Using Probiotics in Intensive Care with Special Reference to Lactobacillus plantarum 299 and 299v

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Using Probiotics in Intensive Care, with Special Reference to Lactobacillus plantarum 299 and 299v

Abstract

Probiotics are Live microorganisms that confer a health benefit on the host.

The composition of the microflora in the gastrointestinal tract is rather constant in healthy subjects, but is easily disturbed by severe illness and the use of antibiotics. The intestinal barrier that separates the luminal contents, with a very high density of microorganisms, from an almost sterile milieu in the gut wall, is disrupted in critically illness. Probiotic bacteria have the ability to counteract those effects on the microflora and the gut wall.

Lactobacillus plantarum 299 and 299v has been shown to adhere to the intestinal mucosa throughout the gastrointestinal tract in healthy volunteers.

L pl 299v was given enterally to critically ill patients and it was verified from biopsies from the rectal mucosa that L pl 299v adheres and colonise also in critically ill patients on antibiotics.

Clostridium difficile is a bacterium that causes diarrhoea and aggressive inflammation in colon and infections with C diff is the most common health care related disease and is almost always related to the use of antibiotics. Probiotics have been used to reduce recurrence of C diff associated disease, and none of critically ill patients given L pl 299v enterally became infected with C diff compared to 19 % in the control group. L pl 299v was also shown to improve the intestinal permeability.

Ventilator Associated Pneumonia is caused by aspiration of infected secretions from the oropharynx. In intubated, mechanically ventilated critically ill patients the oropharyngeal microflora has been deranged and the risk of colonisation with enteric bacteria is high. Oral care procedures reduce the bacterial content in the oropharynx and the risk of developing VAP. L pl 299 was shown to be as effective as the standard oral care procedure using chlorhexidine, in reducing pathogenic.

Susceptibility to antibiotics of probiotics is vital in case one of the seldom appearing infections should occur. Re-isolates of L pl 299v from two studies in intensive care patients on broadspectrum antibiotics were tested against 22 different antibiotics and no change of susceptibility of clinical significance was detected when compared to the original strain.

Key words: Lactobacillus plantarum 299, Lactobacillus plantarum 299, Clostridium difficile, probiotics, ICU,chlorhexidine, critical illness, VAP, CDAD, intestinal permeability, antibiotic susceptibility

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List of Publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I

Adhesion of the probiotic bacterium *Lactobacillus plantarum* 299v onto the gut mucosa in critically ill patients: a randomised open trial
Bengt Klarin, Marie-Louise Johansson, Göran Molin, Anders Larsson, Bengt Jeppsson
Critical Care 2005; 9: R285-R293

II

Lactobacillus plantarum 299v reduces colonisation of *Clostridium difficile* in critically ill patients treated with antibiotics.
Bengt Klarin, Marlene Wullt, Ingrid Palmquist, Göran Molin, Anders Larsson, Bengt Jeppsson
Acta Anaesthesiol Scand.2008; 52: 1096-1102

III

Use of the probiotic *Lactobacillus plantarum* 299 to reduce pathogenic bacteria in the oropharynx of intubated patients: a randomised controlled open pilot study
Bengt Klarin, Göran Molin, Bengt Jeppsson, Anders Larsson
Submitted - ISRCTN00472141

IV

Susceptibility to antibiotics in isolates of *Lactobacillus plantarum* RAPD-type Lp299v, harvested from critically ill patients after administration of the probiotic strain *L. plantarum* 299v.
Bengt Klarin, Claes Schalén, Anders Larsson, Bengt Jeppsson, Göran Molin
In manuscript

Abbreviations

AAD	antibiotic associated diarrhoea
CDAD	*Clostridium difficile* associated disease
CFU	colony forming unit
CHX	clorhexidine
CRRT	continuous renal replacement therapy
C. difficile	*Clostridium difficile*
E. Coli	*Escherichia coli*
ETT	endotracheal tube
GALT	gut-associated lymphatic tissue
GIT	gastrointestinal tract
ICU	intensive care unit
IgA	immunoglobulin A
IL	interleukin
LAB	lactic acid bacteria
LIS	Lung Injury Score
L. plantarum 299	*Lactobacillus plantarum* 299
L. plantarum 299v	*Lactobacillus plantarum* 299v
MIC	minimum inhibitory concentration
MLN	mesenteric lymph node
PPM	potentially pathogenic microbe
PPI	proton pump inhibitor
SCFA	short chain fatty acids
SDD	selective digestive decontamination
TNF-α	tumour necrosis factor alpha
VAP	ventilator associated pneumonia
WBC	white blood cell

Definitions

Probiotics

According to the definition given by FAO, WHO [1] probiotics are *'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'*.

Prebiotics

Substances that are beneficial for the growth of certain species or strains of microorganisms. Prebiotics are primarily soluble food fibres that are ingested and subsequently digested not by the host organism, but by microorganisms in the colon which in turn stimulates the growth of lactobacilli and bifidobacteria in that part of the itestine are stimulated.

Synbiotics

A proper combination of selected microorganisms (probiotics) and prebiotic substances that promote the growth of the selected strains.

Background

Intestinal microflora

People are generally healthy and show no symptoms of infection even though they live in a sort of symbiosis with a wide variety of microorganisms. The main mass of bacteria hosted by mammals, is found in the gastrointestinal tract (GIT). In humans it is estimated that 1 kg of bacteria are found in the GIT. The skin is also a large reservoir for bacteria, and smaller amounts are found in the lungs, the mouth, the nasal cavity, and in the vagina. An adult person is made up of approximately 10^{13} cells but serves as a host for approximately 10^{14} bacteria. It has also been estimated that there are 500 or more species of bacteria in the GIT, that 30–40 species constitute 99% of the faecal microflora [2], and that 500 species (mostly others) are carried in the mouth and oropharynx [3]. The intestinal milieu is complex, especially in the lower GIT, and the multitude of species that was previously only assumed to be present (due to insufficient culturing methods) has more recently been confirmed with the help of new techniques, such as modern DNA tests.

