets, with *Lactobacillus acidophilus* being the most studied species. *Enterococcus faecium* has also been extensively used for piglets and a limited number of studies have reported using bifidobacteria and mixtures of several species of lactobacilli. The dose used is usually in the range of 10^9 - 10^{10} per animal per day and it is often included in the diet at a level of 10^6 - 10^7 per gram feed. A concentration of 10^9 corresponds to approximately 10 - 100 g of digesta for the adult pig and is comparable to the number of lactic acid bacteria in the stomach of the suckling pig. While the dose required for achieving demonstrable effects has been studied to a limited extent for humans and it has been suggested that at least 10^8 cells per day are required (*Gibson* and *Conway*, 1994; *Saxelin*, et al., 1991), the question has not been addressed for piglets.

The efficacy of oral administration of lactic acid bacteria to piglets is often studied in terms of the influence on (a) the stability of the digestive tract microbiota and pathogen invasion, (b) the function and morphology of the digestive tract and (c) performance and health of the pig. The most commonly studied parameter is the latter because of ease of measurement and the commercial interest in the matter. It is generally accepted today that some reports of the prophylactic use of lactic acid bacteria for piglets show no effects, while others report beneficial findings. In a few cases detrimental effects have been reported with decreased weight gain and lower feed conversion noted with dosage of an *L. reuteri* (*Ratcliffe*, et al., 1986) and in one case a higher mortality rate was noted using *Enterococcus faecium* strain M74 (*Kluber*, et al., 1985). One can hypothesis that these detrimental effects may be the result of overdosing since there has been one report that overdosing humans can have a laxative effect (*Gordon*, et al., 1957). In contrast to this one study of *Kluber* and co-workers (1985) using strain M74 , there have been at least six studies reporting improved performance with administration of strain M74 e.g. (*Moen*, 1982). Similarly, oral administration of another *L. reuteri* strain than

Table 2: Criteria used for selecting a probiotic strain for use with piglets

Host origin	
Biological activity against target	
Colonisation potential	
Survival in:	low pH, bile acids, antibiotics, additives
Stability of numbers during:	- preparation
	- storage
Stability of characteristics	

that used by *Ratcliffe* and colleagues (1986), resulted in improved mucosal morphology (*Jonsson* and *Hennings-son*, 1991). Administration of another *Enterococcus faecium* strain, referred to as C68, has resulted in less disease (*Krarup*, 1987) and improved performance (*Maeng*, et al., 1989) by some workers while others fail to show any effect (*Kornegay* and *Thomas*, 1973). This type of inconsistency has also occurred with dosage of *L. acidophilus* with some workers reporting no effect (*Kornegay*, 1985) while several other groups, e.g. *Redmond* and *Moore* (1965) note improved performance and a stabilising effect on the microbiota. Several influencing factors for the inconsistent findings have been presented (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982), namely, the age of the animal, environmental conditions, sensitivity of the gut to disturbances, diet as well as strain variation and variation in the viability of the preparation.

The US markets for animal probiotics have increased about 5-fold over the last decade and this largely reflects the new approaches to probiotics. It can be said that workers have identified the problems associated with probiotics of yesteryear and that there is new hope in the future with the appearance of better strains which are biologically targeted for specific applications.

The work presented here was initiated almost a decade ago when the Swedish government introduced legislation prohibiting the use of low-dose antibiotics in animal feeds and alternative methods were required for the pig industry. On surveying the literature, it appeared that the oral administration of lactic acid bacteria held promise, but that there was no single strain which was clearly significantly better and most strains used at that time failed to produce beneficial effects under some condition. The work presented here can be seen as a case study in which the target was identified and a criteria for selection of a desirable probiotic strain prepared. The mechanisms of action of the selected strains were studied *in vitro* and finally field trials in a commercial piggery were performed.

METHODOLOGY

The strategy used for obtaining a functional probiotic strain for piglets was also followed when selecting a probiotic strain for human usage as included in a recent review (*Conway* and *Henriksson*, 1994). Basically the approach has been to identify the target for the probiotic and then establish a strain

selection criteria as presented in Table 2. Once specific strains were identified, the mechanisms of action was studied *in vitro* and ultimately, *in vivo* field trials for efficacy conducted.

