Acta vet. scand. 1992, *33,* 95-103.

Lactic Acid Bacteria for Mink

Colonization and Persistence of *Enterococcus faecium* Cernelle 68 in the Digestive Tract of Mink

by *Karl Pedersen* and *Mogens Jørgensen*

Royal Veterinary and Agricultural University, Department of Veterinary Microbiology, Copenhagen, Denmark.

Pedersen, K. and M. Jørgensen: Lactic acid bacteria for mink. Colonization and persistence of *Enterococcus faecium* Cernelle 68 in the digestive tract of mink. Acta vet. scand. 1992, 33, 95-103. – A method was developed to follow a lactic acid bacterial strain, Enterococcus faecium Cernelle 68, with respect to adhesion, multiplication, colonization, and persistence in the digestive tract of mink. Also the spread of the strain in the cage was examined. When adding 5×10^9 c.f.u. of a rifampicin resistant mutant per kg feed, high viable counts were registered throughout the digestive tract, apart from the oesophagus. Counts were increasing in the aboral direction, suggesting some multiplication in the intestine. It was possible to detect the strain in the intestinal tract 4 days after discontinuation of administration. Neither culture nor scanning electron microscopy gave evidence to suggest that *E. faecium* Cernelle 68 adhered to the mucosa. The spread of the *E. faecium* strain was observed in the environment. Counts of *E. coli,* lactobacilli, staphylococci, and clostridia were low, and none of these bacteria were constant findings.

probiotics; sticky kits.

Introduction

Diarrhoea in mink may be due to virus or bacteria as well as dietetic or environmental factors and constitute a major problem from birth to pelting *(Jørgensen* 1984). Sticky kits *(Henriksen* 1987) is a multifactorial disease involving intestinal disorders seen in mink kits during the suckling period. The most frequent bacterial findings in the intestinal tract of sticky kits are *E. coli,* staphylococci and streptococci. For several years, feed additives have been used in Denmark during the suckling period to prevent the disease. The use of preparations of lactic acid bacteria is thought to stabilize the intestinal flora *(Fuller* 1989, *Gedek* 1989, *Gilliland et al.* 1980, *Jørgensen* 1988a, *Muralidhara et al.* 1977), and may prevent diarrhoea *(Underdahl et al.* 1982) and reduce side effects to oral administration of antibiotics *(Wunderlich et al.* 1989). A positive effect on litter size, kit growth and kit mortality was recently indicated on 2 Danish mink farms where *Enterococcus faecium* Cernelle 68 (SF68) was added to the feed *(Jørgensen* 1988b). Additional investigations in 1989 using SF68 as prophylaxis against sticky kits showed promising results *(Jørgensen* unpublished). The aim of the present paper was to investigate if *E. faecium* Cernelle 68 would survive the pH barrier of the stomach and if so to examine in which numbers the organism would establish itself in the different parts of the digestive tract.

Materials and Methods

Bacteria

A rifampicin-resistant strain of *Enterococcus faecium* Cernelle 68 was used *(E. faecium* rif^r). It was isolated from gradient plates with maximum rifampicin concentration of 100 µg/ml as a spontaneous mutant from the Cernivet product (Bioferment, Lugano, Switzerland). The mutant was tested for identity with the original strain as judged from cell and colony morphology, fermentation of carbohydrates (API 50 CHL, API System, La Balme-les-Grottes, France), phosphatase, arginine dihydrolase, ornithine decarboxylase, hydrogen sulphide, urease, indole, Voges-Proskauer, and nitrate. Propagation took place in heart infusion broth (Difco), incubated at 37°C for 24 h. The grown-out broth was centrifuged at room temperature in a Sorvall RC-5B Refrigerated Centrifuge at 6,000 rpm, and the pellet was resuspended in fresh heart infusion broth, added 25% sterile glycerol and kept in cryo tubes at –80°C until use. Counting of viable cells took place before and after freezing. The stability of the rifampicin resistance was examined by repeated subculture in heart infusion broth (Difco) without rifampicin. After incubation at 37°C for 24 h subcultures were made on Mitis Salivarius agar (Difco) with and without rifampicin, 100 µg/ml.