Under normal conditions, bacterial counts in saliva are 10^6 to 10^8 colony-forming units (CFU) per millilitre, and *Lactobacillus* spp. often predominate. By comparison, bacterial counts are low in the upper GIT. Gastric aspirates contain 0–10^3 CFU/ml, and increase somewhat in the proximal small intestine (10^3–10^5 CFU/ml) and rise even more farther along the GIT. The highest density of bacteria is found in the colon, reaching levels of 10^{11}–10^{12} CFU/ml, and the flora in that area is dominated by species that are mainly obligate anaerobes, such as *Clostridium* spp., *Bacteroides* spp., fusobacteria, bifidobacteria, and eubacteria [4-7]. Fungal species, mainly yeasts like *Candida albicans*, normally occur throughout the GIT.

The composition and relative abundance of species in the GIT is unique for the individual host, and that pattern is established within the first years of life and depends to some extent on genetic factors but mostly on food intake, ingested bacteria, and other environmental factors. The vast majority of these species are non-pathogenic collaborators that help the host keep the numbers of pathogenic and potentially pathogenic species at levels that will cause no harm. These companions that live in a sort of symbiosis with the host are called commensals (a word indicating the sharing of food, derived from the Latin com mensa meaning *sharing a table*), and they should be thought of as part of homeostasis. Like other intraluminal bacteria, commensals can pass through the mucosa (translocate) [8]. Most translocating commensal bacteria become coated with antibodies (locally produced IgA) and are captured by macrophages within the mucosal layer. However, some of the commensals are phagocytosed by dendritic cells at that site and can remain within those cells for days, where they stimulate the local immune defence and the production of secretory IgA [9-11],

an important factor in the gut barrier function. Unlike pathogenic bacteria commensals are not found beyond the mesenteric lymph nodes (MLNs). Pathogenic and potentially pathogenic microbes (PPMs) that pass the barrier of immune defence in the mucosa and the gut-associated lymphatic tissue (GALT) can cause bacteraemia which can result in sepsis, severe sepsis, or septic shock, and ultimately lead to multi-organ failure and death [12]. The GALT consists of the lamina propria, the intraepithelial lymphocytes, Peyer's patches, and MLNs. Commensal bacteria are considered to be genomically stable, but the large intestine holds every requisite for being an efficient fermenter, and the stationary species are exposed to incoming species. These newcomers may be carriers of genes and plasmids containing genetic material coding for resistance to antimicrobial agents, and transmission of such material can convert strains that were formerly susceptible to being resistant. This has been seen in species such as. *Enterococcus faecalis* [13,14].

Under normal conditions the composition of the microbiota (bacterial and fungal species) is relatively stable, although an increase in *Clostridium spp* and a decrease in bifidobacteria occur with age. However, conditions such as stress, illness, and the use of antibiotics bring about changes that can be rather drastic and it takes months to re-establish the order that prevailed before the insult, especially when antibiotics are involved. That period of time is valid for restoration of the proportions of genera and species as well as for the percentages of resistant strains [15,16], but it is likely that there will be more of resistant strains and in that aspect the balance will not be fully restored. The effects of those changes are most pronounced in the GIT, and symptoms like diarrhoea are frequent, but vaginal fungal overgrowth is another well-known side effect of the use of antibiotics. Diarrhoea is an indication of the imbalance brought about by the conditions mentioned above. Many commonly used antibiotics significantly reduce the number of *Lactobacillus* spp [17-19], which improves the growth conditions for other, less favourable species. Bacteria of the endogenous flora often reside in special niches, where conditions for growth are good or optimal, and where they constitute the resistance to colonisation by transient potentially pathogenic species.

Changes in the intestinal microflora in severe illness

In patients suffering from a severe illness, there are several factors that affect the balance between the bacterial species they carry. Normally, hours to days pass before a person who is not feeling very well contacts the health services. During that period, symptoms such as nausea and vomiting can occur that lead to more or less pronounced dehydration. Conditions like these can also develop in a hospital ward. Dehydration can result in reduced perfusion and lowered oxygenation in the splanchnic region, which will favour the growth of obligate anaerobic bacteria in the GIT. Furthermore, as the availability of substrates decreases, in part due to lack of food intake, the transcription of virulence

factors in pathogenic bacteria is induced [20]. That process increases the aggressiveness of those species, which are thus more likely to cause complicating infections if they translocate in sufficient numbers.

Arrival at a hospital will not solve all the problems in this context, since in many cases it will take another couple of hours of waiting for doctors and laboratory results before a primary diagnosis can be made and the prescribed treatment can be implemented. Even after the correct treatment has been started, it is not unlikely that secondary damage will appear, and it takes time for the gut to recover. Meanwhile it is important to ensure that the hostile environment in the gut, with up to 10^{12} bacteria/ml of luminal contents, is kept well separated from the nearly sterile subendothelial tissue.

The next step in deterioration of the indigenous flora can be the introduction of one or more antibiotics intended to cure the patient.
Illnesses that are due to a bacterial infection can most often be satisfactorily treated with antibiotics aimed at eradicating the pathogens, but that approach can severely disturb the normal proportions of microbiota in the GIT.