In order to isolate a lactobacillus which could be beneficial to piglets, we initially focused on the causative agents for mortality and economic loss in commercial pig raising establishments. Bacterial induced diarrhoea was the largest single contributor to mortality rates and the agent was predominantly enterotoxigenic *Escherichia coli,* with those bearing K88 fimbriae being particularly frequent (*Jonsson* and *Conway*, 1992). Consequently, when using the selection criteria outlined in Table 2 (*Conway* and *Henriksson*, 1994; *Gibson* and *Conway*, 1994), potential strains were screened for biological activity against enterotoxigenic *E. coli* bearing K88 fimbriae. This was measured in terms of inhibition of growth and adhesion of the pathogen. Growth inhibition was assessed by measuring zones of inhibition around a colony on an agar plate (*Conway*, to be submitted), and also by monitoring the growth of the pathogen spectrophotometrically in media containing spent culture supernatant of the isolates mixed with fresh growth media (50:50) (*Rojas* et al., to be submitted). Inhibition of adhesion of the pathogen was investigated using dialysed and fractionated spent culture in an *in vitro* adhesion assay as previously described (*Blomberg*, et al., 1993).

Furthermore since *E. coli* K88 colonise the distal ileum, lactobacillus isolates from the piglet digestive tract were selected for the capacity to colonise the stomach and hence be continually seeded into the distal ileum as viable cells. The diversity of lactobacilli isolated from the various regions of the gut were studied by comparison of the SDS-PAGE protein profiles of lysozyme treated cells.

Colonisation capacity was evaluated by testing the adhesive nature of the isolates in an *in vitro* adhesion assay using radioactively labelled bacteria and epithelial mucosa (*Henriksson*, et al., 1991), bearing in mind the limits of this type of assay and with strict attention to the correct controls necessary for interpreting the data (*Conway* and *Henriksson*, 1994). Survival of the isolates in low pH, bile acids and enzymes was evaluated by appropriate additions to buffered saline and growth media as previously reported (*Conway* and *Henriksson*, 1994; *Gibson* and *Conway*, 1994). The stability of viability was monitored in both the initial material as well as after storage at a range of temperatures by measuring the colony forming units. Even though viability may be maintained over time, we felt the need to also ensure that the characteristics of the strain did not alter and hence when the viability was monitored, so too were the characteristics such as survival, adhesion and effects on pathogens (*Conway* and *Henriksson*, 1994) .

In the first stage of *in vivo* trials (*Conway* and *Rönnow*, to be submitted), piglets were orally dosed with an erythrosine solution immediately prior to oral administration of a preparation of *Lactobacillus murinus* strain C39 suspended in a solution of infant formula. Animals were dosed twice, once in the evening and again next morning, with the *L. murinus* preparation during the first two weeks of life. Rectal swabs were then collected weekly for a 9 week period after which time some piglets from each group were sacrificed. Samples were analysed for the number of colony forming units (CFU) of lactobacilli and coliforms and the ratio of the two calculated for each sample. From the lactobacilli cultured, isolates were randomly picked for studying the pres-

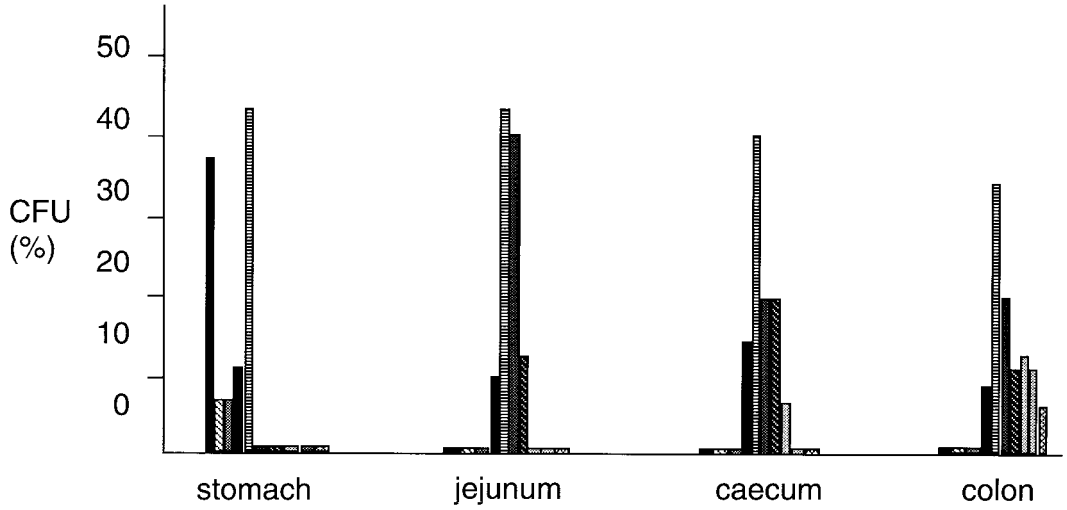

Figure 1: Diversity of lactobacilli in the porcine gastrointestinal tract. Protein profiles, as visualised by SDS-PAGE, of lactobacilli from the various regions of the piglet digestive tract were grouped according to the extent of similarity. Bars shaded in the same pattern represent isolates with similar profiles. The results are presented as the percentage of the viable isolates (colony forming units, i.e. CFU) from each region, which had the particular profile (*Henriksson* et al., 1995).