Experimental design

Fifteen grown scanglow minks were used in the studies. Ten animals were included in the test group, while 5 served as controls. Each animal had its own cage and all were given the same basic diet, a wet feed based on fish, poultry offal, and grain. The energy content of the feed was 171 kcal/100 g. All feed used was delivered at once from the same batch a few days before start of the experiment, examined bacteriologically, weighed out in separate portions for experimental and control group for each day, and kept in plastic bags at –18°C until use. Feed to be used the same day was taken out of the freezer in the morning and thawn in the refrigerator at 5°C. Feeding took place once every 24 h – 150 g per animal. During the first week, to get used to the feed all the animals were given only the basic diet. On days 6 to 13 *E. faecium* rif^r was thawn in the hand immediately before use and subsequently added to the feed of the test group in the amount of 5×10^9 c.f.u. pr. kg feed. One control mink and 2 inoculated mink were killed alternately every 2nd day, starting on day 7. Mink were killed by intracardiac or intrathoracic injection of an aqueous solution of pentobarbital sodium. Sampling from the killed animals and culturing of the samples followed a technique previously described by *Pedersen & Tannock* (1989), slightly modified: After opening into the thoracic and abdominal cavity, the ventricle and rectum as well as approximately 5 cm of the oesophagus, duodenum, mid- and posterior jejunum, respectively, were aseptically ligated and removed for bacteriological examination. The last control mink was killed at day 16 and the last 2 test minks were killed at day 17. Tissue specimens from the oesophagus, duodenum and mid-jejunum were sampled from 11 of the animals for scanning electron microscopy.

Samples of faeces were collected from 2 test animals 4 times during the experiments, and nest material was sampled 5 times from 2 of the cages to be cultured for *E. faecium* rif^r. All samples were kept at 0 – 5°C until immediately before further treatment (approximately 1 – 4 h).

Bacteriological examinations

The tissue specimens from oesophagus,

duodenum, and mid- and posterior jejunum were flushed with 4.5 ml sterile physiologic saline and 0.5 g stomach content was transferred to 4.5 ml sterile saline. Similarly, 0.5 g of feed, faeces and nest material, respectively, was weighed out and transferred to 4.5 ml sterile saline. Tenfold dilutions were made of all samples before plating on agar media.

Enumeration of total streptococci, *E. faecium* rif^r, staphylococci, *E. coli, Lactobacillus,* and sulphite reducing anaerobes *(Clostridium spp)* was carried out in all samples. Total streptococci were counted on Mitis Salivarius agar (Difco) added nalidixic acid (15 µg/ml) instead of tellurite *(Barrow et al.* 1977). *E faecium* rif^r was counted on the same medium added rifampicin, 100 µg/ml. Staphylococci were counted on Baird-Parker medium (Oxoid), *E. coli* on MacConkey agar (Difco) and lactobacilli on Rogosa SL medium (Difco). Sulphite reducing anaerobes were counted in sulphite agar *(Skovgaard* 1958). Rogosa SL plates were incubated anaerobically (GasPak jars, BBL Microbiology Systems, Cockeysville, Md.) 48 h at 37°C, while all other media were incubated aerobically 24 h at 37°C. In addition, feed samples were plated on Plate Count Agar (Oxoid) and incubated aerobically and anaerobically at 37°C for 2 days, 20°C for 3 days or at 5°C for 5 days.

Results from feed, faeces, nest material, and stomach contents are expressed as bacteria per gram material, and those from flushed organs as bacteria per ml flush water.

Scanning electron microscopy (SEM)

Tissue specimens for SEM were removed immediately and kept in a fixative containing glutaraldehyde (Merck) 3%, sodium-cacodylate (Sigma) O.1 M, and $CaCl_2$ (Sigma) 0.15 M in distilled water until further processing. After trimming, tissue samples were dehydrated through a series of acetone baths (25%, 40%, 60%, 90%, 100%, 100% and 100%). Following the last acetone bath, the specimens were critical point dried in a Baltzer CPD 020, mounted on metal stumps, coated with 20 nm gold in a Polaron High Resolution Sputter Coater E5400 and subsequently scanned in a JEOL JSM 840 A scanner.

Results

In all the criteria examined, the rifampicin-resistant strain of SF68 was identical to the parent strain. Subculturing in rifampicin-free media showed that the resistance was a stable trait. Noticeable reduction of the bacterial counts of *E. faecium* rif^r was not seen after the strain had been kept at –18° C.

The bacterial counts from control group and

Table 1. Cell counts for bacteria in various intestinal sections. Control group. The numbers indicate the range of bacteria found in the 5 control mink.