In an illness involving reduced food intake and deviation of perfusion, gut motility will be dampened, and that effect will be accentuated by any sedatives or opiates that are added to the patient's treatment regimen. Prolonged transit time will also lead to a proximal spread of bacteria, with respect to both numbers and species. Consequently, at least in the distal part of the small intestine, the microflora will show similarity to the flora in the colon. Pathogenic bacteria normally found in the colon can be identified in gastric aspirates from critically ill patients as well as from patients with postoperative paralytic or obstructive ileus. In healthy people Gram-negative bacteria are seldom present in the oropharynx of healthy individuals, whereas they can be found in up to 75% of hospitalised patients [21, which is accompanied by a risk of infection in the lower respiratory tract.

An initial phase of illness with compromised perfusion of the splanchnic region can cause dysfunction of the gut barrier, which will lead to an increased risk of translocation of bacteria and endotoxins. Even if the patient receives proper treatment, it will take days before the gut barrier has regained full functionality. Intestinal permeability is increased during critical illness, particular after burns, major trauma and sepsis [22-24] and bacterial translocation has been demonstrated in patients with bowel obstruction [25.26]. An increase in translocation will amplify the risk of secondary infections derived from the gut, and, in patients afflicted with a severe or critical illness, the gut has sometimes been described as an "undrained abscess" [27] or the motor of multi-organ dysfunction syndrome (MODS) [28,29]. In order to attenuate or abolish this threat, non-absorbable anti-microbial agents have been used to decrease the occurrence of facultative aerobic Gram-negative bacteria. This method is

employed in some ICUs and is referred to as selective digestive decontamination (SDD) for reduction of PPMs, and indeed research results have shown that it can lower the rates of infections [30-32], ventilator-associated pneumonia (VAP) [33-34], and mortality [31, 35]. Also for prevention of VAP, a similar procedure limited to the application of antibiotics in the oral cavity has been used with good outcome [36-38]. However, the use of such procedures is limited due to the risk of bacteria developing resistance to antibiotics [31, 39]. This risk may be exaggerated since patients treated in ICUs represent only a small fraction of all patients given antibiotics. Nevertheless, since an increase in multi-resistant bacterial strains constitutes a significant threat to future success of treatment of serious infections, the use of advanced prophylaxis like SDD should be used with cautiousness, and in selected patients.

A number of the first antibiotics identified (e.g., penicillin), some of which are still in use, were actually provided by Mother Nature, and she still offers a multitude of substances to be used as both prophylaxis and adjuvants to therapies against infectious diseases. The production of agents such as bacteriocins that are aimed at inhibiting the growth of species that co-exist with other organisms in the same niches is one example of how we can take advantage of living microorganisms in the never-ending fight against infectious illnesses. Moreover, non-pathogenic species can be added to compete for nutrients and space, which is likely to reduce the numbers and proportions of other microorganisms that are or might be pathogenic.

Adding non-virulent bacteria (and/or fungi) to a setting in which the balance between microbial species has been disrupted can attenuate those disturbances, if that action is taken at an early stage, and the period of pronounced imbalance can be shortened by introducing the non-virulent microorganisms after the onset of symptoms. During the last decades, much research has focused on these problems and the task of finding microorganisms with the proper characteristics. Administration of probiotics—lactobacilli and bifidobacteria—appears to be potentially more beneficial for the microbiological environment as a whole than SDD or other anti-therapies, when the goal is to reduce the growth of pathogens in the gut [5].

Probiotics

Probiotics are living non-virulent microorganisms that, when given in certain amounts, have health-promoting effects on the host. Most probiotics are bacterial strains, although some fungal species also have the desired properties. *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus thermophilus* strains are the main microorganisms in use,and certain strains of *Eschericia coli* (*E. coli*), *Enterococcus feacalis*, and *Bacillus cereus* are examples of other species that have also been investigated as potential probiotics. Among fungi, *Saccharomyces boulardii* has been thoroughly studied and is the only probiotic that is registered as a medicinal preparation in Sweden. It is used as prophylaxis

against antibiotic-associated diarrhoea (AAD) and as an adjuvant to antibiotics for prevention of recurrence of diarrhoea caused by *Clostridium difficile*.

Ingested bacteria face a tough journey on their passage through the GIT. To reach the large intestine, where their main interactive task is performed, they have to resist the effects of gastric juice, bile, and pancreatic juices, and also survive the transport through the small intestine. Besides being alive when they reach the main target (i.e., the colon), bacteria that are claimed to function as probiotics should be able to become established, preferably by adhering to the mucosa, in order to interact optimally with the epithelial cells lining the gut wall. They should also be non-pathogenic, non-toxic, and easy to culture, and, for use in humans, it is also desirable that they are of human origin.

The health-promoting action of probiotics depends on several features. They have to compete with other species for space and nutrients. Their metabolites interact with other bacteria and fungi that are present in the gut, and as well as with the host. Among the metabolites are bacteriocins, which are peptides produced by many bacterial species. Some bacteriocins inhibit the growth of other bacteria that belong to similar species or other genera. Other types of bacteriocins act as signalling molecules in the interaction with other bacteria and with cells of the host organism [40,41]. Specific bacteriocins, such as nisins, have been produced and used as preservatives in the food industry for many years [42].