ence of the administered strain both in rectal samples and throughout the gut of the sacrificed animals. The *L. murinus* strain was positively identified immunologically and morphologically.

In a second field trial (*Conway*, to be submitted), piglets in a commercial piggery were given *ad libitum* access to the *L. fermentum* strain via the creep feed and the weaning feed since the strain was added as a freeze dried supplement to the powdered feed. Erythrosine was orally dosed to the piglets immediately prior to weaning and this was followed by an increased dose of the *L. fermentum* strain. The incidence of diarrhoea and weight gain over the 9 week period were monitored in the negative (no additions to the feeds) and positive (antibiotics added to feeds) control animal and the test piglets.

RESULTS AND DISCUSSION

Reviews of published studies show a positive trend that piglet performance and health may be improved by prophylactic administration of lactic acid bacteria (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982), however, conflicting results have been reported and it is generally agreed that more attention to strain selection could yield more consistent findings (*Conway*, 1989; *Havenaar*, et al., 1992). The work presented here summarises our approach used for developing a functional lactobacillus preparation for prophylactic use in piglets. Since a major cause of economic loss for the pig industry is enterotoxigenic *E. coli* K88 induced diar-

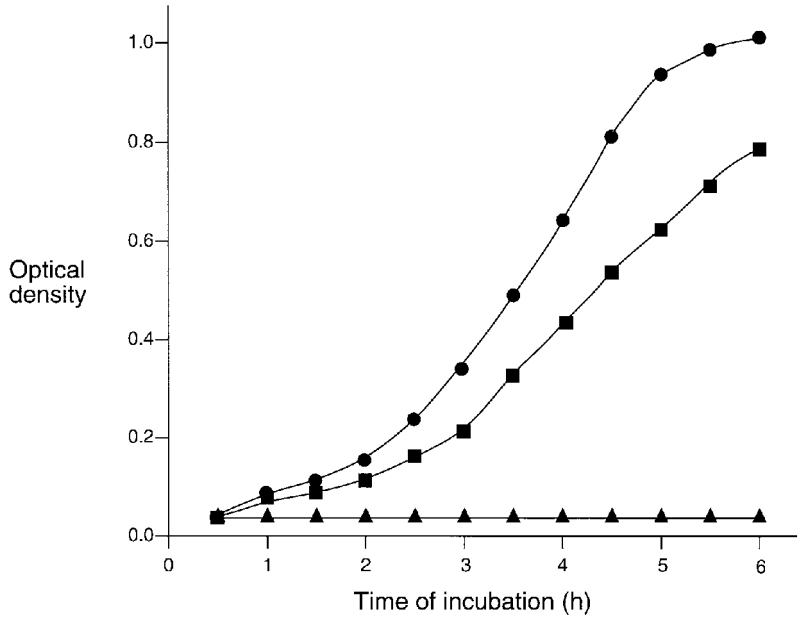

Figure 2: Growth of *Escherichia coli* K88 in brain heart infusion broth (BHI) (circles), BHI + spent culture supernatant of *Lactobacillus fermentum* strain 104 (triangles) or in BHI + dialysed spent culture supernatant of *L. fermentum* strain 104 (squares). Results are expressed as the increase in optical density (590 nm) over 6 hours (*Conway*, to be submitted).

rhoea (*Jonsson* and *Conway*, 1992), the strain selection criteria targeted this pathogen. Consequently, lactobacillus isolates from the porcine digestive tract were screened using the selection criteria outlined in Table 2. In addition, emphasis was placed on selecting strains which could colonise the pars oesophageal region of the stomach since this region is densely colonised by lactobacilli and as epithelial cells are sloughed off from the mucosa, associating lactobacillus cells are carried into the lumenal contents. This process allows a continual inoculum of the lactobacilli to the small intestine which is the site of colonisation of *E. coli* cells bearing K88 fimbriae. An *in vitro* adhesion assay using pars oesophageal mucosa was used to screen for the potential of the isolates to colonise this tissue. Assuming the lactobacillus cells would be more effective if metabolically active in the small intestine, an attempt was made to select strains of host origin with this capacity. Isolates were selected which had the capacity to survive the rigours of the tract e.g. low pH, bile acids, intestinal enzymes, low nutrients.