	Intestinal sections					
Bacterium	Oesophagus	Ventricle	Duodenum	Mid–jejunum	Posterior jejunum	Rectum
Streptococci	40–1.4×10^5	0–600	0–10	0–10	10–4.9×10^3	0–1.7×10^5
E. coli	0	0	0	0	0–100	0
Lactobacilli	0–1.4×10^2	0–2.3×10^3	0–600	0–1.1×10^3	0–4.7×10^3	0–500
Staphylococci	0–10^3	0	0	0	0–100	0–5×10^3
Clostridia	0–40	0–6.4×10^2	0–4.4×10^2	0–40	0–9.1×10^2	10–1.6×10^4

Table 2. Cell counts of bacteria in various intestinal sections. Test group. The numbers indicate the range of bacteria found in the 10 test mink. Counts of streptococci and *E. faecium* rif^r are shown separately.

Bacterium	Intestinal sections					
	Oesophagus	Ventricle	Duodenum	Mid-jejunum	Posterior jejunum	Rectum
E.coli	0	0–5.53102	0–50	0–100	0–1.53104	0–1.73103
Lactobacilli	0–50	2.33102–1.93104	0–53104	0–2.83103	7.83102–5.53103	100–2.03103
Staphylococci	0–1.53102	0–3.63102	0–7.63102	0–50	0–500	0–2.53104
Clostridia	0	0–2.63103	0–53103	0–8.13104	3.33102–6.03104	5.93102–83104

test group are shown in Table 1 & 2. The intestinal flora of all the animals turned out to be sparse and variable. None of the bacteria examined were constant findings. However, it is noticeable that in several of the animals – both in the control and the test group – a non-lactosefermenting bacterium grew on the MacConkey plates, sometimes in even very high numbers. In some animals it was isolated throughout the whole gastrointestinal tract, although only in low numbers in the oesophagus. The highest numbers were seen in the posterior sections of the intestine. When present, the level in the rectum was $10^4 - 2\times10^6$ per ml. The bacterium was identified as an inactive *E. coli (Krieg & Holt* 1984). The counts of this bacteria are not included in Table 1 and 2.

In control minks the counts of streptococci were low. In test minks the counts of *E. faecium* rif^r and total streptococci were similar and high during the whole administration period (Fig. 1). Divergence between the counts of *E. faecium* rif^r and total streptococci was only noticed after the final administration. Apart from the oesophagus, the counts of *E. faecium* rif^r were considerably higher than those of any of the other bacteria. The bacterial counts in the oesophagus were very low – in most of the counts none were detected (data not shown).

In the fecal samples, counts of *E. faecium* rif^r

Table 3. Cell counts of total streptococci and E. faecium rif^r, per gram of nest material from 2 different cages (used for the 2 last killed inoculated mink).

		Number of days after initial administration *)				
		2	4	6	9	11
Cage No. 1	Total streptococci	2.9×10^6	6.2×10^2	7.6×10^4	2.2×10^5	1.5×10^6
	E. faecium rif^r	3.1×10^6	6.6×10^3	7.9×10^4	2.0×10^5	1.1×10^6
Cage No. 2	Total streptococci	5.4×10^3	3.5×10^5	1.7×10^4	2.0×10^6	5.3×10^4
	E. faecium rif^r	5.6×10^3	3.3×10^4	1.3×10^4	2.2×10^6	3.7×10^4