The production of lactic acid and other organic acids lowers the pH in the luminal content, which in turn promotes some other beneficial microbes like bifidobacteria. Some probiotics produce the short chain fatty acids (SCFA) acetic acid, propionic acid, and butyric acid, while others including. *Lactobacillus plantarum* 299 and *Lactobacillus plantarum* 299v generate some of the same metabolites and also stimulate other species to produce SCFA [43,44], which are preferred as substrates by colonocytes and to some extent enterocytes. This improved access to energy substrates will cause the cells lining the mucosa to perform better and will strengthen the intestinal barrier against invading bacteria and bacterial cell wall components such as endotoxins like lipopolysacharide (LPS) from pathogenic Gram-negative bacteria).

The reaction of the general public to the news of the health-bringing properties of probiotics has been overwhelmingly positive. The global probiotic market within the food industry has an estimated yearly turnover of many billions of euros. Regulations concerning probiotics and requirements for documentation of claimed benefits of the products constitute a field of increasing interest for regulatory authorities. Grants from the European Union and other official financial sources support the development of new products, emphasising the importance of the issue. At present, there is no worldwide consensus regarding safety and other issues associated with the use of probiotics.

Unfortunately, many of the products that are marketed as having probiotic features contain strains for which it has been found that few of the bacteria survive the challenge of the hostile milieu in the stomach and proximal intestine. Still, they may have certain positive effects on the host, even if they do not meet the requirements stipulated for true probiotics. Although questions may be raised as to whether the consumed probiotic preparations actually promote the health of people who are already healthy, who also represent the group that consumes most of the sold products, those individuals themselves indicate that they do experience increased well-being. Several controlled trials concerning symptomatic GIT disturbances like irritable bowel syndrome (IBS) [45-47] and other stress-associated conditions have shown that symptoms were reduced or abolished by consumption of probiotic products [48]. Those stress conditions may be a consequence of our modern Western life style, in which a rural, rather monotonous and quiet existence including a diet comprising lots of fibre, bacteria (many of which would be considered probiotic today), and low energy has been replaced with a hectic daily pace and high-energy fast foods processed under strict hygienic control. Those changes in lifestyle over only one or two centuries have been accompanied by very few or no alterations in the human genome or the functionality of the GIT. Therefore, I conclude that probiotics do have a rehabilitating, reconditioning role to play as promoters of the well-being of the healthy "modern" man or woman who has no desire to make any radical changes in his/her lifestyle.

Many investigations have examined the use of different probiotic strains and preparations containing probiotics, but most of those have been interventional studies with rather short periods of observation. Furthermore, investigations have not been conducted to determine whether the good effects seen after intervention with a probiotic product will remain during prolonged intake. Most of the probiotic bacteria in use are of human origin, but many of them are not part of the commensal flora found in most people. Hence, in a healthy subject, introduction of bacteria that are new to the internal ecosystem will disturb the microbiological balance. Over time, a new ecological balance will be created, provided that the strain or strains do become established. However, it is not likely that the new strains will become a part of the commensal family (at least not in adult hosts), and so they will disappear as consumption of the bacteria is discontinued.

Probiotic foods products sold in grocery stores are mostly based on dairy products and often contain more than one bacterial strain. Yoghurt products contain at least *Lactobacillus bulgaris*, and *Streptococcus thermophilus* and a variety of other species are used in other items. Probiotics are also marketed in the form of pellets, suspensions, and freeze dried powders, and many of those dry products can even be purchased on the internet.

The effects of both mono-strain and multi-strain preparations have been assessed in many clinical studies, most of which have focused on infectious and inflammatory parameters in combination with clinical outcome. In a few studies, culture of faeces or other samples from the GIT were performed to monitor survival of the probiotic bacteria, and it is uncertain whether the microorganisms that were used in those evaluations really fulfilled all the criteria of being a probiotic. Nevertheless, the tested products may still have had a positive impact on the well-being and recovery of the participating patients, and that they may also have contributed to restoration of the microbiological flora

Lactobacillus

The genus *Lactobacillus* is large and heterogeneous and about 100 species are recognised to date. They are Gram-positive rods or coccobacilli that are anaerobic or aerotolerant, and they ferment carbohydrates to lactic acid along a sole or a main metabolic pathway, and they do not form spores. Lactobacilli metabolise carbohydrates, and they can be divided into three groups according to whether they perform that task in a homofermantative, facultatively heterofermentative, or heterofermentative manner. Where carbohydrates are available, lactobacilli occur. Consequently they are found in plants, fruits, vegetables, and fermented foods such as diary and meat products, brined olives, sour doughs, sauerkraut, and certain beverages. In the human body, they are found in the GIT and the respiratory and female genital tracts.
Lactobacillus species are integral parts of the healthy human intestinal flora, but they are not the predominating genus on the colonic mucosa—other genera are present at the same level or at higher levels [49-51]. *Lactobacillus* strains are found in every part of the GIT, and they occur in rather high numbers in the oral cavity, where they may be in the majority.

People have exploited fermentation by lactobacilli for centuries as a means of preparing and preserving foods long before the age of refrigerators. In fact, there is evidence that lactic acid fermentation was used even thousands of years ago. During the fermentation process, lactic acid bacteria (LAB) reduce the pH and release substances such as bacteriocins that inhibit the growth of other bacteria, which results in foods that are tasty and contain low counts of PPM

Lactobacillus plantarum

The species *L. plantarum* is facultatively heterofermentative in that it ferments hexoses via glycolysis and pentoses via the phosphogluconate/phosphoketolase pathway, and such metabolisation of carbohydrates results in the production of lactate, acetate, ethanol, and carbon dioxide. *L. plantarum* harbours a larger genome than many other species, which may explain why these bacteria can thrive on plants as well as in animals. *L. plantarum* is generally not considered to be part of the commensal microflora, although strains of this species are often

found in faecal samples from humans. *L. plantarum* is highly tolerant to low pH, and it is often the dominating species in LAB-fermented products, and consequently these bacteria are also very tolerant to gastric juices and are found on the mucosa throughout the GIT.

