A novel enzymic therapy, targeted recombinant β-lactamase, in the prevention of antibiotic-induced adverse effects on gut microbiota

Jaana Harmoinen

Helsinki 2004
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Academic dissertation

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Yliopistopaino
Helsinki 2004
To my family: Antti, Kiia, and Atte

Oma perhe on tärkein, kaikki muu tulee vasta sen jälkeen.

(Pikku G, 2003)
A NOVEL ENZYMIC THERAPY, TARGETED RECOMBINANT β-LACTAMASE, IN THE PREVENTION OF ANTIBIOTIC-INDUCED ADVERSE EFFECTS ON GUT MICROBIOTA

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1 ABSTRACT

The indigenous intestinal microbiota forms a dynamic ecosystem, the equilibrium of which is essential for the host’s well-being. This microbiota has been shown to establish a defense mechanism, termed colonization resistance, against potentially pathogenic microorganisms. Colonization resistance can be markedly diminished by use of either oral antibiotics, which enter the gut directly, or parenteral antibiotics, which are excreted into the intestinal tract via bile or via diffusion through the gut wall. Decreased colonization resistance predisposes the host to health-care related i.e. nosocomial infections and antimicrobial resistance, phenomena that today are significant worldwide problems.

Life-threatening nosocomial infections are usually related to the use of broad-spectrum antibiotics, and disturbance in the patient’s microbiota. Further, development of antimicrobial resistance and transfer of resistance genes are most likely to occur in situations where the antimicrobial agent comes into contact with large amount of bacteria such as in the colon. Emergence of resistance can arise not only in treated individuals but also in the environment and society at large. Multiple means for prevention of these undesirable antibiotic-induced consequences are available inter alia the reduction of antibiotic use, and enhancements of hygiene. However, these means have, at present, proved to be limited. In this thesis, a novel enzymic therapy, oral targeted recombinant β-lactamase, as an approach to prevent the adverse effects during parenteral β-lactam antibiotic treatment is introduced.

Per os administered targeted recombinant β-lactamase is hypothesized to degrade parenteral ampicillin in the intestinal tract without affecting serum antibiotic levels (Study II). In this study, permanent small intestinal fistula, surgically attached to jejunum (Study I), was a key method enabling collection of canine small intestinal content for analysis. The enzymic inactivation of the antibiotic results in prevention of antibiotic-induced changes and selective pressure on canine fecal microbiota, thus preventing emergence of antibiotic resistance (Study III). Oral β-lactamase treatment is also hypothesized to preserve colonization resistance in mice (Study IV) treated with parenteral piperacillin and predisposed to per os administered vancomycin-resistant Enterococcus faecium, extended-spectrum β-lactamase producing Klebsiella pneumoniae or Candida glabrata, all microorganisms which cause severe nosocomial infections in human hospital settings.

Serum and jejunal ampicillin concentrations were determined by high-performance liquid chromatography. Molecular biological methods and conventional bacterial culture were used to
evaluate the fecal microbiota of dogs and mice. Ampicillin-induced selective pressure on canine fecal microbiota was examined by performing an antibiotic susceptibility test on coliform isolates and by determining the portion of the TEM gene (i.e. the concentration of \( \beta \)-lactamase-producing genes) in feces.

The results showed that permanent jejunum fistula did not affect intestinal function, and was a suitable method for collection of small intestinal samples for analysis. The findings also indicated that orally administered targeted recombinant \( \beta \)-lactamase degrades parenteral ampicillin in the canine jejunum in a dose-dependent manner without affecting serum ampicillin concentrations. Subsequent studies demonstrated that oral targeted recombinant \( \beta \)-lactamase prevents ampicillin-induced changes in canine fecal microbiota and significantly reduces development of ampicillin resistance during parenteral ampicillin treatment. Further, oral recombinant \( \beta \)-lactamase was shown to be an effective means of preserving colonization resistance in mice treated with parenteral piperacillin and exposed to aforementioned test organisms per os.

In conclusion, oral targeted recombinant \( \beta \)-lactamase offers considerable opportunities to inhibit antibiotic-induced changes in large intestinal microbiota, occurrence of nosocomial infections and emergence of antibiotic resistance. Further research with larger study populations in both humans and animals is warranted to assess the efficacy of \( \beta \)-lactamase treatment in clinical patients.
2 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BIPS</td>
<td>Barium-Impregnated Polyethylene Spheres®</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CLED</td>
<td>Cystine lactose electrolyte-deficient agar</td>
</tr>
<tr>
<td>CR</td>
<td>Colonization resistance</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum β-lactamase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>p.o.</td>
<td>Per os</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>TSCA</td>
<td>Tryptose sulfite cycloserine agar</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature gradient gel electrophoresis</td>
</tr>
<tr>
<td>TRBL</td>
<td>Targeted recombinant β-lactamase</td>
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<tr>
<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
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3 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers referred to in the text by Roman numerals I-IV:


4 INTRODUCTION

The gastrointestinal tract is a large, complex ecosystem consisting of an enormous amount of anaerobic and aerobic bacteria which preserve gastrointestinal function (Batt, 1996). Although the gastrointestinal tract microbiota normally forms a relatively stable ecosystem (Batt, 1996; Mackie et al., 1999), antimicrobial agents can have several undesirable effects on this ecology (Nord and Heimdahl, 1986; Nord and Edlund, 1990; Levy, 1998; Sullivan et al., 2001). Adverse effects induced by the use of oral and systemic antibiotics include decreased colonization resistance, and selection of antimicrobial resistance with subsequent overgrowth of and/or colonization with microorganisms, such as Enterococcus spp., Staphylococcus aureus, Pseudomonas spp., Klebsiella spp., Clostridium difficile, and Candida (Nord and Edlund, 1990; Du et al., 2002). Further, antimicrobial therapy induces establishment of new resistant strains and transfer of resistant genes (Davison et al., 2000).

The prevention of antibiotic-induced changes in gastrointestinal tract microbiota is important because health care related infections and emergence of antimicrobial resistance pose widespread problems both in human and in veterinary medicine. Virtually every antibiotic can cause alterations in the indigenous gut microbiota, but the antimicrobial spectrum of the drug used is the predominant factor causing such changes (Nakaya, 1982; Grossmsan, 1991).

β-Lactam antibiotics (e.g. penicillin, cephalosporins, carbapenems, and monobactams) are still by far the most widely used antibiotic (Livermore, 1998), whilst various degrees of resistance have emerged against these drugs during the past decades (Houndt et al., 2000; Normand et al., 2000). Bacterial resistance against β-lactam antibiotics is most often mediated by bacterial synthesis of either chromosomally or extrachromosomally produced β-lactamases, enzymes that degrade β-lactam rings of the β-lactam antibiotics (Gulay et al., 2000; Heritage et al., 2001; Sideraki et al., 2001). Numerous different β-lactamases have been described and over 200 have been classified into three main groups (1-3) according to their functional characteristics (Bush et al., 1995). TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated β-lactamases of Gram-negative enterobacteria (Brinas et al., 2000).

In the past, the antibiotic resistance was overcome by the development of new antibiotics. However, the expense, inefficiency, and self-defeating nature of this approach have shifted the interest towards preserving the efficacy of existing drugs by improving treatment protocols to maximize
their lifespan (Huovinen and Cars, 1998; Monnet, 1999; Lipsitch et al., 2002). Novel approaches to deal with antibiotic resistance are, anyhow, required, too (van der Waaij and Nord, 2000).

In this thesis, a novel treatment modality, per os administered enzymic therapy – targeted recombinant β-lactamase (TRBL) – for prevention of β-lactam antibiotic-induced adverse effects on gut microbiota was devised and tested in jejunum fistulated dogs and in mice. The therapeutic background of this innovation is based on the knowledge that systemic β-lactam antibiotics can produce adverse effects on gut microbiota. These effects can be inhibited by an oral treatment of targeted recombinant β-lactamase. Despite the degradation of the antibiotic in the intestine, the antibiotic can still be effective systemically, fulfilling its original purpose: therapy against the microorganism causing the systemic disease. The immense amount of bacteria in the intestines remains, however, unaffected by the antibiotic.
5 REVIEW OF THE LITERATURE

5.1 Gut microbiota

The gastrointestinal tract of animals and humans is colonized by a diverse and metabolically active microbiota called the indigenous, autochthonous or normal microbiota (Batt, 1996). The composition of this microbial community varies between different parts of the gut, but the gastrointestinal tract is considered to be the main reservoir of microorganisms both in humans and in animals (Tancrede, 1992; Strombeck, 1996).

Immediately after birth, fecal, oral, and cutaneous microorganisms from the parents and microorganisms from the environment will be transferred to and colonize in the newborn via neonatal care and feeding (Conway, 1997). The pattern and level of microbial exposure during the neonatal period is assumed to influence development of gastrointestinal tract microbiota (Mackie et al., 1999). Although most bacteria are only in transit through the gastrointestinal tract, i.e. few colonize it permanently, the succession during the first weeks of life produces a complex ecosystem which, according to present knowledge, consists of 300-500 bacterial species (Savage 1977; Hentges, 1983; Berg, 1996; Strombeck, 1996; Conway, 1997). In a healthy gastrointestinal tract, all available habitats and niches are occupied by microorganisms. Certain microbes at various times after birth colonize particular intestinal parts characteristic to that particular habitat and animal host (Smith, 1965; Davis et al., 1977; Benno and Mitsouka, 1986; Zentek 1995 and 2000; Benno et al., 1992; Johnston, 1999; Hopkins and Macfarlane, 2002).

Anaerobic bacteria are the predominant microorganisms in the intestine both in dogs and in humans, outnumbering the aerobes by a factor of $10^2$-$10^4$ (Hentges, 1983; Berg, 1996; Strombeck, 1996; Conway, 1997; Holzapfel et al., 1998; Guaner and Malagelanda, 2003).

Although information on the microbiota in the gastrointestinal tract is sparse and incomplete the stomach and upper parts of the small intestine, are postulated to contain low numbers ($10^1$-$10^3$ CFU/g) of microorganisms (Strombeck, 1996). Some microorganisms adhere to the mucosa, but most are only in transit through the stomach and upper small intestine. The microbial population in transit, i.e. the allochthonous, nonindigenous microbiota, can be derived from, for example, the environment or food (Mackie et al., 1999).

The numbers of microorganisms in the stomach and proximal small intestine are restricted mainly because of low pH and peristalsis of the gut (Batt, 1996; Berg, 1996; Strombeck, 1996; Conway,
The predominant microbes in the upper part of the gastrointestinal tract both in humans and in canines are acid-resistant species such as clostridia, lactobacilli, *E. coli*, streptococci, and yeasts (Batt, 1996; Berg, 1996; Strombeck, 1996; Conway, 1997; Mackie et al., 1999).

The quantity of microorganisms increases distally, being $10^2$-$10^8$ CFU/g in the jejunum and ileum, with the composition of microbiota approaching that of the colon (Strombeck, 1996). The most common species of humans and dogs are coliforms (*E. coli*), enterococci, lactobacilli, streptococci, *Bacteroides*, bifidobacteria, and fusobacteria (Holzapfel et al., 1998; Johnston, 1999).

The large intestine harbors a tremendous number of microorganisms ($10^{10}$-$10^{13}$ CFU/g). A large proportion of fecal mass (approximately 60%-75%) is assumed to consist of bacteria (Dunne, 2001; Guaner and Malagelanda, 2003). The large intestine’s slow motility and very low redox potential enable the survival of these microorganisms (Batt, 1996; Berg, 1996). Virtually all (99.9%) colonic microbiota are obligate anaerobes (Hentges, 1983; Batt, 1996; Berg, 1996; Strombeck, 1996). The predominant genera both in humans and in canines are *Bacteroides* and *Bifidobacterium*. However, fusobacteria, enterobacteria, clostridia, *Veillonella*, lactobasilli, *Proteus*, staphylococci, and *Pseudomonas* are also found in large numbers. Yeasts and protozoa are present as well (Strombeck, 1996; Holzapfel et al., 1998; Guaner and Malagelanda, 2003).

Although most of the microorganisms existing in the gastrointestinal tract remain, at present, unidentified, the indigenous intestinal microbiota has been established to play several significant roles in maintaining the host’s health. For example, the intestinal microbiota contributes to digestion of food, metabolism of endogenous and exogenous compounds, immunopotentiation, and prevention of colonization by pathogens (Batt, 1996; Berg, 1996; Conway, 1997; Cummings, 1997; Hooper and Gordon, 2001).

The host, its diet, and the microorganisms themselves all influence the activity and lifespan of the microbiota. As a result, an ecological equilibrium prevails in a healthy gastrointestinal tract amidst numerous symbiotic and competitive interactions. The gastrointestinal tract microbiota is considered to be normal when beneficial rather than deleterious host-microbe interactions predominate (Ewing and Cole, 1994).