The data presented in Figure 1 groups, according to the SDS-PAGE protein profiles of lysozyme-treated cells, lactobacillus isolates from various regions of the gastrointestinal tract. From these data, it is apparent that some groups of lactobacilli were localised in the upper regions of the tract, while others were only found in the lower regions. One profile (histogram with shaded horizontal lines, Figure 1) was detected throughout the tract and an isolate consistent with this profile should therefore have a greater chance of colonising the entire tract (*Henriksson* and *Conway*, 1995).

Biologically activity was initially screened by overlaying the pathogenic *E. coli* K88 strain on colonies of the

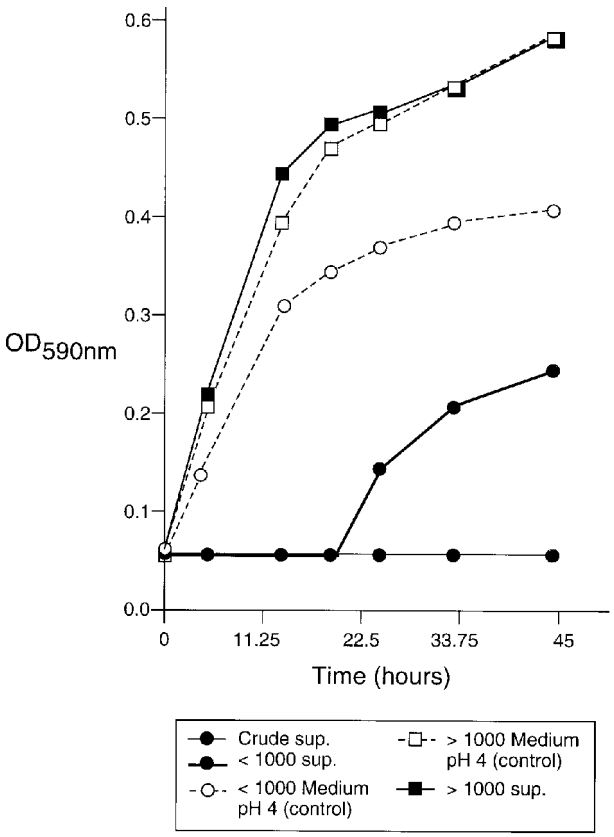

Figure 3: Growth of *Escherichia coli* K88 in modified brain heart infusion broth (MBHI) containing: a 1:1 dilution with spent culture supernatant of *L. fermentum* 104, or culture supernatant dialysate (<1000 Mr), or MBHI broth dialysate (<1000 Mr) adjusted to yield pH 4, or MBHI broth retentate (>1000 Mr) at pH 4, as well as retentate of MBHI spent culture supernatant. Results are presented as the optical density (590 nm) of the growth media (*Rojas* et al, to be submitted).

lactobacillus isolates. Zones of inhibition of growth varied from nothing to 35 mm in diameter for the strains studied. Some isolates producing large zones were further screened for the presence of growth inhibitors in the spent culture supernatant. An isolate identified as *Lactobacillus fermentum* was shown to produce spent culture supernatant that totally inhibited the growth of *E. coli* K88 when combined with fresh culture medium (Figure 2). It was further shown that the retentate after dialysis (membrane cut-off 8-10 K) as had some growth inhibitory activity (Figure 2). This activity was destroyed by protease and heat treatment, however, was not stable with time. Even primary cultures stored at -70°C for 2 years did not retain the activity and one can suggest that the activity may be restored with animal passage as can occur for virulence factors not expressed on subculturing. It has subsequently been shown that the lower molecular weight components (<1000 Mr) were also bacteriostatic and that this effect was enhanced when the smaller molecular weight components were included with the higher molecular weight (>1000) components (Figure 3). This activity was pH sensitive and one can question