Lactobacillus plantarum 299 (DSM 6595) and 299v (DSM9843)

L. plantarum 299 and *L.* 299v are genomically closely related Gram-positive rod shaped bacteria that are facultatively anaerobic and heterofermentative. These two strains were identified in human mucosal samples among several other potential probiotic *Lactobacillus* strains [52]. In another study [53], 19 of those strains were fermented separately in oatmeal gruel and then combined in a mixture that was given to healthy volunteers. Biopsies were taken from the upper jejunum and the rectum before consumption of the test product, and again one and 11 days after administration was terminated. In samples taken on day 11 after administration of the test mixture, *L. plantarum* 299 and 299v were identified in 11 of 13 subjects, whereas other strains were found only in scattered samples. This finding, together with the results of sensitivity tests performed *in vitro* using human gastric juice and bile [54-56], demonstrates that these two strains must be regarded as true probiotics.

Expression of a mannose-specific adhesin has been identified in *L. plantarum* 299 and 299v [57], as well as certain other strains in the same *L. plantarum* subgroup, and it is believed that the capacity for attachment provided by that protein is involved in the ability of the bacteria to colonise the intestine. This mechanism is also used by pathogenic bacteria [58,59], and thus the *L. plantarum* strains can displace the harmful species

L. plantarum 299 and 299v have been studied in many settings, in humans as well as in animal models. In a rat model of colitis, *L. plantarum* 299v has been shown to reduce intestinal permeability and bacterial translocation, and decrease mucosal injury [60,61]. In addition, the ability of *L. plantarum* 299v to adhere to the intestinal mucosa prevents bacterial translocation in septic rats [62], and pretreatment with *L. plantarum* 299v prevents increased intestinal permeability induced by *Escherichia coli* [58].

Not only has *L plantarum* 299v been reported to adhere to the mucosa in the small intestine and in the rectum [53], but also to the mucosa of the tonsils [63]. The cited studies were performed on non-antibiotic-treated healthy volunteers, and it is not known whether *L. plantarum* 299v would also be able to become established and adherent in seriously ill patients who are receiving antibiotic treatment and have a deranged microbial flora.

Probiotics and patient safety

Strains of species that are used as probiotics are assumed to be non-pathogenic. There are a wide variety of starter cultures available on the market, all of which are generally regarded as safe (GRAS). Many strains have been tested regarding their viability and usefulness as probiotics, and *in vitro* assays have also been applied to several strains (primarily *Lactobacillus* spp.) to determine their ability to inhibit the growth of pathogenic bacteria. However, *ex vivo* tests and tests in healthy volunteers cannot guarantee that complications will not occur when a probiotic or a mixture of probiotics is used in a specific clinical setting. Unfortunately, such a problem did arise in a study in which a mixture of probiotics was given to patients with pancreatitis, because increased mortality and several cases of bowel ischaemia were observed in the group given the active bacteria treatment [64]. The mixture administered in that investigation was based on thorough and systematic research [65.66], but other questions have been raised regarding the procedures used to administer the mixture, indicating that they may very well have been the main reason for the failure, and not the probiotics per se. That particular study is given further consideration in the discussion section.

Although there are only a few reports in the literature that concern systemic infections with *Bifidobacterium* spp. [67], much more information has been published about infections (e.g., bacteraemia, sepsis, and endocarditis) involving *Lactobacillus* spp., including the strains that are used as probiotics [8, 68-72]. When the probiotic *L. rhamnosus GG was* introduced in diary products in Finland, no increase in *Lactobacillus* bacteraemia was observed, whereas there was a very rapid increase in use of the GG strain [73].

Furthermore, in a survey conducted in Stockholm, Sweden, over a period of more than six years [74] it was found that the fraction of bacteraemia caused by lactobacilli remained the same, and none of the *Lactobacillus* strains used as probiotics were identified in the area covered by the microbiological laboratory responsible for the analyses.

The incidence of infections with *Lactobacillus* spp. is low, as indicated by data showing that they represent < 1% of positive findings in blood cultures, and 0.2% might be a more accurate figure. It is plausible that the number of cases reported is erroneous because some findings of *Lactobacillus* are discarded as contaminants, or the bacteria are never identified due to an inappropriate culture medium or procedure. In general, *Lactobacillus* bacteraemia is rare, and cases positive for *L. plantarum* are even more uncommon.

Lactobacillus spp. are part of the microbiological flora to which we are exposed, and strains indistinguishable from specified probiotic strains have been identified in blood cultures and other sites of infection. Nonetheless, there is no convincing evidence that the use of probiotics is accompanied by an increased

risk of infection in either healthy or sick people, with some exceptions. Almost all reports of systemic infections with lactobacilli refer to patients that in some aspect have had an inadequate immune defence. *L. plantarum* 299v and *L. rhamnosus* GG have been shown to have beneficial effects in both children and adults infected with HIV [75,76], and no adverse events were observed in those studies. Probiotics are used by healthy people as well as in hospitalised patients, and the selection pressure of antibiotics in relation to those bacteria is low in the general public, but higher in care settings, especially in the treatment of critically ill patients.

Probiotics and the critically ill patient

Over the past decade a number of studies have been performed focusing on the effects of probiotics given to critically ill patients [77-82]. The results have varied, and most of the observations have been favourable for patients given such active treatment, although the differences have not always reached statistical significance. The negative outcome for the patients in the above-mentioned study of probiotics in pancreatitis [64] was very disappointing, and led to a halt in all investigations involving probiotic treatment of critically ill patients in the Netherlands. This was a drastic decision, and I believe such research must be continued. The protocol and results of the Dutch study should be analysed with an open mind, and all reasonable explanations for the increased morbidity and mortality should be considered and measures taken to avoid those factors in similar investigations in the future.