### 5.2 Colonization resistance

One important function of autochthonous intestinal microbiota in the gastrointestinal tract is to provide a natural defense against colonization and translocation by exogenous potentially
pathogenic microorganisms or against the overgrowth of indigenous opportunistic. This feature was introduced in 1971 by van der Waaij et al. and coined colonization resistance (CR). It is considered to be a function of normal intestinal microbiota (Tancrede, 1992) and to be related to the population of nonpathogenic indigenous gut microbiota normally residing in the gastrointestinal tract of humans and animals (van der Waaij, 1971; Vollaard and Clasener, 1994). CR is suggested to be related to both anaerobic and aerobic components of gastrointestinal tract microbiota (van der Waaij et al., 1972; Hentges et al., 1985). However, it is not entirely clear which bacterial groups in the indigenous microbiota are involved (Edlund and Nord, 1991; Williams, 2003).

Several anatomical and physiological features of the gastrointestinal tract also influence colonization resistance (van der Waaij et al., 1972; Snel, 2003; Williams, 2003). These include an intact mucosa, salivation, swallowing, secretion of immunoglobulin A, production of gastric acid, and normal gastrointestinal motility. These factors, by limiting the adhesion of potentially pathogenic microorganisms to the mucous membranes and their increase in the gastrointestinal tract, maintain the CR together with the normal gastrointestinal microbiota (van der Waaij, 1989; Vollaard and Clasener, 1994).

Although all features or bacterial groups impacting CR are not yet identified, the most significant cause of decreased CR is known to be the administration of antibiotics (Nord et al., 1984b).

5.3 Obtaining samples from the gut

The host’s well-being is dependent on microbial interactions, especially within the gut (Batt, 1996; Berg, 1996; Conway, 1997; Cummings, 1997; Hooper and Gordon, 2001). However, information on the environment within the small bowel, and the pharmacokinetic properties or effects of drugs in the upper gut is rather limited, the main obstacle being in gaining regular access to small bowel content and mucosa (Zentek, 1995, 2000).

Different techniques have been used to collect intestinal fluid from animals and humans, including laparatomy, endoscopy, and self-opening capsules, and in animals alone, cannulation (Jones et al., 1971; Gurnsey et al., 1986; Brass and Schunemann, 1989; St. Jean et al., 1989; Delles et al., 1994; Hill et al., 1996; Stozzer et al., 1998). There have been many difficulties with these methods. Both laparatomy and endoscopy require general anesthesia in dogs. Patients must fast before the operations, and because of this, the volume of the samples has been minimal (German et al., 2003). Both methods are also time-consuming and do not allow frequent sampling over a longer time period. It is not possible to follow up changes in intestinal content with any of these methods. With
the cannulation models, complications due to the presence of the foreign material of the fistula in the intestine have been numerous, including leakage and/or tissue rejection, abscess, cannula extrusion, and ulceration of the skin (Hill et al., 1996). Because plugs are needed to prevent leakage, collars are required to protect the implants and animals must be housed singly.

5.3.1 Permanent jejunal fistula in dogs

Because of the numerous disadvantages and complications related to previous fistula models, Wilsson-Rahmberg and Jonsson (1997) devised a method for long-term access to the canine small intestine. This fistula method is based on the principle of biological one-way nipple valves that occur naturally in animals and humans in places such as the lower esophageal sphincter, where leakage is not desirable.

In this nipple valve method, the fistula is created from a segment of the dog’s own intestine (e.g. from part of the jejunum) by an intussusception. One end of the fistula is inserted into the intestine and the other end extrudes through the skin to the outside of the body, enabling the sampling of intestinal content.

This fistula provides an easy and repeatable way of obtaining small intestinal content samples. Simply by inserting a catheter or tube through the valve into the intestinal lumen, it is possible to extract samples several times a day. Continuous sampling, lasting several hours, is also possible, and sampling can be performed on a conscious dog, with minimal discomfort to the animal (Wilsson-Rahmberg and Jonsson, 1997).

5.4 Methods of investigating gastrointestinal motility

Intestinal surgery (e.g. fistula operation) as such can potentially affect gastrointestinal transit time (Husebye, 1995), but also disturbances in the composition of intestinal microbiota may contribute to functional bowel symptoms (Maxwell et al., 2002), and the delay of intestinal transit. There are several methods employed to measure gastrointestinal tract motility. However, no practical method is available to quantify transit time in all patients and under a range of circumstances (Guilford and Strombeck, 1990). Limitations exist in all the methods for studying small intestinal motility. Gastrointestinal scintigraphy i.e. evaluation of gastric emptying, small bowel and colonic transit after a radio labeled food, is the current gold standard for diagnosing problems in gastrointestinal function. However, it has a serious limitation in dogs; dogs usually have to be tranquillized for the procedure, which can affect intestinal motility (Guilford and Strombeck, 1990). Further, barium
contrast imaging merely provides an overall impression of the gastrointestinal tract and is thus a relatively insensitive method, and breath hydrogen tests, which measure the metabolism of fermentable substrates (primarily carbohydrates) by the gastrointestinal microbiota (Summers and Soffer, 1992), are easy to perform in humans but are less practical in dogs.

In recent decades, radiopaque markers, such as Barium-Impregnated Polyethylene Spheres®, have been used to measure gastric emptying in humans (Guilford and Strombeck, 1990). In dogs, BIPS (Chemstock Animal Health Ltd, Christchurch, New Zealand) are used to diagnose gastrointestinal motility disorders (Frazer et al., 1996). Unlike liquid barium, BIPS mimic the normal passage of food through the intestines, and thus, have proved to be sensitive markers for gastric emptying in dogs (Guilford and Strombeck, 1990). Today, BIPS are considered to be both easy to use and helpful in diagnosing functional or physical partial obstructions in dogs.

5.5 Methods of investigating gastrointestinal microbiota

5.5.1 Conventional bacterial culture

The gastrointestinal microbiota has traditionally been investigated by conventional bacterial culturing. Because certain gastrointestinal tract bacteria have fastidious growth requirements, the use of appropriate media and anaerobic techniques, especially when culturing samples in which the organisms are present in low numbers, is essential for optimal results (Corpet, 1993). Prior to culture, samples are diluted and inoculated on several selective and nonselective agars. Nonselective media are used to estimate the total numbers of anaerobic and aerobic bacteria in a sample, whereas selective agars are chosen species-specific (O’Sullivan, 1999, 2000).

Although, culture-dependent methods are powerful means for evaluating gastrointestinal microbiota (O’Sullivan 1999, 2000; Edlund and Nord, 2001), they have disadvantages. The culture results are acknowledged to represent only a small proportion of the bacterial diversity (Olsen, 1990) since several bacteria that can be seen by direct microscopic examination of diluted fecal samples cannot be cultured (Guaner and Malagelada, 2003). Further, culture methods are laborious and have technical limitations (Welling, 1997). All bacterial species cannot be cultured by standard techniques, and the actual selectivity power of the medium used is not always known (Corpet, 1993; Welling, 1997; O’Sullivan, 1999, 2000). Thus, a number of culture-independent techniques have been developed to detect target bacteria in complex ecosystems and to complement microbiological
information yielded by conventional approaches (Wang et al., 1996; Ranjard 2000; Vaughan et al., 2000; Dunne, 2001; Tannock, 2002).

5.5.2 Molecular biological methods

In molecular methods, the microbes are grouped according to their genes (Muyzer, 1999). In genetic fingerprinting technique (Hugenholtz et al., 1998; Muyzer and Smalla, 1998; Muyzer, 1999; Zoetendal et al., 1998; Vaughan et al., 2000; Tannock, 2002) all nucleic acids are extracted from the sample (e.g. from feces), and a variable region of the 16S ribosomal ribonucleic acid (rRNA) gene is amplified by polymerase chain reaction (PCR). The PCR products are analyzed by gel electrophoresis, in which the double-stranded 16S ribosomal deoxyribonucleic acid (rDNA) fragments migrate through a polyacrylamide gel and become partially denatured. The denaturation in Denaturing Gradient Gel Electrophoresis (DGGE) is caused by chemical conditions and in Temperature Gradient Gel Electrophoresis (TGGE) by increasing temperature. Finally, in ideal situation, each bacterial type is represented by a DNA fragment in band profiles obtained from the sample. The band profiles are computerized and similarities between profiles calculated.

Although neither DGGE nor TGGE allows discrimination between species, these methods have been shown to be well suited for comparing large sets of samples, including fecal samples, from the same individual and for detecting similarities and changes between samples. Further, the genetic fingerprints provide complex band profiles, and reflect the predominant bacteria in the sample (Zoetendal et al., 1998). The profile represents 90-99% of the total bacterial community (Zoetendal et al., 1998; Ranjard et al., 2000).

There are also limitations related to both DGGE and TGGE. Different sample handling procedures and the extraction of DNA from bacterial cells in the samples can affect species composition (Muyzer and Smalla, 1998). To avoid these sources of error, the samples should be handled, stored, and extracted by the same procedure. Moreover, the amount of the microorganism limits the analysis because only organisms present in relatively high concentrations ($\geq 10^8$ CFU/g) are represented on the gel (Muyzer, 1999).

Regardless of these limitations, both DGGE and TGGE are well-established methods for studying changes in natural microbial communities. These techniques have been shown to be reliable and reproducible (Muyzer, 1999). Samples taken at different time intervals can be analyzed
simultaneously; thus, similarities and/or differences in the microbial community after an environmental change, such as antimicrobial administration, can be analyzed (Muyzer et al., 1998).

5.6 Antibiotic resistance

Today, antibiotic resistance is considered a threat to public health (Levy, 1998; Huovinen, 1999; Davison et al., 2000) since a great number of microorganisms in humans and animals, and both in hospitals and in the community thwart treatment because they are resistant to one or several antibiotics (Levy, 2002).

From the evolutionary perspective, antibiotic resistance is a natural consequence of bacterial cell adaptation upon exposure to antimicrobials (Sèveno et al., 2002), thus being an unavoidable phenomenon (Courvalin and Trieu-Cuot, 2001). It is a direct competitive advantage conferred by the resistant phenotype (Sèveno et al., 2002).

Although, it is generally accepted that the main risk for the increase in resistance is the use of antimicrobials, development of resistance depends on the microorganism involved and on the drug used i.e. on the spectrum of the drug (Andremont et al., 2001). Other predisposing factors for emergence of antibiotic resistance include increased severity of illnesses, more severe immunocompromise, newer devices and procedures, and resistance in the community (Patterson, 2001). Of concern is also the observation of the transfer of resistant bacteria from animals to humans. For example, multi-drug resistant zoonotic bacteria e.g. Salmonella and Campylobacter have emerged in many regions of the world (van den Bogaard and Stobberingh, 1999).

5.6.1 Intrinsic and acquired resistance

Bacteria can be naturally resistant to some antibiotics i.e. bacteria can have intrinsic resistance properties (Stark et al., 1993; Davison et al., 2000; Sorum and Sunde, 2001; Newman and Seidu, 2002). Intrinsic resistance is genus or species-specific and based, for instance, on lack of target sites for the antimicrobial agent (Schwarz and Chaslus-Dancla, 2001). In acquired resistance microorganisms have become resistant to antibiotics through chromosomal mutations or by acquisition of resistant traits from another microorganism (Davison et al., 2000; Handal and Olsen, 2000; Prescott, 2000).

Mutation is the single form of acquired resistance. It is a spontaneous but generally gradual, stepwise process and transfer of mutations takes place during multiplication (vertical transfer)
Acquired resistance, however, is most often associated with the acquisition of mobile elements, which contain antibiotic resistance genes (Davison et al., 2000; Schwarz and Chaslus-Dancla, 2001).

In transformation, bacteria acquire segments of free DNA from the environment. For instance, the death of one bacteria and the release of its DNA makes it available to another cell, usually among closely related genera (Handal and Olsen, 2000). In transduction, exogenous DNA is incorporated into a bacteriophage and transferred to another bacterium (Sèveno et al., 2002).

In conjugation, a donor bacterium attaches to a recipient in the mating process and transfers resistance genes to this recipient, which now becomes a potential donor (Handal and Olsen, 2000). This mechanism occurs between bacterial strains of the same species, within species of the same genus, or even between species belonging to different families (Sèveno et al., 2002). Via conjugation, bacteria can transfer genetic material found on plasmids and transposons. Plasmids are small loops of extrachromosomal circular DNA that may contain antibiotic resistance gene (R-plasmids), and can be easily transferred between bacteria. Plasmids replicate independently of bacterial chromosomal DNA (Schwarz and Chaslus-Dancla, 2001; Sèveno et al., 2002). Transposons (“jumping genes”) are short sequences of DNA that can easily move from plasmid to plasmid, from plasmid to chromosome, from chromosome to plasmid, or between a plasmid and a bacteriophage (Livermore, 2000; Schwarz and Chaslus-Dancla, 2001). Unlike plasmids, transposons are not able to replicate independently (Davison et al., 2000; Schwarz and Chaslus-Dancla, 2001).