*) Final administration: 7 days after initial administration

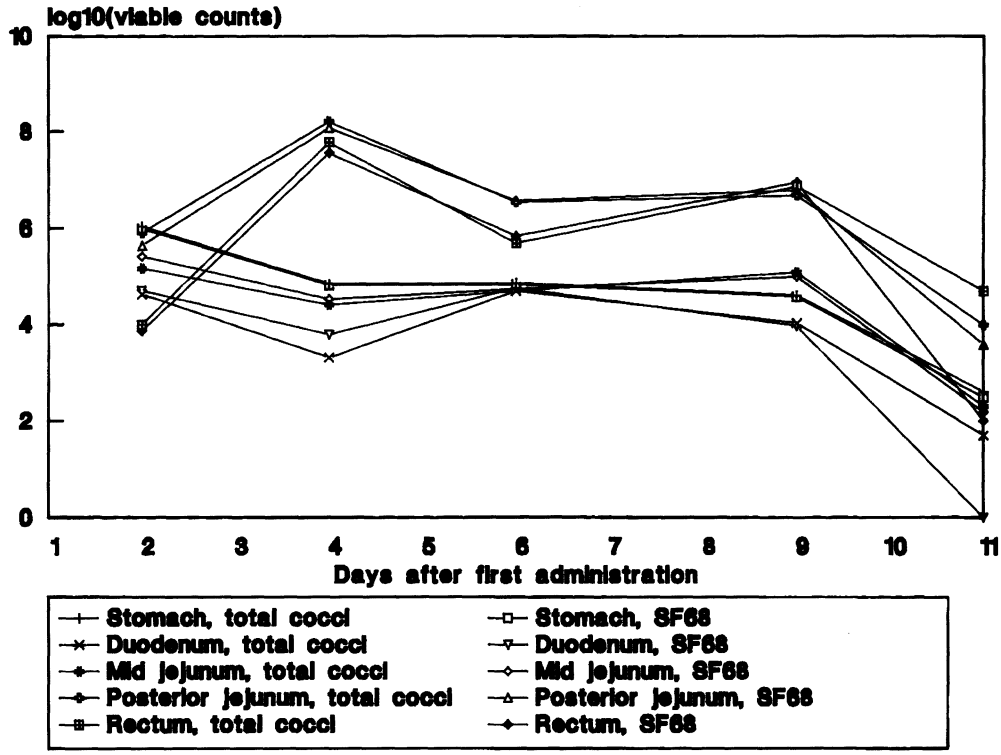

Figure 1. Counts of *Enterococcus faecium* (SF68) rif^r at different sites in the gastrointestinal tract of test mink. Each point marks the average of 2 animals. The final administration of the strain took place on day 7.

ranged between 3.4×10^7 –2.2×10^8 during the test period. Two and 4 days after the final administration counts of *E. faecium* rif^r were 2.1×10^7 and 1.4×10^5 c.f.u. per gram, respectively.

Counts of *E. faecium* rif^r in feces compared with the number of bacteria ingested showed that up to 10–15 times as many bacteria were excreted as were ingested, but usually the figure was somewhat lower.

Counts of streptococci in nest material varied considerably (Table 3). The nest material was not renewed during the study.

Scanning electron microscopy showed no differences between inoculated and control animals. No bacteria were found to adhere to the mucosa, not even in the oesophagus (Fig. 2). A few bacteria were observed in the intestinal content or in mucus (Fig. 3).

The feed only contained low numbers of the bacteria examined. Counts of streptococci were 7.2×10^3 c.f.u. per gram, and counts of *E. coli,* lactobacilli, and clostridia were 10^2, 2.2×10^3, and 5.0×10^2, respectively. *Staphylococcus aureus* was not detected. The main part of the flora was facultatively anaerobic, psychrophilic bacteria.