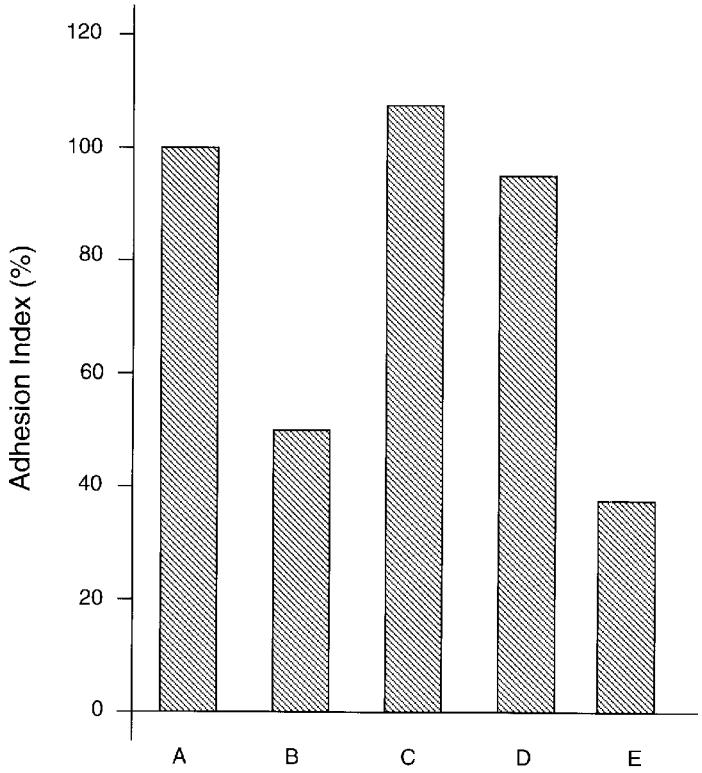

Figure 4: Adhesion of *Escherichia coli* K88 fimbriated cells to immobilised porcine ileal mucus in the presence of buffer (A; control), *Lactobacillus fermentum* strain 104 spent culture fluid (B) and fractionated spent culture fluid of strain 104 yielding up to 10 K (C), 10-30 K (D) and >30 K (E) relative molecular weight. Results are expressed as a percentage of the adhesion obtained for the control (A) (*Blomberg* et al, 1993; *Conway* unpublished data).

whether this activity functions in the gut.

The potential for the lactobacillus cells to protect the host from pathogen invasion can also be discussed in terms of the effects of lactobacillus metabolites on adhesion of the pathogen to the epithelial mucosa, since it has been established that this is a pre-requisite for colonisation by the pathogen. Using an *in vitro* adhesion assay, it has been established that the presence of spent culture supernatant of the lactobacilli of porcine origin inhibits the adhesion of the *E. coli* K88 fimbriated cells (Figure 4). In this study (*Blomberg* et al., 1993), it was also shown that the interference was demonstrable if the mucosal surface, but not the bacterial surface, was treated with the spent culture supernatant prior to the assay. Furthermore fractionation of the supernatant revealed that the activity was detectable in the retentate with an Mr of greater than 30 K. It was concluded that component(s) interfered with the interaction between the K88 fimbriae and the mucosal receptor and the mechanism is undergoing further investigation.

Two strains, identified as *L. murinus* and *L. fermentum*, showed inhibition of growth of the *E. coli* K88 cells in the primary screen using the overlay technique (*Conway*, to be submitted), and also adhered well to non-secreting stomach tissue in the *in vitro* adhesion