There is also a class of small mobile genetic elements, distinct from transposons, called integrons/gene cassettes. Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination (Schwarz and Chaslus-Dancla, 2001). The antibiotic resistance genes that integrons capture are located on gene cassettes. Integrons can be located in chromosomal DNA, but are more often located in plasmids or transposons (Schwarz and Chaslus-Dancla, 2001).

Genetic exchange is a very significant phenomenon. The transferred genetic material responsible for resistance can reproduce and then spread to other cells. This may lead to a marked increase in resistance. For example, minor differences in molecular structure can result in dramatic differences in the function of the β-lactamase enzyme (Livermore, 2000). Because bacteria can collect multiple resistance traits over time, they can become resistant to many different families of antibiotics.
Multiresistant bacteria may persist in the host or in the environment in the absence of antibiotic selection, and they may act as reservoirs for resistant genes that can later spread to other bacteria (Prescott, 2000).

5.6.2 Mechanisms of resistance

Antibiotic resistance may appear via a number of mechanisms. These mechanisms include enzymatic inactivation of the drug (e.g. β-lactamases); decreased permeability of the organism to the drug (common in Gram-negative bacteria in which a change in porins or transport proteins occurs); active expulsion of the drug (e.g. cell efflux pump for tetracyclines); alteration in target receptors (e.g. β-lactams and altered penicillin binding proteins (PBPs); development of alternate metabolic pathways (e.g. trimetoprin and plasmid-mediated dihydrofolate reductase production), and altered enzymes which can function in the presence of the drug (e.g. aminoglycoside resistance in enterococci). Other drug resistance mechanisms comprise increased formation of the metabolites competing with the drug, overproduction of a target enzyme, and changes in ribosomal protein structure (Ewing and Cole, 1994; Prescott, 2000). Multidrug resistance can arise if all or even some of these mechanisms occur simultaneously.

5.6.3 Determination of resistance

Testing the susceptibility of bacteria to antimicrobials is used to guide the choice of treatment for individual patient, but par excellence susceptibility testing is fundamental for determination of antibiotic resistance and for resistance surveillance studies (Greenwood, 2000, Davison et al., 2000).

Bacterial susceptibility against different antimicrobials can be tested by several different methods in vitro. These include dilution methods (e.g. agar and broth dilution), disc diffusion method, and other methods (e.g. E-test®) (Greenwood, 2000). Although these methods differ from each other, they all use the inhibition of the bacterial growth as end point. Qualitative test results are reported in terms of susceptibility category (i.e. susceptible, intermediate or resistant) and quantitative results (µg/ml) as the minimal inhibitory concentration (MIC) (Walker, 2000). MIC is defined as the lowest drug concentration that inhibits the visible bacterial growth after an overnight incubation (Davison et al., 2000; Andrews, 2001).

The most recent approach for detection of resistance is by molecular biology based methods for identification and characterization of known genes encoding specific resistance mechanisms
These genotypic methods are important supplements to traditional phenotypic methods (Greenwood, 2000). Thus, in the future, it may be feasible to combine susceptibility tests with molecular based methods, to provide data for clinicians, as well as for surveillance and monitoring programmes (Davison et al., 2000; Greenwood, 2000).

5.7 Nosocomial infections

Infections acquired during or immediately after hospitalization are known as nosocomial or healthcare-associated infections (Farr, 2002). Today nosocomial infections, by far the most common complication affecting hospitalized patients, are often related to the use of broad-spectrum antibiotics such as β-lactam antibiotics. Nosocomial infections can be caused by microorganisms that have lived in harmony with the host for many years without any deleterious effects and only become harmful when the microbial ecosystem is disturbed in some way (Mackie et al., 1999; Guarner and Malagelada, 2003), or a healthcare procedure has impaired the host’s defense (Farr, 2002; Sèveno et al., 2002). Such microorganisms as Enterococcus spp., Staphylococcus aureus, Pseudomonas spp., E. coli, Klebsiella spp., or Candida are known to be contagious and cause severe nosocomial infections (Farr, 2002; Ray and Donskey, 2003). As an example, antibiotic intervention destabilizes the normal intestinal microbiota and destroys e.g. commensal antibiotic-susceptible enterococci. Antibiotic-resistant enterococcal strains can now colonize the niche that lacks commensal enterococci, and the gastrointestinal tract then serves as a source of infection for other parts of the patient, e.g. bloodstream, wounds, etc. (Gilmore and Ferretti, 2003).

Microorganisms in the gastrointestinal tract can be affected by antibiotics due to the incomplete absorption of an orally administered antibiotic, secretion of a parenteral antibiotic via bile into the intestines, or secretion of a drug concentrated in the intestinal mucosa (Nord and Heimdahl, 1986; Edlund and Nord, 2000). Several β-lactam antibiotics have been reported to produce derangements in gastrointestinal tract microbiota (Sullivan et al., 2001). Vancomycin, ampicillin, and third-generation cephalosporins are known to promote vancomycin-resistant enterococci (VRE) infections (Donskey et al., 2000a, 2000b) or persistent high-density VRE colonization (Donskey et al., 1999). Among the risk factors predisposing to infections with Candida glabrata and extended-spectrum β-lactamase-producing Klebsiella pneumoniae is the use of broad-spectrum antibiotics that disturb the composition of the indigenous gastrointestinal tract microbiota, permitting these microorganisms to thrive (Samonis et al., 1994; Kullberg and Oude Lashof, 2002).
The most important sites of nosocomial infections are the urinary tract, surgical wounds, the respiratory tract, and skin. Nosocomial infections afflict two to five million patients in United States annually and contribute to death of approximately 88,000 deaths (Peterson and Noskin, 2001). In Finland over 50,000 nosocomial infections occur per year in human hospitals, and these contribute to death of over 2000 patients (Lyytikäinen et al., 2003).

Nosocomial infections are reported also in veterinary medicine, and are likely to increase in prevalence as intensive care practices increase in many animal hospitals. However, exact worldwide statistics of nosocomial infections in veterinary hospitals are not available. In Finland, approximately 10% of class I and II surgical wounds in small animal unit of Veterinary Teaching Hospital in University of Helsinki are infected annually (Rantala, M. 2004, personal communication, retrospect study concerning year 2000). Nosocomial infections in veterinary medicine are of concern, since these infections may be transmitted to humans, too. For example in Finland, in large animal unit of Veterinary Teaching Hospital in University of Helsinki, hospital acquired *Salmonella Typhimurium* epidemic occurred infecting also two humans (Kallio et al., 2004).

Since, nosocomial infections and antimicrobial resistance can be considered as a major public health importance, and development of such adverse effects a complex “chain reaction”, both national and global infection control programs for prevention and control of nosocomial infections and prevention of emergence of antimicrobial resistance are elaborated both in human and in veterinary medicine (e.g. World Health Organization’s strategies [www.who.int/csr/resources/]; community strategy against antimicrobial resistance [http://europa.eu.int/scadplus/leg/en/cha], or FINRES-Vet, [www.eela.fi]).

### 5.8 β-Lactam antibiotics

β-Lactam antibiotics (e.g. penicillins, cephalosporins, monobactams, and carbapenems) belong to a family of antibiotics characterized by a β-lactam ring. The integrity of the ring is necessary for the mode of action – interference in the biosynthesis of the bacterial cell wall (Handal and Olsen, 2000).

The spectrum of activity of β-lactams ranges from narrow (penicillin) to very broad (carbapenems). β-Lactams are considered to be clinically safe and reliable, hence they are widely used in human and in veterinary medicine (Livermore, 1998; Handal and Olsen, 2000). β-Lactam antibiotics account for 50% of global antibiotic consumption in humans (Livermore, 1998). Worldwide data of
\( \beta \)-lactam use in veterinary medicine are not available, but in Finland they account 60% of antimicrobial consumption (Koppinen et al, 2003).

The widespread clinical use of \( \beta \)-lactams has resulted in the emergence of \( \beta \)-lactam-resistant organisms. Resistance to \( \beta \)-lactam antibiotics can be due to decreased uptake, increased efflux, modified target, or especially due to production of inactivation enzymes i.e. \( \beta \)-lactamases (Livermore, 1995; Handal and Olsen, 2000).

### 5.9 \( \beta \)-Lactamases

\( \beta \)-Lactamases constitute a heterogeneous group of enzymes differing in the species of bacteria producing them. \( \beta \)-Lactamases can be of plasmid or chromosomal origin, constitutively expressed (i.e. stable and unchanged \( \beta \)-lactamase production) or inducible. These enzymes alter the chemical structure of the \( \beta \)-lactam antibiotic irreversibly, abolishing its antibacterial activity (Livermore, 1995; Handal and Olsen, 2000). Their mechanism of action can differ, as well as the spectrum of antibiotics they are capable of hydrolyzing (Bush et al, 1995).

In Gram-positive bacteria, \( \beta \)-lactamases are located mostly in cell wall and excreted outside of the cell, whereas in Gram-negative bacteria, they are secreted into the periplasmic space i.e. the same place that is the site of action of the \( \beta \)-lactam antibiotic; thus a relatively small amount of enzyme can generate a sufficiently high concentration of enzyme to overwhelm susceptible antibiotics (Livermore, 1995).

Over 200 distinct \( \beta \)-lactamases have been isolated, and on the basis of their functional characteristics (substrate spectra and inhibition profile), the enzymes have been divided into three major groups (Bush et al, 1995). Group 1 represents cephalosporinases which are insensitive to inhibition by clavulanic acid. Group 2 penicillinases, cephalosporinases, and broad-spectrum \( \beta \)-lactamases are generally inhibited by active site-directed \( \beta \)-lactamase inhibitor (clavulanic acid). Group 3 contains penicillinases, cephalosporinases, and carbapenemases which are not inhibited by clavulanic acid (Bush et al, 1995).

The most wide-spread of \( \beta \)-lactamase are those classified as TEM-type \( \beta \)-lactamases. TEM-1 is the most commonly isolated \( \beta \)-lactamase from human clinical isolates and produced by both Gram-positive and Gram-negative bacteria (Livermore, 1995; Payne et al., 2000). For instance, TEM-1 \( \beta \)-lactamase is the prevailing \( \beta \)-lactamase found in *Enterobacteriaceae*. Resistance in 50-96% of
ampicillin-resistant *E. coli* clinical isolates is due to TEM-1 β-lactamase (Huovinen et al., 1988; Newman and Seidu, 2002).

Production of β-lactamases is the most common mechanism of resistance to penicillins, thus, over the years penicillins have been replaced by other antibiotics (Wright, 1999). However, β-lactam antibiotics are likely to remain as a form of antimicrobial therapy also in the future (Moreillon and Majcherczyk, 2000).

### 5.10 The fight against resistance

It has become increasing clear over the last decade that the future of antibiotic treatment depends on the evolution of resistance, both among pathogens targeted by therapy and among commensal microbiota (Andremont et al., 2001). It is also agreed that the development of resistance can limit the effective period of a new antibiotic (van der Waaij and Nord, 2000; Lipsitch et al., 2002). As a consequence of the emergence of antibiotic resistance, multiple and often complex recommendations to reduce this phenomenon have been made (Lipsitch et al., 2002). For example, in hospital settings, reduction in the use of some or even all antimicrobial drug classes combined with increased use of prophylactic antimicrobials to reduce colonization, antibiotic cycling in a temporal sequence, simultaneous use of different antimicrobials for different patients, screening of colonized patients to prevent spreading, and hygiene and management approaches have been instigated (Burke et al., 1998; John et al., 2000; Farr, 2002; Lipsitch et al., 2002). In animal husbandry, diminishing the need for antibiotics is possible by improvement of animal husbandry systems, feed composition and eradication of or vaccination against infectious diseases. Abolishing the use of antibiotics as growth promoters in animals bred for food would decrease the use of antibiotics in animals on a worldwide scale by nearly 50% (van den Bogaard and Stobberingh, 1999). Further, amelioration of gut flora by orally administered probiotics (Orrhage and Nord, 2000) or other bacteriotherapy (Huovinen 2001; Tagg and Dierksen, 2003) and exploitation of the antibacterial characteristics of bacteriocins or antimicrobial peptides (Joerger, 2003) have aroused interest as a means of reducing antibiotic use or even substituting for them in the fight against antibiotic resistance both in human and in veterinary medicine.

Because of the failure to significantly reduce antimicrobial use and the subsequent emergence of antimicrobial resistance, a novel approaches to deal with this problem are invariably and urgently required (van der Waaij and Nord, 2000). The importance of studies on antimicrobial inactivation within the digestive tract has been highlighted. de Vries-Hospers et al. (1993) has suggested the
possibility of using antibiotic-inactivating molecules as an oral treatment adjusted to the antibiotics used in therapy, and Van der Waaij and Nord (2000) have presented the concept of intra-intestinal inactivation of antimicrobial agents as part of the solution for antimicrobial resistance.