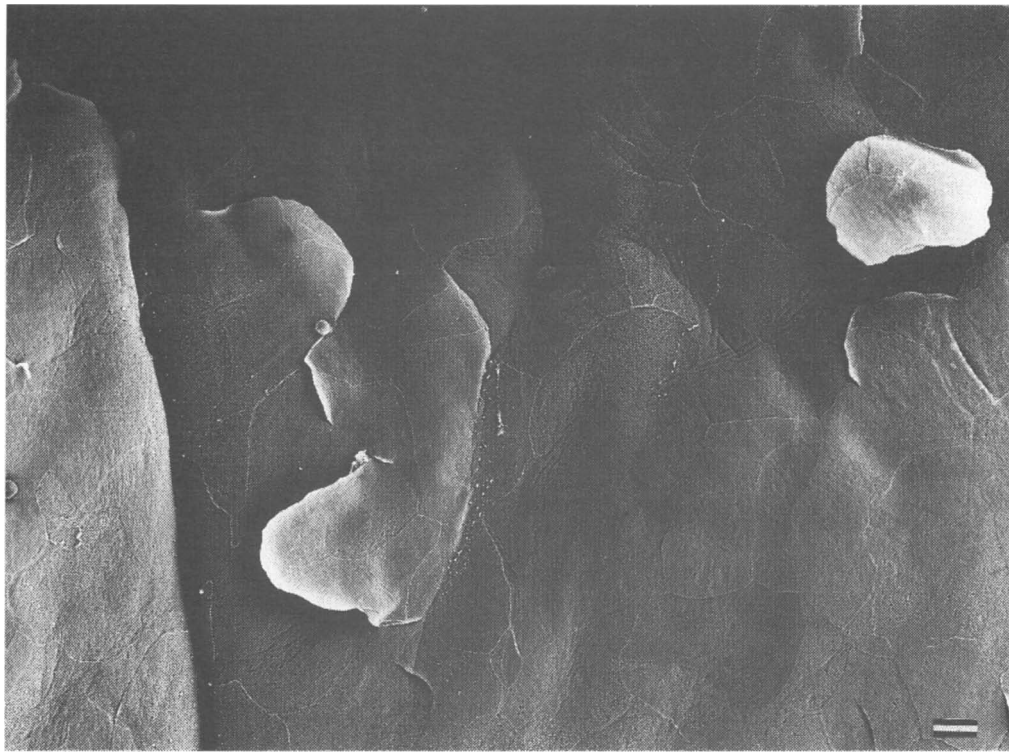

Figure 2. Scanning electron micrograph showing the squamous epithelium of the oesophagus from a mink fed *E. faecium* rif^r. No bacteria can be observed adhering to the epithelium. Bar = 10 µm. × 430.

Conclusion and discussion

Apart from *E. faecium* rif^r, the intestinal flora of mink was sparse and variable, even in the posterior sections of the intestine. This is in contrast to a number of other animal species, including man, in which about 10^{12} bacteria per g content is frequently found in the colon *(Simon & Gorbach,* 1981). The low numbers of bacteria observed were probably mainly due to the short time of passage of the feed through the intestinal tract. In mink this may make it difficult for an autochtonous flora to develop. The results indicate that none of the examined bacteria were members of an autochtonous intestinal flora, but passants originating from the feed or the environment. The ecology and significance of the inactive *E. coli* colonizing the gut of some of the animals is unknown. Its presence was not associated with any symptoms of disease.

Apart from *E. faecium* rif^r, it was not possible to demonstrate any significant differences between inoculated and control animals. The presence of even high counts of *E. faecium* rif^r did not seem to influence the counts of the other types of bacteria. It should be stressed, however, that investiga-

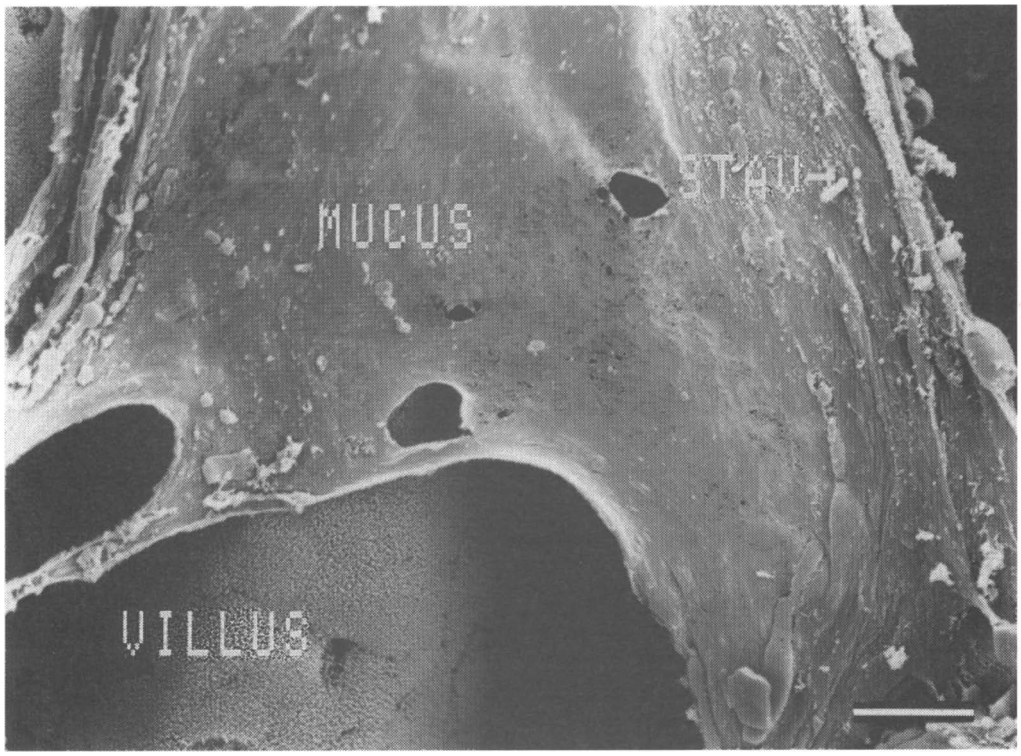

Figure 3. Scanning electron micrograph showing a villus, partially covered by a mucus layer, from the jejunum of a mink fed *E. faecium* rif^r. No bacteria are adhering to the intestinal epithelium. One rod–shaped bacterium is seen (arrow) trapped in the mucus. Bar = 10 µm. × 1200.