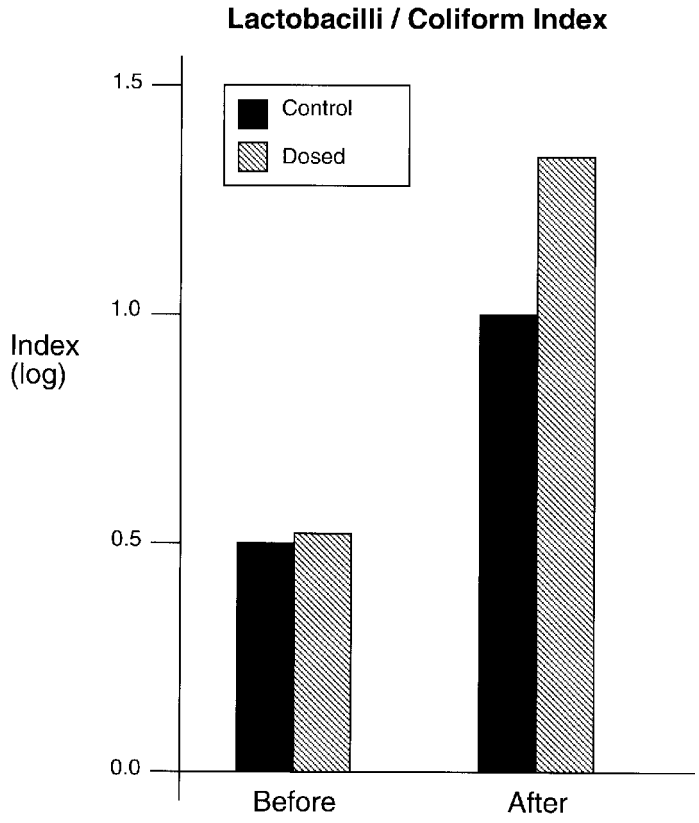

Figure 5: Ratio of lactobacilli to coliforms in the faeces of piglets before and after dosing with *Lactobacillus murinus*. Results are presented as the ratio of the colony forming units (CFU) of lactobacilli to CFU coliforms using log CFU per g faeces (*Conway* and *Rönnow*, to be submitted).

assay (*Henriksson* and *Conway*, 1992; *Henriksson*, et al., 1991). These strains survived low pH, bile acids and nutrient deprivation (data not presented), and were investigated in more detail. When *L. murinus* was sub-cultured in laboratory media, some decrease in adhesive capacity was noted. In contrast, *L. fermentum* exhibited stable adhesive characteristics. When cell wall extracts were reductively methylated, a carbohydrate with affinity for the porcine gastric squamous tissue could be identified (*Henriksson* and *Conway*, 1992). This carbohydrate did not adhere to gastric mucus from the secreting region and it was suggested that proteins could be involved in association of the strain with mucus (*Henriksson*, 1993). From the data, one can hypothesise that *L. murinus* and *L. fermentum* have the potential to colonise the digestive tract of piglets and be biologically active against *E. coli* K88 bearing strains.

In the first stage of the *in vivo* trials, piglets were dosed with *L. murinus* during the first 2 weeks of life and the lactobacilli and coliforms in rectal swabs were quantified. As can be seen in Figure 5, the lactobacillus:coliform ratio was greater after oral dosage. The lactobacilli cultured from the rectal samples, as well as isolates from the lumenal contents and mucosal homogenates of sacrificed 9 week old piglets, were subcultured and immuno-

Table 3: The weight gain recorded for piglets orally dosed with lactobacillus

Group	Weight increase of test group (%)		
	0-37 d	0-63 d	37-63 d
Negative control	35*	72*	55*
Positive control	32	28	4

* Significantly different from control, $p<0.05$
No. of litters per group = 8

Results are expressed as the percentage weight gain in the test group relative to that noted for the negative and positive controls, the latter group receiving antibiotics. Piglets were weaned at 37 days and the results are presented for the entire period of study (0-63 days), pre-weaning (0-37 days) and post-weaning (37-63 days) (*Conway*, to be submitted).

logically screened for the presence of the introduced *L. murinus*. This strain was regularly detected in the rectal swabs for the 9 weeks of the study and in lumenal and mucosal samples from the 9 week old pigs (*Conway* and *Rönnow*, to be submitted). It was concluded that the criteria used for strain selection yielded a strain with the capacity to colonise the piglet digestive tract for extended periods of time.

In the second stage of the field trials, the *L. fermentum* strain was orally dosed to piglets in a commercial piggery. In this study the parameters investigated include the effects on the incidence of diarrhoea and on the weight gain over a 9 week period. As can be seen in Table 3, the piglets receiving the *L. fermentum* (test group) significantly gained more weight then did piglets in the negative control group which received no supplements and also gained more weight than did those in the positive control group receiving an antibiotic supplement. Consistently, no post-weaning diarrhoea was detected in the litters receiving the *L. fermentum*, while 80% of the negative control litters suffered diarrhoea (Table 4). It was concluded that the criteria used for strain selection (Table 2), yielded isolates which had the capacity to colonise the digestive tract and the results provide indirect evidence that the strains also exerted biological activity on the pathogenic *E. coli* K88 when orally dosed.

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Table 4: The incidence of diarrhoea in piglets before and after weaning at 37 days for negative and positive controls (receiving antibiotics) as well the test group being orally dosed with lactobacilli

Group	No. of litters	Before weaning	After weaning
Neg. control	15	47 %	80 %
Pos. control	15	33 %	27 %
Test	8	25 %	0 %

Results are expressed as the number of sows with diarrhoea in the litter as a percentage of the total number of sows.

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