5.11 β-Lactamase treatment

In animal husbandry, an antibiotic inactivation-based treatment modality to destroy penicillin residues in milk during and after antibiotic treatment of cows is already available. Antibiotic residues are destroyed adding a commercially available β-lactamase enzyme to milk (Kroycka-Dahl et al., 1985; Suomen Eläinlääkkeet, 2003). As a result, calves can be fed milk in which no active antibiotic remains.

In this thesis, a new approach to the antibiotic inactivation-based modality, an orally administered therapy - targeted recombinant β-lactamase (TRBL) - is devised and tested in dogs and mice. The purpose of this β-lactamase treatment modality is to degrade intestinal antibiotic during parenteral treatment with a β-lactam antibiotic e.g. ampicillin or piperacillin.

Proportion of parenteral ampicillin and piperacillin is excreted into the bile (Mandiola et al., 1972; Morris et al., 1983). The antibiotics then enter via bile the upper part of the small intestine, and a portion of the drug may also enter the intestines via diffusion through the gut wall.

Some of this intestinal antibiotic is reabsorbed, and the remainder drifts aborally into the intestines causing changes in microbiota and inducing emergence of antibiotic resistance (Figure 2a). The nonabsorbed portion of the antibiotic in the gut is the object of the recombinant β-lactamase action – the enzyme meets the β-lactam antibiotic and, being a β-lactamase, degrades its β-lactam ring (Figure 1). After the β-lactam ring is degraded, the antibiotic is nonfunctional, i.e. no active antibiotic is left in the gut. Thus, the microbiota remains unchanged and selective pressure is averted (Figure 2b).

Figure 1. Schematic molecular structure of basic penicillin. The arrow indicates the β-lactam ring that will be degraded by TRBL. To get various extended-spectrum penicillins R site is replaced with different molecules.
Figure 2a. Parenteral antibiotic treatment without oral TRBL. Active antibiotic enters the gut and causes changes on the microbiota, and induces the development and spread of antibiotic resistance (R).

Figure 2b. Per os administered TRBL degrades the nonabsorbed portion of ampicillin, i.e. no active antibiotic is left in the gut, thus subverting changes on microbiota and emergence and spread of antibiotic resistance. Rounds in stomach illustrate TRBL pellets on their way into the gut.
6 AIMS OF THE STUDY

The aims of this study were

I. to evaluate the feasibility of permanent jejunal fistula for small intestinal sampling by:

   a) Determining does the surgery effect on intestinal function, i.e. on transit time and microbiota composition

II. to evaluate the abilities of oral recombinant $\beta$-lactamase treatment to:

   a) Degrade parenteral ampicillin in the canine jejunum without affecting serum antibiotic concentration.

   b) Prevent ampicillin-induced changes in the canine gut microbiota and inhibit emergence of ampicillin resistance.

   c) Inhibit fecal colonization of mice exposed per os to vancomycin-resistant Enterococcus faecium, extended-spectrum $\beta$-lactamase-producing Klebsiella pneumoniae, or Candida glabrata in conjunction with parenteral piperacillin treatment.
7 MATERIALS AND METHODS

7.1 Experimental design

Flowcharts 1-4 present the experimental designs and sample collection procedures of Studies I-IV. More detailed information on study protocols and methods is given below.

Ethical approval

The experimental protocols were approved by the local ethics committee for animal experimentation in Helsinki, Finland (Studies I, II, and III) and in Cleveland, OH, USA (Study IV).

Clinical condition and housing

All of the animals were clinically examined prior to the experiments and declared healthy. None (dogs or mice) had previous exposure to any antimicrobials.

Between experiments dogs were allowed to socialize with each other and participate in outdoor activities in group. During the experiments the dogs were housed and treated singly. For outdoor activities all the dogs used the same pen. Coprophagy was avoided by cleaning feces from the premise after each dog.

Mice were housed in individual cages with plastic filter tops to prevent cross-contamination among animals.

7.1.1 Evaluation of the permanent jejunal fistula operation (Study I)

Fistula operation

In Study I, permanent jejunal fistulas were surgically attached 60 cm distal to the pylorus to seven laboratory beagles using a method devised by Wilsson-Rahmberg and Jonsson (1997). The dogs (6 males and 1 female) were obtained from the National Laboratory Animal Center (University of Kuopio, Finland). Ages of the dogs ranged between 1 and 2 years and weights between 9 and 14 kg.

The surgery was done under general anesthesia through a midline abdominal incision. An approximately 25-cm segment of the small intestine was separated by dividing the mesentery between the mesenteric vessels. An end-to-end anastomosis was performed on the free ends of the intestine.
An intussusception was made in the isolated intestinal segment. The invagination was fixed with deep, wide, interrupted sutures. The nipple was inserted and sutured in an end-to-side anastomosis to the small intestine. A measuring tape was used to determine the exact place for the fistula at the beginning of the operation.

The proximal end of the segment was pulled out of the abdominal cavity through a hole made perpendicular to the skin and abdominal muscle layers. The end of the nipple was sutured to the parietal peritoneum and abdominal muscles. Finally, the abdominal incision was closed.

No prophylactic or postsurgical antibiotics were used. Buprenorphine 0.03 mg/kg (Temgesic®, Reckitt & Colman, Hull, England) was used for analgesia. Recovery time for complete healing of the fistula was four weeks. During this time the dogs wore Elizabethan collars and were singly housed and walked to avoid licking of the nipple valve by themselves or by other animals. Postoperative feeding, commencing the day after surgery, consisted of small portions of regular dog food (Baron Complete, Raisio Feed Ltd, Raisio, Finland) provided frequently. At one month postsurgery, the feeding routine returned to normal. At this time, the collars were removed and the dogs were allowed to socialize with one another.
Intestinal motility

To ensure that the fistulation did not affect intestinal transit time, the small intestinal transit was determined by using Barium-Impregnated Polyethylene Spheres\textsuperscript{®} (BIPS, Chemstock Animal Health Ltd, Christchurch, New Zealand) two weeks before and five weeks after the operation. Each dog was fed 10 large and 30 small BIPS markers with a small portion (25\% of daily calorie requirement) of food. The dogs were radiographed 8 and 12 h after feeding. Motility was assessed by calculating the orocolic transit percentages using the following formula:

\[
\text{Orocolic transit } \% \text{ of BIPS} = \frac{\text{max} - \text{O.C.}}{\text{max}} \times 100\%
\]

, where max is the maximum number of BIPS fed to the dog (10 large and 30 small) and O.C. is the number of BIPS in the radiographs which had not reached the colon.

Jejunal and fecal microbiota

To determine whether the fistula operation had an effect on the microbiota of the small intestine or feces, samples from four dogs were analyzed by bacterial culture. Samples were collected immediately before the operation and eleven weeks post-surgery.

Fecal samples were extracted manually per rectum. Jejunal samples were taken intraoperatively by aspirating aseptically using a catheter and a syringe. Postsurgical jejunal samples were obtained by inserting a silicon tube through the nipple valve into the jejunum when the dog was awake and standing, i.e. without any sedatives or other medications.

Flowchart 1. Study I protocol.

<table>
<thead>
<tr>
<th>Intestinal motility (BIPS)</th>
<th>Fistula operation and samples for bacterial culture</th>
<th>Intestinal motility (BIPS)</th>
<th>Samples for bacterial culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 2 weeks</td>
<td>0</td>
<td>+ 5 weeks</td>
<td>+ 11 weeks</td>
</tr>
</tbody>
</table>

Flowchart 1. Study I protocol.
7.1.2 Dose-response effect of TRBL on jejunal ampicillin (Study II)

Study II examined the capacity of oral targeted recombinant β-lactamase enzyme (TRBL) to degrade intravenous ampicillin in the canine jejunum dose-dependently. All of the beagles that survived from Study I (5 males and 1 female) and three other beagles (all males) obtained from the Faculty of Veterinary Medicine (University of Helsinki, Finland) were included in this study (n=9). Ages of the dogs ranged between 1 and 2 years and weights between 9 and 14 kg.

Due to the limited number of laboratory beagles, several dose-response studies were conducted to get 5-6 animals treated per dose. The different treatment groups in Study II are presented in Table 1.

<table>
<thead>
<tr>
<th>number of dogs/treatment</th>
<th>TRBL mg/kg p.o.</th>
<th>Amp. mg/kg i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>0.003</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>0.03</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1. Study II treatment groups. TRBL p.o. = targeted recombinant β-lactamase enzyme dosed per os; Amp. i.v. = ampicillin dosed intravenously.

Dogs were fed (Pedigree®, Fortivil 400 g, Waltham®, Masterfoods Oy, Helsinki, Finland) 30 min before and TRBL administered 3 min before intravenous ampicillin medication (Flowchart 2). Serum, jejunal, and fecal samples were collected for ampicillin analysis, and jejunal and fecal samples for β-lactamase analysis. Serum samples were taken from the vena cephalica. Jejunal samples were collected by inserting a silicon tube through the nipple valve into the jejunum, and fecal samples were taken from defecated stool before any treatment and on the following morning after the treatment. Serum and jejunal sampling started 15 min after ampicillin administration and continued for 5 h.

<table>
<thead>
<tr>
<th>Fecal samples</th>
<th>Feeding</th>
<th>TRBL p.o.</th>
<th>Amp. i.v.</th>
<th>Sampling starts</th>
<th>Sampling ends</th>
<th>Fecal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 30 min</td>
<td>3 min</td>
<td>0</td>
<td>+ 15 min</td>
<td>+ 5 h</td>
<td>≥ 24</td>
<td></td>
</tr>
</tbody>
</table>

Flowchart 2. Study II protocol.
7.1.3 Prevention of ampicillin-induced changes on canine fecal microbiota by TRBL (Study III)

Study III evaluated the capacity of oral TRBL to prevent antibiotic-induced changes on the canine gut microbiota during parenteral ampicillin administration. To yield information about the lower part of the small intestine, permanent jejunal fistulas were surgically attached 170 cm distal to the pylorus to a total of 18 male laboratory beagles obtained from Harlan-Winkelmann GmbH (Borchen, Germany) and the National Laboratory Animal Center (University of Kuopio, Finland). The dogs’ ages ranged from 1 to 3 years and their weights from 12 to 19 kg.

The three different treatment groups in Study III are presented in Table 2.

<table>
<thead>
<tr>
<th>number of dogs/group</th>
<th>TRBL mg/kg p.o</th>
<th>Amp. mg/kg i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.6</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Study III treatment groups. TRBL p.o. = targeted recombinant β-lactamase enzyme dosed per os; Amp. i.v. = ampicillin dosed intravenously.

Dogs were treated four times a day. Total duration of the treatment was 14 days. Dogs were fed (Pedigree®, Fortivil 400 g, Waltham®, Masterfoods Oy, Helsinki, Finland) 30 min before each intravenous ampicillin/placebo medication. TRBL/placebo was administered twice, 10 and 3 min before each ampicillin/placebo administration (Flowchart 3).

Samples were collected before (day 0), during (day 1, day 4, day 10, and day 14), and after (day 28) the treatment period. Serum samples were taken from the vena cephalica. Jejunal samples were collected into sterile containers by inserting a silicon tube through the nipple valve into the jejunum. Serum and jejunal samples were collected once on each sampling day, 1 h after the first antibiotic administration of the day (Flowchart 3). Fecal samples were taken from fresh defecated stool (within 1 h of defecation). Samples were collected into sterile containers and frozen at -70°C until prepared or sent for analysis. Persons running the tests did not know from which group the samples originated.
Flowchart 3. An example of a daily timetable for one of the 18 dogs in Study III.

7.1.4 Preservation of fecal colonization resistance in mice by oral β–lactamase treatment (Study IV)

Study IV determined whether oral β-lactamase treatment preserves colonization resistance in mice treated with subcutaneous piperacillin and exposed to one of the following per os administered microorganisms: vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamase-producing Klebsiella pneumoniae, or Candida glabrata. Female Sprague-Dawley CF-1 mice, 8-12 animals/treatment group, each animal weighing 25-30 g, were purchased from Harlan (Indianapolis, IN, USA).

The combinations of subcutaneous (s.c.) and oral treatments used in Study IV are presented in Table 3.
Three important microorganisms that colonize the human intestinal tract during hospitalization were studied. VRE strain C68 is a vanB-type vancomycin-resistant *Enterococcus faecium* strain isolated from the wound of a patient in Cleveland, OH, USA. The minimum inhibitory concentrations of vancomycin, piperacillin, and ampicillin for this VRE strain C68 were 512, 1250, and 256 µg/ml, respectively (Donskey et al., 1999). *Klebsiella pneumoniae* strain P62 was a bloodstream isolate that produces an SHV-type extended-spectrum β-lactamase (ESBL). The minimum inhibitory concentrations (MICs) for P62 were determined by E-test® (AB BIODISK, Piscatway, NJ, USA) and confirmed using a macrodilution technique in a Mueller-Hinton broth (Becton Dickinson, Sparks, MD, USA). The MICs for P62 were 16 µg/ml for ceftazidime and 4 µg/ml for piperacillin/tazobactam. The *Candida glabrata* strain used was isolated from the bloodstream of a patient at the Louis Stokes Cleveland Veterans Affairs Medical Center (Cleveland, OH, USA).