A review of the literature on use of prebiotics, probiotics, and synbiotics in adult ICU patients was published in Clinical Nutrition 2007 [83] it was concluded:

"The use of pre- pro- or synbiotics in adult critically ill patients confers no statistically significant benefit in the outcome criteria studied. There is currently a lack of evidence to support the use of pre- pro- or synbiotics in patients admitted to adult ICUs, and a large well-designed trial is needed in this area. "

However, most of the studies that have been conducted have been small or fairly small, probably due to practical difficulties. Some limited investigations with positive outcomes were reported after the mentioned review was published [81, 82].

Some of the studies involving patients admitted to ICUs have evaluated the effects of *L. plantarum* 299 and 299v [77, 78, 80]. For example, in Scarborough, in the United Kingdom, the MacFie group has studied *L plantarum* 299v (in the form of ProViva) and also other probiotic preparations [79] in surgical and intensive care patients. Furthermore, in studies performed in an intensive care setting, most of the admissions included in the investigations have been postoperative cases with rather low APACHE II scores, which might explain why the differences found were not always statistically significant. The

soundness of continuing clinical investigations of *L. plantarum* 299v has even been questioned based on the negative results of the cited UK study [84]. Notably, when the total numbers of probiotic bacteria administered in those studies are added together, they should be considered as somewhat low, and, in addition, most of the patients included were not very ill, and consequently it must have been more difficult for the investigators to identify differences in disturbances that were not very pronounced. Our research group has thoroughly evaluated the results of other investigations and concluded that there are no reasons not to continue the use of *L. plantarum* 299 and 299v strains in the intensive care setting.

Antibiotic associated diarrhoea (AAD)

Treatment with antibiotics, especially broad-spectrum agents, is accompanied by changes in the microbiological flora of the body, and it is in the GIT that the impact of those disturbances becomes most evident. AAD and *Clostridium difficile*-associated disease (CDAD) [85–88] are common side effects and it has been reported that the incidence of AAD in hospitalised patients given antibiotics is 3–29% [89] and that 20–60% of those cases are due to *C. difficile* [90]

Clostridium difficile and *Clostridium difficile* associated disease (CDAD)

C. difficile is an anaerobic spore-forming species that is probably responsible for most of the health-care-related bacterial infections. The spores can survive for long periods of time and are difficult to eradicate [91], and consequently they represent a threat for re-infection or transmission to caregivers or patients sharing the same facilities.

A few percent of the population are carriers without symptoms, and since the intestinal flora suppresses the growth of *C. difficile*, the hosts are not aware of the potential risk of disease or spreading the bacteria to others. Many strains of *C. difficile* produce toxins A and B, which damage the gut mucosa, especially in the colon. Such injury ranges from mild inflammation to toxic megacolon, sepsis, and death. In severe cases, colectomy is the radical life-saving therapy. Different strains produce different amounts of these two toxins. One strain, PCR ribotype 027, generates up to 100 times higher levels than most other *C. difficile* strains and has emerged as a serious threat to health care after outbreaks in North America [85,92,93] and some European countries [94]. *C. difficile* ribotype 027 was already present in "historical" isolates collected in Sweden between 1997 and 2001 [98,99], and, has in recent years caused some cases with severe illness in this country. Infections with PCR ribotype 027 lead to greater morbidity and mortality [85,95-97] compared to other strains of *C. difficile*. Epidemic strains have been reported to exhibit increased sporulation

[99], which elevates the severity of the problem, since it means a greater risk of spreading and survival of the bacteria.
The incidence of CDAD in the general population is increasing [85,89,90,100], as is the number of deaths reported to be due to *C. difficile* infection [101]. Besides the use of antibiotics, increasing age and length of hospital stay are risk factors for becoming infected.
CDAD is usually treated with oral metronidazole or vancomycin, and that strategy in combination with probiotics (*Saccharomyces boulardii* or *Lactobacillus* spp.) has been found to reduce the recurrence rates [102, 103]. In one of the cited studies [103], recurrence was 36% in patients given *L. plantarum* 299v and metronidazole compared with 67% with a group given only the antibiotic.

CDAD in the intensive care setting

Case reports [92, 104, 105] and retrospective investigations [93. 94] have been published, but, to my knowledge, there have been no studies of the incidence of *C. difficile* infections in ICUs, even though that rate is probably higher in patients in such facilities than in the general hospital population. One study of the use of probiotic preparations as prophylaxis against *C. difficile* infection and CDAD has been published [106]. Briefly, commercially available yoghurt was given to hospitalised, antibiotic-treated patients, and no cases of *C. difficile* infection appeared in that group, whereas 17% of a group given control product had CDAD. Since *L. plantarum* 299v had been found to be effective in reducing recurrence of infection with *C. difficile* [103], it seemed logical for us to use that *Lactobacillus* strain for the purpose of preventing *C. difficile* infections in ICU patients treated with antibiotics.