tions for *Bacteroides* and *Eubacterium spp.* which are known to constitute a major part of the intestinal flora of humans and a number of animals *(Savage* 1977, *Simon & Gorbach* 1981) were not performed.

The scanning electron microscopy did not show any bacterial adhesion in the intestinal tract. In a number of other animals, e.g. pigs, mice and rats, a close colonization of lactic acid bacteria in the oesophagus *(Fuller et al.* 1978, *Tannock et al.* 1987, *Pedersen & Tannock* 1989) is invariably seen, just as certain other bacteria – most of which have not yet been identified – are seen adhering in different intestinal sections of various animal species *(Savage* 1977, 1984). A similar colonization with lactic acid bacteria in the crop of poultry has been described *(Fuller* 1973, 1989). The adhesion of *Enterococcus faecium* in the duodenum of poultry has also been reported *(Fuller et al.* 1981).

The method used made it possible to follow the colonization with a specific bacterial strain in the animals as well as in the environment. The aim was to find out whether and to what extent *E. faecium* rif^r could be reisolated in the various compartments of the digestive tract. The strain was able to survive

the pH–barrier of the stomach and to some extent multiply on its passage through the intestine. High counts of *E. faecium* rif^r were obtained, in some cases exceeding 10^8 bacteria per ml, outnumbering the counts of all other bacteria investigated. The counts of the strain were reduced after withdrawal from the feed, but 4 days later it was still found in faecal samples in numbers around 10^5 cells per gram. Thus, the colonization of the intestinal tract was probably not permanent, but lasted a few days. Considering the limited time of intestinal passage in the mink, it may seem surprising that the *E. faecium* rif^r strain could be reisolated so long after discontinuation. Analyses of the nest material showed relatively high counts of the strain, indicating that it was spread and survived in the cage.

The rifampicin resistance was found to be stable. This has also been reported by other researchers *(Compeau et al.* 1988). They described rifampicin resistance as a chromosomal and very stable trait of bacteria. An alternative to using antibiotic resistance as a marker might be in situ hybridization using DNA probes, a technique which, however, is not fully developed in this area *(Tannock* 1989).

The experiments described here do not allow conclusions to be made as to whether *E. faecium* SF68 or probiotics as such has any beneficial function in the intestinal tract of mink or might be able to prevent outbreaks of sticky kits or other gastrointestinal diseases. However, the results concerning the colonization and persistence of this strain encourage further studies in order to substantiate a possible beneficial effect of lactic acid bacteria in minks.

Acknowledgement

The financial support from Lundbeckfonden, Denmark, is gratefully appreciated.

References

Barrow PA, Fuller R, Newport MJ: Changes in the microflora and physiology of the anterior intestinal tract of pigs weaned at 2 days, with special reference to the pathogenesis of diarrhoea. Infect. Immun. 1977, *18,* 586–595.

Compeau G, Al–Achi BJ, Platsouka E, Levy SB: Survival of rifampicin–resistant mutants of Pseudomonas fluorescens and Pseudomonas putida in soil systems. Appl. environ. Microbiol. 1988, *54,* 2432–2438.

Fuller R: Ecological studies on the Lactobacillus flora associated with the crop epithelium of the fowl. J. appl. Bacteriol. 1973, *36,* 131–139.

Fuller R: A review. Probiotics in man and animals. J. appl. Bacteriol. 1989, *66,* 365–378.

Fuller R, Barrow PA, Brooker BE: Bacteria associated with the gastric epithelium of neonatal pigs. Appl. Environ. Microbiol. 1978, *35,* 582–591.

Fuller R, Houghton SB, Brooker BE: Attachment of Streptococcus faecium to the duodenal epithelium of the chicken and its importance in colonization of the small intestine. Appl. environ. Microbiol. 1981, *41,* 1433–1441.

Gedek B: Intestinal flora and bioregulation. Rev. sci. tech. Off. int. Epiz. 1989, *8,* 417–437.