To confirm that the protective effect of the β-lactamase required a biologically active enzyme, one group of mice in the VRE experiments was treated with piperacillin and oral β-lactamase that was inactivated by adding a 100-fold millimolar excess of tazobactam, an effective inhibitor of β-lactamase. At 24 and 12 h prior to oral inoculation of test organisms, mice were treated with piperacillin/placebo s.c. according to their respective protocols (Table 3 and Flowchart 4). Oral inoculation of microorganisms, PBS and β-lactamase was performed using a stainless steel feeding tube (Perfektum, Popper & Sons, New Hyde Park, NY, USA). $10^4$ colony-forming units (CFU) of VRE, and $10^8$ CFU of ESBL-producing *K. pneumoniae*, and *C. glabrata* were inoculated. Fresh stool samples were collected 1, 3, and 6 days after oral inoculation.

### Table 3. Treatment combinations used in Study IV. PBS = phosphate-buffered saline.

<table>
<thead>
<tr>
<th>Subcutaneous</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (0.2 ml)</td>
<td>PBS (0.5 ml)</td>
</tr>
<tr>
<td>piperacillin (10.7 mg)</td>
<td>PBS (0.5 ml)</td>
</tr>
<tr>
<td>piperacillin (10.7 mg)</td>
<td>β-lactamase (0.5 ml)</td>
</tr>
</tbody>
</table>

35
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>- 24 h</td>
<td>- 12 h</td>
<td>0</td>
<td>+ 1 day</td>
<td>+ 3 days</td>
<td>+ 6 days</td>
</tr>
</tbody>
</table>


7.2 Drug substances

7.2.1 Injectable drug substances

Na-Ampicillin (A-PEN® inject 1 g, Orion Pharma, Espoo, Finland) was used as intravenous antibiotic treatment in Studies II and III.

NaCl (Natrosteril® 9 mg/ml 0.9% infusion, 100 ml, Orion Pharma, Espoo, Finland) was used as intravenous placebo medication in Study III.

Piperacillin (Piperacillin sodium salt, Sigma-Aldrich Company, St. Louis, MO, USA) was used as subcutaneous antibiotic treatment, and PBS (phosphate-buffered 0.9% saline, pH 7.4) as subcutaneous placebo treatment in Study IV.

7.2.2 Oral drug substances

For Studies II, III, and IV, recombinant β-lactamase, a penicillinase containing the PenP protein of Bacillus licheniformis 749/C (Neugebauer et al., 1981), was received from Ipsat Therapies Oy (Espoo, Finland). A purified recombinant β-lactamase was freeze-dried and used, in Studies II and III, as the biologically active substance in manufacturing enteric-coated pellets (Röhm GmbH&Co KG, Darmstadt, Germany).

Two pellet formulations differing from each other with the thickness of the outer layer of the pellet were used - in Study II pellets with thin layer, and in Study III pellets with ticker layer. For oral administration (Study III), TRBL and placebo pellets were packed into hard gelatin capsules (Capsugel, size 0, University Pharmacy, Helsinki, Finland) according to the dose and treatment for
each individual dog. Pellets without the enzyme were used as an oral placebo treatment in Study III, and in Study II, empty gelatin capsules were fed as oral placebo treatment.

In Study IV, recombinant β-lactamase was used as a liquid formulation in 20 mM phosphate buffer, pH 7.4.

7.3 Ampicillin assay

Ampicillin analysis was done in Yhtyneet Laboratoriot Oy/United Laboratories Ltd (YL, Helsinki, Finland) by high-performance liquid chromatography (HPLC). The method for ampicillin determination is a modification of a method described previously by Vree et al. (1978). The quantitation limit was 1.0 µg/ml for serum and 0.5 µg/ml for jejunal and fecal samples.

7.3.1 Determination of ampicillin from serum samples

After collection, the blood samples were kept at room temperature for 30 min before centrifugation (room temperature 1000 g, 15 min), after which the serum was divided into three parallel cryotubes and placed into liquid nitrogen, and frozen at -70°C until transported for analysis. One of the triplicates was for ampicillin analysis, with the others serving as extra samples for further analysis, if needed.

7.3.2 Determination of ampicillin from jejunal and fecal samples

Jejunal samples were placed into liquid nitrogen immediately after collection and frozen at -70°C until prepared for analysis. Samples were prepared as soon as possible after each experiment. To prepare them for antibiotic and β-lactamase analysis, they were melted at room temperature in groups of 10-20 samples, mixed in a vortex, and centrifuged (4°C, 1800 g, 15 min). After centrifugation, the samples were placed into an ice bath and preparation began immediately. The supernatant was pipetted into a cooled glass beaker, diluted with cooled NaCl (Braun® 9 mg/ml, 0.9%, 100 ml, B. Braun Medical Oy, Espoo, Finland), and filtered through 0.22-µm filters into Eppendorf vials (three tubes from each sample, 200-500 µl/vial). The dilutions were taken into consideration in calculating the final results. After dividing the samples into three parallel tubes, they were put into a -70°C freezer. One of the triplicates was taken to Yhtyneet Laboratoriot Oy/United Laboratories Ltd for ampicillin analysis, one was for β-lactamase analysis, and the third was stored for possible further analysis.
To prepare fecal samples for antibiotic analysis they were melted, in groups of five samples, at room temperature. After melting, 0.5-1.5 g of stool was weighed into a cooled mortar, minced with a fivefold amount of cooled NaCl (Braun® 9 mg/ml, 0.9%, 100 ml, B. Braun Medical Oy, Espoo, Finland), vortex-mixed, and centrifuged (4°C, 1800 g, 20 min). After centrifugation, fecal samples were handled as described above for jejunal samples.

7.4 β-Lactamase assay

7.4.1 Determination of β-lactamase activity from the β-lactamase solution and from enteropellets

β-Lactamase activity from the β-lactamase solution and from β-lactamase containing enteropellets was determined at Ipsat Therapies Oy/Ltd (Helsinki, Finland).

Activity in the β-lactamase solution was determined spectrophotometrically by using chromogenic cephalosporin nitrocefin as a substrate (O’Callaghan et al., 1972; Simons et al., 1975; Livermore and Brown, 2001). β-Lactamase samples were either directly mixed with nitrocefin solution (Oxoid) or first diluted in 100 mM sodium phosphate buffer, pH 7.0, to obtain a linear kinetic reaction. Absorbance was measured spectrophotometrically.

β-Lactamase was extracted from the pellets with 20 mM sodium citrate buffer (pH 6.5), occasionally shaking the mixture at room temperature for 60 min. Insoluble material was removed by centrifugation (room temperature 8000 g, 10 min.). The β-lactamase activity of the supernatant was determined spectrophotometrically by using nitrocefin (Oxoid) as a substrate.

In both determinations absorbance change was converted into µg/ml (O’Callaghan et al., 1972) and further into units (Simons et al., 1978).

7.4.2 Determination of β-lactamase activity from jejunal and fecal samples

β-Lactamase activity was determined in Study II from the jejunal and fecal samples spectrophotometrically with a microtiter plate assay by using nitrocefin as a substrate (O’Callaghan et al., 1972). To determine β-lactamase activity, control samples were prepared both in buffer (0.1 M sodium phosphate buffer, pH 7.0) and in sample matrices. Jejunal and fecal control samples were prepared from zero samples of each dog. Blank samples with known concentrations of β-lactamase and β-lactamase in buffer were always analyzed on the same plate as the samples.
Ten microliters of samples and controls were pipetted onto the microtiter plate. Nitrocefin was extracted and placed into microtiter plate wells with a Labsystems Multidrop instrument. The plate was then transferred to a Labsystems Multiskan reader. Finally, kinetic measurements were carried out.

7.5 Analysis of microbiota

7.5.1 Bacterial culturing (Studies I, III, and IV)

In Study I, the samples were placed immediately after collection into anaerobic bags (Anaerocult P mini, Merck, Darmstadt, Germany) and transported to the laboratory (Technical Research Centre of Finland, VTT, Espoo, Finland). The total numbers of aerobes and anaerobes were cultured in fecal samples, as were lactic acid-producing bacteria (LAB) and Clostridium perfringens-like bacteria, which were chosen as indicator bacteria. Samples were processed in an anaerobic chamber, serially diluted \((10^{-1} \text{ to } 10^{-7})\) in prereduced peptone-yeast extract broth (pH 7.0), and quantitatively cultured on the following media: Brucella agar supplemented with sheep blood and K1-vitamin to determine total anaerobes and Tryptose Sulfite Cycloserine agar (TSCA, Merck) for Clostridium perfringens-like bacteria. The plates were incubated in anaerobic jars filled with mixed gas (85% N\(_2\), 10% CO\(_2\), 5% H\(_2\)) by an evacuation-replacement method (Anoxomat, Mart, Lichtenvoorde, the Netherlands) at 37°C for 5-7 days. For culture of LAB and total aerobes, samples were plated on Rogosa agar (Oxoid, Hampshire, England) and nutrient agar, respectively, under aerobic conditions. Rogosa plates were incubated in jars containing anaerobic bags (Anaerocult A, Merck) at 37°C for 3-4 days. Nutrient agar plates were incubated in ambient air at 37°C for 2 days. From Brucella, nutrient agar, and Rogosa plates, all colonies were enumerated. From TSCA plates, black-pigmented colonies were counted as C. perfringens-like bacteria.

In Study III, fecal samples were placed into liquid nitrogen to ensure expeditious and complete freezing of the sample immediately after collection. The samples were then moved to a -70°C freezer, after which they were transported frozen to National Public Health Institute (Helsinki, Finland) for culturing. For standard culture techniques, fecal samples were thawed, serially diluted \((10^{-1} \text{ to } 10^{-7})\) in prereduced peptone-yeast extract broth (pH 7.0), and quantitatively cultured using selective and nonselective agar media. Plates were incubated in anaerobic jars filled with gas mixture (80% N\(_2\), 10% CO\(_2\), 10% H\(_2\)), in 5% CO\(_2\) atmosphere or in ambient air at 37°C. Total counts and main groups of aerobic and anaerobic bacteria and yeast were enumerated (detection
limit 100 CFU/g) and identified by conventional methods; only predominant bacterial groups were recorded.

To determine the susceptibility of the coliform strains, 10 colonies of Gram-negative bacteria per sample were picked at random from different dilutions of blood and CLED plates where optimal growth was detected. All visible phenotypes were picked. The bacteria were identified using established methods (Farmer, 1999). The susceptibilities of the isolates to ampicillin, cefotaxime, and meropenem were determined using the disk diffusion method approved by the National Committee for Clinical Laboratory Standards (NCCLS).

In Study IV, fresh fecal samples from mice were quantified using standard microbiological techniques as described previously (Donskey et al., 1999). To quantify the VRE, *K. pneumoniae*, and *C. glabrata* strains, the samples were plated on Enterococcal agar (Becton Dickinson, Sparks, MD, USA) supplemented with vancomycin 20 µg/ml, MacConkey agar (Difco Laboratories, Detroit, MI, USA) supplemented with ceftazidime 10 µg/ml, and Sabourad dextrose agar (Becton Dickinson) supplemented with piperacillin/tazobactam 16 µg/ml and linezolid 2 µg/ml, respectively. For each experiment, several stool isolates were subjected to identification, and susceptibility testing according to the NCCLS guidelines for dilution antimicrobial susceptibility tests.

### 7.5.2 Molecular diagnostic methods

#### 7.5.2.1 Temperature gradient gel electrophoresis, TGGE (Study III)

Temperature gradient gel electrophoresis was performed on canine fecal samples (Study III) to evaluate changes in the indigenous microbiota during experiment period (Microscreen, Groningen, The Netherlands). TGGE method was briefly as follows: Fifty milligrams (wet weight) of fecal sample was homogenized, and DNA was isolated. Primers P16S968GCF and P16S1401R were used to amplify the V6 to V8 regions of the bacterial 16S rRNA gene. The GC clamp in primer P16S968GCF creates PCR products suitable for separation by TGGE (Muyzer et al., 1993). Polymerase chain reaction (PCR) was performed with Invitrogen’s Taq DNA polymerase kit. The samples were amplified in a PTC-200 PCR system (MJ-Research). The TGGE MAXI system (Biometra, Germany) was used for sequence-specific separation of PCR products. Electrophoresis was performed and the gel was stained with AgNO3 and developed (Cairns and Murray, 1993). For computer analysis, images were scanned, saved in TIFF format, and analyzed with GelCompar II software (Applied Maths, Belgium).
7.5.2.2 Denaturing gradient gel electrophoresis, DGGE (Study IV)

To evaluate changes in the indigenous microflora in mice (Study IV), DGGE of PCR-amplified bacterial rRNA genes was performed on stool samples collected after inoculation of test organisms (Case Western Reserve University, Cleveland, OH, USA). Genomic bacterial DNA was extracted from 200 mg of stool using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. PCR was done according to the technique of Muyzer et al. (1993) by using Taq DNA polymerase (Promega, Madison, WI, USA) on a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Norwalk, CT, USA). DGGE was performed using a Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The PCR products were electrophoresed on polyacrylamide gels. Gels were stained with SYBR Green (BioWhittaker Molecular Applications, Rockland, ME, USA) and photographed under UV transillumination. Stained gels were analyzed with Molecular Analyst 1.12 software (Bio-Rad). Similarities between DGGE profiles were assessed by calculating similarity indices, and corresponding dendrograms showing the relationships between the DGGE profiles were constructed.