Ventilator associated pneumonia (VAP)

VAP is a common complication in intensive care patients on mechanical ventilation, and it is associated with prolonged ICU and hospital stays, additional costs, and high mortality [107]. The risk of developing VAP increases by 1% with each additional day of mechanical ventilation [107, 108].
The major cause of VAP is aspiration of either potentially pathogenic microorganisms from the oropharynx or fragments of biofilms from the endotracheal tube (ETT). With the use of specially coated ETTs, formation of such biofilms can be delayed, but not prevented [109,110]. Selective decontamination achieved with antibiotics in the oral cavity alone [111-112], or throughout the GIT [114,115], has been shown to lower the incidence of VAP and reduce mortality. However, the use of such procedures is limited due to the risk of bacteria developing resistance to antibiotics [116, 117]. In recent meta-analyses, it was concluded that oral decontamination with chlorhexidine (CHX) can prevent VAP [118], but that strategy does not reduce the time on ventilator, the length of stay (LOS) in the ICU, or mortality [119]. Thus, there is a need for

alternative approaches that are intended to lower the oropharyngeal load of pathogenic microorganisms as a means of decreasing the risk of VAP.

In recent years orally administered probiotics have been shown to reduce bacteria and yeasts in biofilms on vocal prostheses [120, 121]. Therefore, we hypothesised that swabbing the oral mucosa with probiotics would be an effective (and microbiologically attractive) method of reducing pathogenic oral microorganisms in tracheally intubated, mechanically ventilated, critically ill patients.

Other investigators had previously observed that *L. plantarum* 299v adhered to the tonsils in healthy volunteers [63], but it was not known whether that strain, or the genomically closely related *L. plantarum* 299, can become established in the oropharynx and suppress the growth of PPMs. Accordingly, we were anxious to investigate that new and challenging perspective of the use of probiotics.

Susceptibility to antibiotics

Antibiotics and probiotics are being widely used. by the general public. With the intention of counteracting the side effects of antibiotics, probiotics are recommended by many physicians and are often chosen by health care facilities. A number of investigations have focused on probiotics and critically ill patients, but few if any, before two of the present studies (Papers I and II), have explored the impact of administered probiotics on the intestinal flora or the patterns of susceptibility of the treatment bacteria to antibiotics.

Antibiotics, often those that are broad-spectrum agents, are vital components of the treatment of ICU patients. Exchange of genetic material is an ongoing process in the GIT, where some species are more prone to both import and export such material, including genes coding for resistance. In the ICU environment, there is an increased risk of selection of bacteria that are resistant to antibiotics due to the high selection pressure of those agents. To counteract AAD, probiotics are used in many ICUs. With the growing abundance of bacteria that are resistant to antimicrobial agents, there is a concomitant increase in the risk that other bacteria will incorporate genetic material containing coded information for antibiotic resistance. Administered probiotics and other transiting bacteria are exposed to the risk of transfer of resistance genes, as well as other genes from the rest of the microbial species present in the gut.

Aims

The main objective of the research underlying this thesis was to investigate the usefulness and safety of the two genomically closely related probiotic strains *L. plantarum* 299 and 299v in the intensive care setting. More specifically, the aims of the four studies that were conducted were as follows:

- To study the feasibility and safety of useing *L. plantarum* 299v in a fermented oat meal gruel administered enterally to critically ill patients;
- to determine whether *L. plantarum* 299v can also adhere to and become established on the rectal mucosa in critically ill, antibiotic-treated patients;
- to investigate the influence of *L. plantarum* 299v on the intestinal microflora and inflammatory parameters;
- to explore the ability of *L. plantarum* 299v to reduce or inhibit colonisation with *C. difficile* in critically ill antibiotic treated patients;
- to ascertain whether enteral administration of *L. plantarum* 299v can improve intestinal permeability;
- to find out whether *L. plantarum* 299 in an oral care procedure in mechanically ventilated critically ill patients is safe and can be used as an alternative to the antiseptic chlorhexidine;
- to study the influence of *L. plantarum* 299 on the oropharyngeal microflora in intubated, mechanically ventilated critically ill patients.;
- to investigate whether the susceptibility of *L. plantarum* 299v to antibiotics would change upon exposure to such agents in the GIT.

Study designs and methods

The present research included three clinical studies (Papers I–III) and one laboratory investigation (Paper IV). Two of the clinical studies (Papers I and III) were approved by the Human Ethics Committee at Lund University, and a third (Paper II) was approved by the Human Ethics Committees at Lund University and Gothenburg University, and by the Swedish Medical Products Agency.

Studies I and III were performed in the ICU of Lund University Hospital, and study II was conducted at the following facilities: the ICU of Lund University Hospital; the ICU of the Department of Infectious Diseases and the general ICU of Malmö University Hospital; the Neurosurgical ICU of Sahlgrenska University Hospital in Gothenburg; the ICU of Karlskrona County Hospital.

In all three studies GCP/ICH was applied, and the work was done in compliance with the Helsinki Declaration. Informed consent was obtained from the patients or their next of kin.

Patients were included in the studies and procedures were started within 24 hours of admission to the respective ICU. The study designs and inclusion and exclusion criteria used in the clinical investigations are presented in Table 1.

Study products

The study products used in all three clinical investigations were provided by Probi AB, Lund, Sweden. A few patients were able to drink the study products, although in the majority of cases the products were administered through a nasogastric tube. Organisation of the clinical investigations and the composition and volumes of the control products administered are summarised in Table 2.