Gilliland SE, Bruce BB, Bush LJ, Staley TE: Comparison of two strains of *Lactobacillus acidophilus* as dietary adjunct for young calves. J. Dairy Sci. 1980, *63,* 964–972.

Henriksen P: A survey of the syndrome "Sticky kits" in mink. Dansk Vet.Tidsskr. 1987, *70,* 580–583.

Jørgensen J: Mink production. Det kgl. danske Landhusholdningsselskab, Copenhagen, 1984.

Jørgensen M: Probiotic – a survey. An alternative to antibiotics in the feed of fur–bearing animals. Scientifur 1988a, *12,* 247–249.

Jørgensen M: Probioticum *(Streptococcus faecium* Cernelle 68 – SF68) for improvement of health and well–being with mink and fox. Scientifur 1988b, *12,* 250–256.

Krieg NR, Holt JG: Bergey's Manual of Systematic Bacteriology, vol. I. Williams and Wilkins, Baltimore 1984, pp. 408–601.

Muralidhara KS, Sheggeby GG, Elliker PR, England DC, Sandine WE: Effect of feeding lactobacilli on the coliform and *Lactobacillus* flora of intestinal tissue and feces from piglets. J. Food Protect 1977, *40,* 288–295.

Pedersen K, Tannock GW: Colonization of the por-

cine gastrointestinal tract by lactobacilli. Appl. environ. Microbiol. 1989, *55,* 279–283.

Savage DC: Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 1977, *31,* 107–133.

Savage DC: Adherence of the normal flora. In: Boedeker EC (ed.): Attachment of organisms to the gut mucosa, vol. I. CRC Press Inc., Florida, 1984, pp. 3–10.

Simon GL, Gorbach SL: Intestinal flora in health and disease. In: Johnson LR (ed.): Physiology of the gastrointestinal tract, Raven Press, New York, 1981, pp. 1361–1380.

Skovgaard N: About specific enumeration of clostridia in food. Proc. VIIIth Nordic Veterinary Congress, Helsinki, 1958, pp. 766–772.

Tannock GW: Biotin–labelled plasmid DNA probes for detection of epithelium–associated stains of lactobacilli. Appl. environ. Microbiol. 1989, *55,* 461–464.

Tannock GW, Blumershine R, Archibald R: Demonstration of epithelium–associated microbes in the oesophagus of pigs, cattle, rats and deer. FEMS microbiol. Ecol. 1987, *45,* 199–203.

Underdahl NR, Torres–Medina A, Doster AR: Effect of *Streptococcus faecium* C–68 in control of *Escherichia coli*–induced diarrhea in gnotobiotic pigs. Amer. J. vet. Res. 1982, *43,* 2227–2232.

Wunderlich PF, Braun L, Fumagalli I, Apuzzo VD, Heim F, Karly M, Lodi R, Politta G, Vonbank F, Zeltner L: Double–blind report on the efficacy of lactic acid–producing enterococcus SF68 in the prevention of antibiotic–associated diarrhoea and in the treatment of acute diarrhoea. J. int. med. Res. 1989, *17,* 333–338.

Sammendrag

Mælkesyrebakterier til mink. Kolonisering og persistens af Enterococcus faecium i tarmkanalen hos mink.

En metode blev udviklet til at følge en specifik mælkesyrebakterie med hensyn til adhæsion, opformering, kolonisering og persistens samt indflydelse på den øvrige flora i tarmkanalen hos mink. Også stammens spredning i redemiljøet blev undersøgt. Den anvendte stamme var en rifampicinresistent mutant af *Enterococcus faecium* tildelt forsøgsmink i mængden 5×10^9 c.f.u. pr. kg foder. Høje kimtal kunne genfindes i hele fordøjelseskanalen undtagen oesophagus, og kimtallene steg i aboral retning. Bakterien kunne detekteres 4 dage efter sidste tildeling. Der var hverken ved dyrkning eller scanning elektron mikroskopi noget, der tydede på, at den tildelte stamme adhærerede til mucosa. Ingen af de bakterier, der blev dyrket for, viste sig at være konstante fund.

(Received November 28, 1990; accepted July 24, 1991).

Reprints may be requested from: Karl Pedersen, Royal Veterinary and Agricultural University, Department of Veterinary Microbiology, Bülowsvej 13, DK-1870 Frederiksberg C, Denmark.