7.5.2.3 TEM gene analysis (Study III)

The emergence of ampicillin resistance in dogs of Study III was evaluated by examining feces using quantitative PCR for the detection of TEM β-lactamase genes (Microscreen, Groningen, The Netherlands).

To determine the concentration of the TEM gene in samples, the number of TEM-producing organisms was compared with the total number of bacteria in the fecal samples, as determined by performing quantitative PCR on the 16S rRNA gene. Amplification reactions were monitored by using SYBR® Green (Molecular Probes, AA Leiden, the Netherlands) as a fluorogenic marker. The total count of bacteria was set at 100%.

7.6 Statistical analysis

In Study I, the Wilcoxon signed-rank test was used to evaluate differences in bacterial counts before and after the surgery. Before the statistical analysis, all bacterial counts were log-transformed. A count of <10^3 was rounded up to 10^3.

In Study II, analysis of variance was performed by using the S-plus 2000 Mixed Effects Linear Models program to calculate the significance in the ampicillin and β-lactamase concentrations
between each treatment group at each time-point.

In Study III, the outcome variables of interest were the fecal TGGE similarity percentages, the amount of the TEM gene, and the serum and jejunal ampicillin concentrations. Treatment group and sampling time were independent variables.

These data analyses were conducted with the Statistical Analysis System (SAS®, version 8.1, SAS Institute Inc, 2000). PROC MIXED in SAS was used to analyze the repeated measurements of fecal TGGE percentages. Due to non-normal distribution of data for the ampicillin concentrations and levels of the TEM gene, nonparametric methods were applied to compare values between treatment groups.

For TGGE similarity percentage results, the covariance of repeated measurements within dogs over time was accounted for by using a first-order autoregressive correlation pattern, which indicated that adjacent observations were more closely correlated than observations made farther apart. An interaction term between treatment and time was also included in the model. Least-square means for each treatment and time were compared using Tukey’s adjustment for multiple comparisons.

For ampicillin concentrations and TEM gene levels in which data were nonnormally distributed, nonparametric analysis of variance was performed to analyze the effect of the intervention. Data from all time-points were initially pooled and analyzed together. If the results showed a significant ($p<0.01$) difference between treatments, data from each time-point were analyzed separately. Then, if a significant overall difference between groups was observed at any sampling point, the groups were further compared pairwise, also using nonparametric ANOVA to reveal which groups differed from each other. Results were considered statistically significant when $p<0.01$. For statistical analysis, the ampicillin concentration and the amount of the TEM gene were assigned values of zero when they were below detection level.

For the counts of total anaerobic and aerobic bacteria, statistical analyses were performed using SPSS10 software for windows. To compare the concentrations between the groups Spearman’s rho t-test was used. A p-value < 0.05 was considered significant.

In Study IV, analysis of variance was performed to compare the densities of test organisms among various treatment groups. DGGE similarity indices were compared using Student’s $t$ test. Computation were performed using Stata software (version 5.0, Stata, College Station, TX, USA). A p-value < 0.05 was considered to be significant.
8 RESULTS

8.1 Permanent jejunal fistula operation (Study I)

8.1.1 Recovery of the dogs from the operation

A nipple valve for permanent intestinal access was surgically inserted into seven dogs (Study I) and another three dogs (used in Study II) 60 cm distal to the pylorus, and into 18 additional dogs (used in Study III) 170 cm distal to the pylorus.

Except for transient anorexia in some dogs in the first 2-3 days after surgery, no complications were seen during recovery. There was no leakage from the valve and no signs of infection.

Approximately four months after the surgery (Study I), one dog had to be euthanized because of leakage of small intestinal content from the nipple valve that could not be fixed. At necropsy, adhesions between the nipple, small intestine, and peritoneum were observed, and the intussusception had straightened itself.

Over five years, six of these fistulated dogs have been euthanized for reasons unrelated to the fistulas. In the remaining dogs, the function of the nipple has been excellent. No leakage of intestinal content from the fistulas has occurred. Small intestinal content has been collected by inserting a silicon tube through the nipple valve in these dogs dozens of times.

The small intestinal content is very liquid from a fistula inserted 60 cm distal to the pylorus, but it is more solid 170 cm distal to the pylorus. According to our experience, when the tube is inserted into the fistula placed 60 cm distal to the pylorus, the small intestinal content always runs out passively. Due to the more solid small intestinal content, sometimes the sample has to be “milked” from the 170 cm fistula by moving the tube back and forth within the fistula. Anyhow, the samples, in both cases, can be collected by 4-6 h after feeding.

8.1.2 Effects on intestinal motility

The results of motility analyses (Study I) showed that in all dogs most of the Barium-Impregnated Polyethylene Spheres® (BIPS) had passed through the stomach and small intestine and reached the colon on average in 12 h, and in six dogs, the mean transit percentages remained practically the same before and after the surgery (93% vs. 83%). In the seventh dog, the orocolic transit percentage changed markedly following the operation (100% -> 22.5%). This dog was the one subsequently
euthanized because of leakage of the fistula and adhesions between the nipple, small intestine, and peritoneum.

In the additional dogs used in Study II, as well as in all dogs used in Study III, small intestinal motility was tested with BIPS before and after the operation. In all of these dogs, motility was within the same limits as for the six healthy dogs of Study I.

8.1.3 Effects on intestinal microbiota

When comparing the fecal bacterial culture (done for four dogs) results before and after the fistula operation in Study I, no change was observed in counts of total anaerobes and lactic acid-producing bacteria in any of the dogs. A decrease was seen in *C. perfringens*-like bacteria and total aerobes in fecal samples of three dogs (publication I).

In small intestinal samples, counts of LAB increased after fistulation in three of four dogs. Counts of total aerobes, by contrast, decreased (two dogs) or remained unchanged (two dogs).

8.2 Effects of TRBL on serum ampicillin concentrations (Studies II and III)

In Study II, serum ampicillin was detected in all of the dogs during the experimental period, with the maximum concentration being reached 15 min after intravenous dosing. No significant difference was present in serum ampicillin concentrations between treatment groups ($p=0.32$).

In Study III, serum ampicillin concentration was high throughout the treatment period (day 1-day 14) in both of the ampicillin-treated groups (Figure 4). No significant difference was present in serum ampicillin concentrations between these two groups ($p=0.98$).

![Figure 4. Mean serum ampicillin concentrations ± SEM in different treatment groups of Study III during the treatment period d1-d14. Solid circle: Ampicillin 40 mg/kg i.v. + TRBL p.o. (n=6); Solid square: Ampicillin 40 mg/kg i.v. + placebo p.o. (n=6); Solid triangle: placebo i.v. + placebo p.o. (n=6). d=day, SEM=standard error mean.](image-url)
8.3 Effects of TRBL on intestinal ampicillin concentrations (Studies II and III)

In Study II, a dose-response effect of oral TRBL on ampicillin concentrations in the jejunal samples was observed. The highest concentration of secreted ampicillin was detected in the placebo group within 30 min of intravenous antibiotic administration.

The lowest dose of TRBL (0.003 mg/kg) reduced the jejunal ampicillin concentration below that of the placebo. However, in these two groups (placebo and TRBL 0.003 mg/kg), jejunal ampicillin remained elevated for the first 1.5-2 h of sampling. With a dose of 0.03 mg/kg of TRBL, there was negligible elevation in ampicillin concentrations 15 min after ampicillin administration. Finally, with the highest TRBL dose (0.3 mg/kg), the jejunal ampicillin concentration dropped below detection level (0.5 µg/ml) throughout the 5-h study period.

In Study III, some fluctuation in the jejunal ampicillin concentrations occurred in the ampicillin + placebo-treated group. However, jejunal ampicillin was elevated throughout the treatment period in this group (Figure 5).

In the ampicillin + TRBL-treated group, jejunal ampicillin concentration was below the detection level, except on day 10, when an increase occurred due to an elevated jejunal ampicillin concentration in one of the six dogs in this group. Ampicillin was, however, below detection level in all of the other jejunal samples for this dog.

The overall difference in jejunal ampicillin concentrations during the treatment period (day 1 – day 14) between the ampicillin + TRBL and the ampicillin + placebo-treated groups is significant at \( p<0.0001 \).

No ampicillin was found in fecal samples (Studies II and III).

![Figure 5. Mean jejunal ampicillin concentrations ± SEM in different treatment groups of Study III during the experimental period d0-d28. Solid circle: Ampicillin 40 mg/kg i.v. + TRBL p.o. (n=6); Solid square: Ampicillin 40 mg/kg i.v. + placebo p.o. (n=6); Solid triangle: placebo i.v. + placebo p.o. (n=6). d=day, SEM=standard error mean.](image)
**Jejunal β-lactamase concentrations (Study II)**

In Study II, β-lactamase concentrations determined in the jejunal samples of the different treatment groups were in good agreement with the concentrations obtained from the jejunal ampicillin assays, as here, too, a dose-dependent effect was observed, the highest TRBL dose causing the greatest elevation in jejunal β-lactamase concentration.

No active β-lactamase was detected in the fecal samples (Study II), either in the zero samples or in the samples taken the following morning (data not shown).

### 8.4 TRBL in prevention of ampicillin-induced changes in canine intestinal microbiota (Study III)

#### 8.4.1 Fecal bacterial culturing

In conventional bacterial culture, the total counts of aerobic ($\log_{10} 9.1$ vs. 10.2, $p=0.003$) and anaerobic ($\log_{10} 10.3$ vs. 10.9, $p=0.041$) bacteria were lower in the ampicillin + placebo group than in the ampicillin + TRBL group during the intervention (Figure 6). The counts of especially streptococci, enterococci, clostridia and anaerobic gram-positive cocci decreased, and the proportions of both aerobic and anaerobic gram-negative rods increased (data not shown). Otherwise, no marked changes were recorded. Dogs receiving ampicillin + TRBL or placebo alone had only minor overall changes and some occasional changes within a single species.

![Figure 6](image_url)  
**Figure 6.** Number ($\log_{10}$CFU/g) of total anaerobes and aerobes in the different treatment groups during the experimental period. Treatment period day 1–day 14. d=day.
8.4.2 Comparison of TGGE similarity percentages between treatment groups

The mean fecal TGGE similarity percentage (%) decreased significantly in the ampicillin + placebo-treated group during the treatment period, whereas in both placebo + placebo and ampicillin + TRBL groups it remained virtually stable for the entire experimental period (Table 4.).

Fecal similarity % during the treatment period (on day 4, day 10, and day 14) in the ampicillin + placebo group differed significantly from that of the placebo + placebo ($p<0.0001$) and the ampicillin + TRBL ($p<0.0001$) groups. The overall difference in mean fecal similarity % between the two latter groups was not significant ($p=0.54$).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>day 0</th>
<th>day 4</th>
<th>day 10</th>
<th>day 14</th>
<th>day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>amp + placebo</td>
<td>100 ± 0</td>
<td>38.3 ± 7.1</td>
<td>53.1 ± 8.8</td>
<td>52.4 ± 7.3</td>
<td>71.0 ± 1.9</td>
</tr>
<tr>
<td>amp + TRBL</td>
<td>100 ± 0</td>
<td>85.2 ± 3.8</td>
<td>85.2 ± 3.8</td>
<td>82.7 ± 2.5</td>
<td>80.7 ± 3.0</td>
</tr>
<tr>
<td>placebo + placebo</td>
<td>100 ± 0</td>
<td>83.0 ± 3.3</td>
<td>80.8 ± 4.6</td>
<td>78.3 ± 5.2</td>
<td>77.0 ± 4.1</td>
</tr>
</tbody>
</table>

Table 4. Mean TGGE similarity % ± SEM in each treatment group during the experiment period (day 0–day 28). Treatment period day 1–day 14. SEM=standard error mean.

8.5 TRBL in prevention of ampicillin-induced selective pressure on canine fecal microbiota (Study III)

8.5.1 Amount of TEM gene

The TEM gene level was very low (8/18 dogs) or undetectable (10/18 dogs) on day 0 in all of the dogs. Dogs receiving ampicillin + placebo developed a significant increase in TEM gene levels during the treatment period compared with dogs receiving ampicillin + TRBL ($p<0.001$) or placebo + placebo ($p<0.001$). The difference in the percentage of TEM genes between the two latter groups was not significant ($p=0.38$).

Two weeks (day 28) after the treatment period, the TEM gene concentrations returned to near baseline levels in all treatment groups.

One of the six dogs in the ampicillin + TRBL group had developed a transient elevation in jejunal ampicillin concentration on day 10, coinciding with a reduction in the fecal TGGE index and an
elevation in the proportion of TEM genes. By day 14, these changes had returned to previous baseline levels.

### 8.5.2 Susceptibility of fecal coliforms

A total of 862 coliformic colonies were isolated from 90 samples (9.6 isolates/sample). *Escherichia coli* was the predominant species (99% of all isolates). Other organisms included *Escherichia vulneris* (4 isolates) and *Klebsiella pneumoniae* (3 isolates).

At baseline, the majority (69-83%) of isolates in all groups were sensitive to all of the tested antibiotics (ampicillin, cefotaxime, and meropenem).

In the ampicillin + placebo group, one dog harbored ampicillin-resistant *E. coli* at baseline, but as the intervention started, all six dogs became ampicillin-resistant *E. coli* carriers. The proportion of ampicillin-resistant strains of all isolated fecal coliforms increased dramatically, from 2% at baseline to 98% on day 4, remaining high throughout the intervention. Two weeks after the intervention (day 28), the proportion of ampicillin-resistant strains was still 65% in this study group (Figure 7).

![Figure 7](image.png)

**Figure 7.** Effect of enterocoated β-lactamase pellets on the proportion (%) of ampicillin-resistant strains of coliform isolates in different treatment groups during the experimental period. Treatment period day 1-day 14. d = day.
In the ampicillin + TRBL group, three dogs harbored ampicillin-resistant *E. coli* at baseline, but no new dogs became carriers during the intervention. Thus, the proportion of fecal ampicillin-resistant strains at no time exceeded 46%. On day 28, the distribution of the different resistance patterns was restored and was similar to the baseline situation.

In the placebo + placebo group, two dogs were carriers of ampicillin-resistant *E. coli* at baseline, and three new dogs became carriers during the intervention. The proportion of fecal ampicillin-resistant strains grew steadily, from 16% on day 0 to 50% on day 28.

Other resistance patterns (ampicillin-susceptible strain with resistance to some other antibiotic(s)) were rare in all groups throughout the intervention (data not shown).

### 8.6 Oral β-lactamase in prevention of fecal colonization resistance in mice (Study IV)

Prior to inoculation, none of the mice had detectable levels (level of detection, ~2 log\(_{10}\) CFU/g stool) of the tested microorganisms; vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamase producing *Klebsiella pneumoniae*, and *Candida glabrata*.

Mice treated with subcutaneous piperacillin developed high-density stool colonization with each of the tested microorganisms, whereas saline controls and mice treated with subcutaneous piperacillin in conjunction with 6 mg/kg of oral β-lactamase did not (*p*<0.002 for each of the test organisms). β-lactamase treatment induced a greater than 10 000-fold reduction in the density of VRE or *Klebsiella pneumoniae* in stool (mean densities, >10\(^8\) CFU/g of stool versus < 10\(^4\) CFU/g).

When the enzyme was inactivated with tazobactam, the protective effect of the β-lactamase was eliminated; the mean density of VRE colonization associated with piperacillin treatment in conjunction with the tazobactam-inactivated enzyme did not differ from the mean density associated with piperacillin treatment alone (*p*=0.69). The efficacy of the β-lactamase treatment was also lost by boiling the enzyme for 30 min or by altering the timing of administration such that piperacillin was administered 6 h prior to each dose of β-lactamase.

The protective effect of β-lactamase was dose-dependent; when the β-lactamase dose was reduced (6 mg/kg -> 1 mg/kg), mice had higher densities of VRE than saline control mice (*p*=0.03). However, these mice had significantly lower densities of VRE than piperacillin-treated mice (*p*<0.001).
9 DISCUSSION

9.1 Fistula operation

Our understanding of the gastrointestinal tract environment, particularly factors affecting small intestines, is still largely in its infancy (Zentek, 2000) although it is known that several factors can affect gastrointestinal tract equilibrium (Mackie et al., 1999).

The main obstacle hindering the analysis of small bowel content is gaining regular access to the small intestine. In these studies, permanent jejunal fistulas were surgically attached to laboratory beagles, enabling collection of small intestinal samples for analysis.

Gastrointestinal motility

Motility disorders that are sometimes seen after intestinal surgery are one potential etiology for disturbances in gastrointestinal tract microbiota (Husebye, 1995), and vice versa. The results for canine gastrointestinal transit time before and after the surgery for six of seven dogs in Study I correlate well with the findings of Iwanaga et al. (1998), which show only modest deviation in small bowel transit time in healthy dogs. The orocolic transit percentage changed markedly following the operation in one dog. This dog had to subsequently be euthanized because of leakage of the fistula and adhesions between the nipple, small intestine, and peritoneum. Surgery can therefore change intestinal motility profoundly if complications, such as adhesions, occur. The euthanized dog must have had the adhesions already at the time of the motility test, although clinical signs did not appear until four months after surgery.

These data indicate that it is impossible to judge the immediate success of the operation from clinical signs alone. Thus, determining possible alterations caused by fistula surgery to intestinal motility is essential before beginning any trial with fistula-operated dogs. Dogs with abnormal transit time should be excluded from trials.

Jejunal and fecal microbiota

Microbiota from jejunal and fecal samples of four dogs was analyzed by conventional bacterial culturing before and after the fistula operation in Study I.

Bacterial counts of fecal samples, both before and after the nipple valve operation, were virtually identical; the subtle differences can be considered to be normal variation. Results from the small intestinal bacterial cultures were not as constant. This could be explained by differences in sampling
techniques during and after the operation, by natural variation between individual samples, and at least to some extent, by the propulsive functioning of the small intestine.

The results did, however, indicate that the nipple valve does not cause small intestinal bacterial overgrowth because the total aerobic bacterial count had not increased in samples taken after surgery. Moreover, the operation had no effect on small intestinal permeability (unpublished data).

In conclusion, barring complications, the surgery does not affect gastrointestinal transit time. Because anesthetizing the animal is not necessary, post-feeding samples can also be collected. Due to the more solid composition of the small intestinal content, the samples are, sometimes, only trickling from the fistula located 170 cm distal to the pylorus. In these cases the tube has to be moved back and forwards in the fistula, which might irritate the mucosa slightly causing minor bleeding. Anyhow, the samples can be collected without any aspiration, and our five years of experience with the fistula, has convinced us that the nipple valve method provides a suitable means of sampling small intestinal content for analysis.

9.2 Targeted recombinant β-lactamase

Targeted recombinant β-lactamase (TRBL) is a novel treatment modality intended for use in conjunction with β-lactam antibiotics to preserve gastrointestinal tract microbiota consortium. Since, TRBL is dosed per os in a controlled release pellet formulation, before antibiotic administration, it is available in the gut to degrade the portion of the antibiotic that reaches there. Inactivation of the antibiotic enables inhibition of antibiotic-induced adverse effects on the gut. This treatment principle was preclinically tested in fistula-operated beagles and in mice.

9.2.1 Serum drug levels

Nitrocefin assay as such is not suitable for determining β-lactamase activity from serum samples (O’Gallaghan et al., 1972), thus the nonabsorption of β-lactamase was proved indirectly by determining serum antibiotic levels in each canine TRBL experiment. This was done, although biological enzymes are not expected to penetrate from the gastrointestinal tract to the parenteral system.

The results proved that TRBL was not absorbed from intestines to systemic circulation, since no significant differences were seen in serum ampicillin concentrations between the ampicillin-treated groups – neither in Study II, wherein the pellet formulation with a thinner outer layer was used, nor
during multiple-dose treatment with TRBL over a period of two weeks (Study III). This is a noteworthy result signifying that this combination treatment (i.v. antibiotic and p.o. TRBL) can maintain the systemic concentration of the antimicrobial agent invariable in dogs. Further, in toxicological studies done with TRBL in minipigs (unpublished data, Scantox, Denmark), in which very high doses of β-lactamase were used, no β-lactamase was detected in plasma samples analyzed by HPLC; thus, absorption of the enzyme into systemic circulation is not likely in humans either. Confirmation that this treatment does not affect serum antibiotic levels of clinical patients is, however, needed.

9.2.2 Jejunal and fecal drug levels

In Study II, ampicillin was measurable from jejunal samples of the dogs receiving the oral placebo already 15 min after intravenous ampicillin administration. This supports the pharmacokinetic data showing that after parenteral injection ampicillin is distributed rapidly and widely, resulting in a high concentration of the drug in bile (Mandiola et al., 1972). In Study II, TRBL degraded ampicillin in the jejunum in a dose-dependent manner.

In Study III, the mean jejunal ampicillin concentration was elevated in the ampicillin + placebo group throughout the 14-day treatment period. However, the highest jejunal ampicillin concentrations were measured at the beginning of the antibiotic intervention (day 4). The jejunal ampicillin concentration can be hypothesized to have been highest in the ampicillin + placebo group during the first few days of treatment because jejunal bacteria did not produce β-lactamases to degrade ampicillin.

In fecal samples, no ampicillin (Studies II and III) or β-lactamase (Study II) was present. According to our in vitro studies, the half-life of β-lactamase in jejunal samples is approximately 4 h (unpublished data). The experiment protocol of Study II, in which the fecal samples were collected ≥ 24 h after TRBL administration, could thus explain the absence of β-lactamase activity in fecal samples.

What explains the lack of ampicillin in the fecal samples? Antibiotic absorption to fecal compounds known to decrease the amount of active antibiotic in gut (de Vries-Hospers et al., 1993) could explain its absence in fecal samples. Also difficulties in detecting ampicillin in feces may have impeded. When a fresh canine stool was spiked with ampicillin, and immediately minced with a pestle in a saline solution, the immediate recovery of the spiked amount, as analyzed by HPLC,
varied between 1% and 60% (unpublished data). The timing of the sampling may have also contributed to the absence of ampicillin in fecal samples, especially in Study II where fecal samples were collected $\geq 24$ h after ampicillin administration.

Furthermore, enzymic (i.e. $\beta$-lactamase production in normal intestinal microbiota) inactivation of $\beta$-lactam antibiotics inside the gut may significantly reduce the availability of an active drug for analysis (Edlund et al., 1994; van der Waaij and Nord, 2000). This inactivation has been found in fresh feces of healthy human subjects (Welling et al., 1990) and to vary significantly between individuals (Veringa and van der Waaij, 1984). It can be hypothesized that intestinal bacteria produced $\beta$-lactamases, especially, at the end of the ampicillin treatment period in Study III; thus, enzymic inactivation interfered the problems in measuring ampicillin from canine feces, at least in this particular study.

In the literature, several studies in which ampicillin has been measured by microbiological methods from serum (Kager et al., 1983), bile and saliva (Vree et al., 1978), intestinal mucosa (Kager et al., 1983), or feces (Daikos et al., 1964; Kager et al., 1982) exist. However, there are no study available in PubMed, in which ampicillin has been assayed from fecal samples by HPLC. One can therefore hypothesize that it would have been possible to measure ampicillin from the fecal samples by some method other than HPLC.

To summarize, considering the difficulties in detecting ampicillin and $\beta$-lactamase in fecal samples, our beagle dog model proved very useful in demonstrating that ampicillin was actually degraded by TRBL in the small intestine.

9.2.3 TRBL prevented ampicillin-induced changes on canine fecal microbiota

Antibiotics inside the intestinal tract cause profound disruption of the indigenous microbiota (Nord et al., 1984; Nord and Edlund, 1990; Monroe and Polk, 2000; Sullivan et al., 2001) resulting in a number of adverse effects such as antibiotic-associated diarrhea and development of antibiotic resistance (Nord and Heimdahl, 1986). For instance, ampicillin represents a significant risk factor for altering the gut microbiota (Nakaya et al., 1982), and inhibits both anaerobic and aerobic human fecal flora in vitro (Hazenberg et al., 1983). Ampicillin therapy has been associated with a significant decrease in human fecal flora in vivo, too (Kager et al., 1983). Moreover, it is reported that the fecal microbiota composition is nearly normalized within 2 weeks after ampicillin intervention (Nakaya et al., 1982; Nord et al., 1984b). Bacterial culturing results obtained from
Study III supported these findings, as both anaerobic and aerobic bacterial counts were decreased in the ampicillin + placebo-treated group during the treatment period. In samples taken 2 weeks after ampicillin treatment, fecal microbiota composition mainly resembled the baseline situation.

The temperature gradient gel electrophoresis (TGGE) results support the culture results as here too the most significant change in microbiota was seen in the ampicillin + placebo treated group during the antibiotic intervention. The overall mean fecal TGGE similarity percentage in this group decreased significantly during the treatment period, particularly at the beginning of the treatment (day 4). After the intervention, fecal TGGE similarity percentages approached the percentages determined before the intervention.

The significant decrease in fecal TGGE similarity % during the first days of antibiotic intervention in ampicillin + placebo group in Study III was most probably because the fecal bacteria were still susceptible to ampicillin at the beginning of the study. Since the β-lactamase activities were not analyzed, it can only be hypothesized that prolongation of the antibiotic induced β-lactamase production within intestinal bacteria in the ampicillin + placebo group. As a consequence, the amount of intestinal ampicillin decreased; thus, the changes in microbiota also decreased and the TGGE similarity % increased slightly after day 4 of the intervention.

Similar results have been obtained from a mice study, in which parenteral ampicillin was shown to change the microbiota and decrease colonization resistance (van der Waaij et al., 1972), in the beginning of antibiotic treatment. If the treatment was exceeded, the effect on colonization resistance reduced because of a strongly β-lactamase-secreting bacterial strain in the gut flora. This indicates that bacterial enzymes can degrade various antibiotics in the gut such that deleterious effects on colonization resistance are diminished (Veringa and van der Waaij, 1984; Edlund et al., 1994).

From an ecological point of view, the resistant microorganisms in the gut, i.e. bacteria that produce β-lactamase enzymes to inactivate β-lactam antibiotics, have an advantageous effect since the enzymic activity prevents the decrease of colonization resistance during antibiotic treatment, thus protecting against invading potentially pathogenic microorganisms (van der Waiij, 1986).

Although this kind of natural protection of gut microbiota occurs during multiple-day antibiotic treatment, the first few days of antibiotic intervention are critical because the changes in microbiota, and the development and dissemination of resistance to antibiotics can already be detected within a few days after onset of therapy (Hoiby, 2000). With here introduced TRBL treatment modality the
changes in indigenous microbiota can be prevented from the beginning of the antibiotic intervention.

**Exceptional dog in ampicillin + TRBL group**

In Study III, one dog in the ampicillin + TRBL-treated group had an elevated jejunal ampicillin concentration on day 10. This was most probably caused by a transient disturbance in passage of food and ingesta (and thus TRBL), although no clinical signs were observed.

The fecal TGGE similarity % on that sampling day in this dog was considerably decreased compared with the situation on other days. This attests to the TGGE analysis method being sensitive to detection of antibiotic-induced changes in the fecal microbiota. It also confirms data on the adverse effects of unabsorbed or, in this case, undegraded antibiotic on the gut.

In this individual, the share of the TEM gene was also significantly increased compared with the other experiment days. This supports previous data that ampicillin alters the gut microbiota expeditiously and profoundly (Nakaya et al., 1982), that it is an important risk factor for development and spread of antibiotic resistance (Burman et al., 1992), and that antibiotic resistance can be detected soon after antibiotic exposure (Hoiby, 2000).

The TGGE similarity percentage being increased in the subsequent sample affirms that long-term preservation of the composition of fecal microbiota with TRBL is possible despite a transient elevation in the jejunal ampicillin concentration and a change in microbiota. Although the subsequent sample was take at day 14, and there is no data available of the situation in the gut between these two sampling points (day 10 and day 14), this is a significant result because transient disturbance in intestinal function is also commonly seen in hospitalized patients.

### 9.2.4 TRBL prevented ampicillin-induced selective pressure on canine fecal microbiota

Even normal intestinal flora contains antibiotic-resistance bacteria to various degrees (Sorum and Sunde, 2001). For instance, high frequencies of antimicrobial resistance have been encountered in enterobacteria (Österblad et al., 2000), and the carriage of ampicillin-resistant *E. coli* among healthy persons and domestic animals has shown to be over 50% (Moss et al., 1984; Tvede et al., 2001). Of concern is, however, the continual exchange of microbes among people that facilitates transmission of resistance genes among hospitalized patients (Donskey et al., 2000a). Hence, in a hospital setting a few heavily colonized patients can contaminate a whole ward or even several wards (Levy,
2002). Accordingly, it is accepted that the future of antibiotic treatment depends on the evolution of resistance both among the pathogens and among the commensal microbiota (Twomey, 2000; Andremont et al., 2001).

An ideal approach to determine the effect of an antimicrobial agent on the composition of gastrointestinal tract microbiota and to assess antimicrobial resistance would be the enumeration of anaerobes (Corpet, 1993). However, technical problems in culture make this approach inaccessible. Thus, although aerobes constitute only a small portion of the gastrointestinal tract microbiota, the determination of resistant populations of aerobes has proved to be a sensitive assessment of microbiota modifications by antimicrobials (Corpet, 1993). Determination of the proportion of resistant enterobacteria (e.g. *E. coli*) appears to be a simple and sensitive method to define changes in gastrointestinal tract microbiota under antibiotic treatment (Corpet, 1993). However, it is not recommended to rely upon a single method for determining the share of antibiotic resistance (Heritage et al., 2001). Thus, two different methods to evaluate ampicillin-induced selective pressure were used in Study III: PCR to assess TEM gene levels in fecal samples, and susceptibility pattern determination of fecal coliform isolates.

*TEM gene analysis*

The TEM gene test relies on the endogenous presence of ampicillin-resistance genes. The starting level of the TEM gene was very low in 44% of the dogs and undetectable in 56% of the dogs. During the intervention the share of the TEM gene increased significantly in ampicillin + placebo-treated dogs as compared with the other two treatment groups.

A comparison of the fecal TGGE similarity % with the proportion of the TEM gene clearly indicated that if a decrease occurred in the similarity percentage, a concomitant increase was observed in the amount of the TEM gene; thus, when the composition of the population changed due to the presence of ampicillin, it had a direct effect on TEM gene levels. This was also seen in the exceptional dog in ampicillin + TRBL group.

These results indicate that when ampicillin-resistant bacteria had a selective advantage, i.e. during the ampicillin intervention in ampicillin + placebo group, ampicillin resistance increased. When this selective pressure was decreased, also the proportion of ampicillin-resistant bacteria decreased. Consequently, the TEM gene level decreased. In all treatment groups, the amount of the TEM gene in Study III resumed levels close to baseline after the treatment period.
Susceptibility of coliform isolates

The susceptibility test results support the TEM gene results and indicate that TRBL decreased the selective pressure significantly during the ampicillin intervention. The minor increases in the proportion of ampicillin-resistant coliforms that were observed in the placebo + placebo and ampicillin + TRBL groups were potentially due to cross-contamination from the heavily colonized ampicillin + placebo group, as the dogs were not completely isolated from each other, were handled by the same personnel, and used the same pen for outdoor activities, though the feces were always cleaned away from the premise after each dog, and the personnel disinfected their hands frequently.

To summarize, the results obtained from Study III, wherein the both traditional conventional and new molecular methods were used to evaluate the ampicillin-induced changes and selective pressure on gut microbiota indicated that with oral targeted recombinant β-lactamase treatment these adverse consequences of antibiotic treatment can be averted. However, more studies are needed to confirm that the microbiota in hospitalized patients can be preserved during long-term hospitalization because factors other than antibiotic therapy may cause significant alterations to the indigenous microbiota (Vollaard and Clasener, 1994). Since most severe ill hospitalized patients might be fed intravenously with nutrient substitutes, the capacity of TRBL to preserve indigenous microbiota under fasted conditions should also be tested.

Further work is also needed to determine the antibiotic concentrations and microbiota from intestinal biopsies since bacterial adhered to the intestinal epithelium are also important in maintaining gastrointestinal equilibrium (Berg, 1996). Determining that TRBL treatment does not cause any adverse effects, such as immunological reactions in the gut, is, essential, too. Although immunological effects are unlikely, since most of the Gram-negative bacilli produce β-lactamases to some extent (Brinas et al., 2000). Furthermore, TRBL contains the PenP protein of *Bacillus licheniformis* 749/C, and bacillus species, other than *Bacillus cereus* and *anthracis*, are generally considered to be inconsequential (Rowan et al., 2003). Hence, β-lactamase *per se* is not anticipated to be harmful to the gastrointestinal tract.

### 9.2.5 Oral β-lactamase preserved fecal colonization in mice

Nosocomial infections occur worldwide and are among the major causes of death and increased morbidity among hospitalized patients (Patterson, 2001). Although, patients are exposed to a variety of microorganisms during hospitalization, contact between the microorganism and patient does not
by itself necessarily result in the development of nosocomial infection. However, antimicrobial resistance emerges in populations with a high frequency of infections, due to underlying patient status or interventions compromising patients host defense, resulting in a high rate of antibiotic use (Patterson, 2001). Selective pressure exerted by antimicrobials in the intestinal tract contributes to the acquisition and overgrowth of antimicrobial-resistant microorganisms (Donskey et al., 1999). Antibiotic-resistant microorganisms, such as extended-spectrum β-lactamase producing *Klebsiella pneumoniae* or vancomycin-resistant enterococci (VRE), are significant causes of health care related infections among hospitalized patients (Patterson, 2001). A strong association between the use of broad-spectrum cephalosporins and both antibiotic-resistant *Klebsiella pneumoniae* and VRE have been demonstrated. Further, piperacillin, is known to inhibit intestinal anaerobes, decrease colonization resistance, and predispose to nosocomial infections (Donskey et al., 2000a).

In mice, antibiotic treatment induced high-density stool colonization of VRE, whilst if VRE was inoculated before any antibiotic intervention or antibiotics with less potent against anaerobes were used, stool colonization of VRE was not observed (Donskey et al., 1999) This was also demonstrated in Study IV, which showed that mice pretreated with piperacillin developed high-density stool colonization after ingestion of VRE, *Candida glabrata*, and extended-spectrum β-lactamase producing *Klebsiella pneumoniae*. By contrast, in mice pretreated with piperacillin in combination with oral β-lactamase, preservation of fecal microbiota during antibiotic treatment was observed. These results demonstrate that oral β-lactamase treatment preserves colonization resistance of mice during parenteral piperacillin treatment, and suggest that this treatment modality could provide a new strategy to limit the spread of nosocomial pathogens in humans. This is a significant finding, since the main application of TRBL therapy is, presently, aimed to be used in hospital settings during parenteral antibiotic treatments. Any impact on preventing serious nosocomial infections and stemming the steady rise in antibiotic resistance will be welcomed particularly by immunocompromised patients. The substance of results from Study IV is broadened against the knowledge that colonization of hospital patients by resistant opportunistic pathogens is considered difficult to prevent because of shared environment, equipment, and nursing staff, and the use of antibiotics that destroy the normally protective bacterial flora in patients (Prescott, 2000).

Since β-lactamase treatment was not effective when the enzyme was inactivated by tazobactam or heat, it is unlikely that nonspecific effects related to administration of the enzyme contributed significantly to the treatment effect. Hence, it can be concluded that TRBL treatment as such is effective.
In the future, it would be interesting to develop enzymes with greater activity against more widely used parenteral β-lactam agents, such as cephalosporins, and against β-lactam antibiotics administered per os, as well as β-lactam β-lactamase inhibitor combinations. Moreover, it would be challenging to determine whether similar strategies could be used to inactivate non-β-lactam classes of antibiotics in the intestinal tract.

To recapitulate, since bacterial resistance poses a serious threat worldwide, every effort should be made to prevent development and spread of this problem, and based on here reported preclinical mice and canine studies oral β-lactamase treatment would offer one option for thwart this problem.

If the results obtained from these preclinical studies can be repeated in clinical studies, this would offer considerable possibilities for healthcare. These would be achieved for instance by reduced numbers of nosocomial infections, the subsequent need for prolonged hospitalization, and the reduced acquisition and spread of antibiotic resistance.
10 CONCLUSIONS

I. Evaluation of the feasibility of the permanent jejunal fistula led to the following conclusions:

The permanent jejunal fistula does not disturb normal intestinal functions. Thus, it can be considered to be a suitable method for obtaining representative small intestinal samples for pharmacokinetic and microbiological analyses. The fistula has proven to be reliable over time, and dogs tolerate the fistula and sampling well.

II. Evaluation of the properties of targeted recombinant β-lactamase led to the following conclusions:

1. Orally administered targeted recombinant β-lactamase degraded the portion of parenteral ampicillin that entered the jejunum dose-dependently. No disadvantageous effect of the β-lactamase doses used was observed on canine serum ampicillin concentration in the single-dose study (II) or in the 14-day multiple-dose study (III).

2. Intravenously administered ampicillin caused significant disruption in canine intestinal microbiota and induced the emergence of ampicillin resistant bacteria. With oral targeted recombinant β-lactamase these antibiotic-induced adverse effects were prevented.

3. Mice treated with subcutaneous piperacillin developed high-density stool colonization with each of the tested microorganisms i.e. vancomycin-resistant Enterococcus faecium, extended-spectrum β-lactamase producing Klebsiella pneumonia, or Candida glabrata. With per os administered β-lactamase enzyme fecal colonization resistance was preserved, thus fecal colonization with above mentioned microorganisms impeded.
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