Table 1
Designs and inclusion and exclusion criteria of the three clinical studies

Randomised, open, single centre study	**Randomised, double-blind, placebo-controlled multicentre study**	**Randomised, open, single centre study**
Paper I	**Paper II**	**Paper III**
Age≥ 18 years	Age≥ 18 years	Age≥ 18 years
Presumed need for intensive care for 3 days or more	Presumed need for intensive care for 3 days or more	Presumed need for mechanical ventilation for more than 24 hours
Anticipated to tolerate enteral feeding	No known positive test for *Clostridium difficile* during the week before enrolment	Not suffering from pneumonia at ICU admission
Indication for broadspectrum antibiotics	Expected to tolerate enteral feeding started within 24 h of ICUadmission	No fractures in the facial skeleton or the base of the skull
No significant coagulation disorder or thrombocytopenia	Not allergic to any of the components of the study product	No oral ulcers
	Not moribund	Not moribund
	Patient was excluded if enteral feeding could not be started within 24 h	Not immune deficient
	Patient positive for *C. difficile* on inclusion sample was excluded from the analyses of that species	Not carrier of HIV or viral hepatitis

Table 2
Content and protocol for administration of the study products in the three clinical investigations

	Paper I	Paper II	Paper III
Study product	Oatmeal gruel fermented with **Lp 299v**	Oatmeal gruel fermented with **Lp 299v**. Fruit juices added	Suspension of **Lp 299**
Bacterial content	10^9 CFU/ml	$8x10^8$ CFU/ml	
Amount given	* 50 ml q6h for3 days, then 25ml q 6h	100 ml q12h for 3 days, then 50ml q12h	10^{10} CFU twice a day
Control product	No control product	Oatmeal gruel with added lactic acid and fruit juices	Chlorhexidine solution 0.1 mg/ml

The first two patients received 50 ml q 6h throughout the study.
See comments in the text.

Microbiological analyses

Department of Clinical Microbiology

Specimens of blood, urine, and tracheal secretions, and samples from wounds or other relevant locations were sent for culture weekly or when clinically indicated. All tips of removed central venous catheters and on suspicion of infection, also arterial lines were sent for microbiological analysis.

Samples collected from the participating patients either due to clinical indications or specifically for the present investigations (including analysis for *C. difficile* in Paper II) were analysed at the clinical microbiology laboratories of the hospitals involved according to routine methods. In one of the studies (Paper III), the results of the analyses of specimens from the oropharynx were validated as being sputum samples and were analysed according to the protocol for samples from the lower respiratory tract. All results from these laboratories were available to the attending physicians.

Probi AB

All samples that were sent for analysis at the research laboratory at Probi AB, Lund, Sweden were blinded to all of their personnel.
In study I samples from the rectal mucosa were washed three times in a solution (0.9% NaCl, 0.1% peptone, 0.1% Tween, and 0.02% cysteine) before dilution and inoculation.

The samples that were sent for analysis at the research laboratory at Probi AB, Lund, Sweden, were blinded to all personnel at that facility. In the first study (Paper I) samples from the rectal mucosa were washed three times in a solution consisting of 0.9% NaCl, 0.1% peptone, 0.1% Tween, and 0.02% cysteine before dilution and inoculation. Viable bacteria counts were done as follows: on Rogosa agar (Oxoid) incubated anaerobically at 37 °C for three days for lactobacilli (including Lp299v); on Violet Red Bile Glucose agar (Oxoid) incubated aerobically at 37 °C for 24 hours for *Enterobacteriaceae*; on Perfringens agar base (Oxoid) + TSC Selective Supplement (Oxoid) incubated anaerobically at 37 °C for three days for sulphite-reducing clostridia; in Raffinose-*Bifidobacterium* medium (RB) incubated anaerobically at 37 °C for three days for bifidobacteria; on Slanez&Bartley agar (Oxoid) incubated aerobically at 44 °C for two days for enterococci; on Wilkins-Chalgren agar (Oxoid) incubated anaerobically at 37 °C for three days for total anaerobic bacteria; on Wilkins-Chalgren agar (Oxoid) + Gram-negative supplement incubated anaerobically at 37 °C for three days for Gram-negative anaerobic bacteria. Furthermore, representative colonies of *L. plantarum* 299 and 299v were selected and purified on Rogosa agar for subsequent identification by randomly amplified polymorphic DNA typing (RAPD) [122].

Chemistry

Samples for of routine chemical analysis were taken at inclusion and thereafter once a day. Samples for blood gas analysis were collected several times a day and the analyses were performed at the point of care.

In the study reported in Paper II, samples for analysis of cytokines were also taken at inclusion and then daily. The analyses were performed at the Department of Experimental Surgery, Malmö University Hospital, using human ELISA sets for TNF-α, IL-1β, IL-6, and IL-10 (BD Biosciences, San Diego, CA, USA).

Nutrition

In the three clinical studies (Papers I–III), enteral formula administered through a nasogastric tube constituted the main source of nutrition for the ICU patients, and this was started as soon as circulatory and respiratory functions had been stabilised. According to the protocols of the participating departments,

nasogastric administration was initiated at a low rate and then increased stepwise.There were no significant differences between the active treatment and control groups in the three studies with regard to delivered volumes or total energy content.

Medication

All medications given to the participating patients were recorded. Antibiotics were prescribed on empirical grounds by the physician in charge, and changes in the regimens were made in consensus with a consultant physician at the departments of infectious diseases at the respective hospitals. According to the protocols used in two of the studies (Papers I and II), the participants received metoclopramid (Primperan®, Sanofi, Paris, France) and sodium picosulphate (Laxoberal®, from Boehringer Ingelheim, Ingelheim, Germany; or Cilaxoral® from Ferring, Malmö, Sweden). In the first study (Paper I), the patients were also given cisapride (Prepulsid®, Janssen-Cilag) after the first two patients given active treatment had developed bowel distension. Adjustments in this medication were made to correspond with the frequency of bowel movements.

Methods of specific relevance for the individual studies

PAPER I

Biopsies

Within 24 hours of admission of the patients to the ICU of Lund University Hospital (always before administration of the study product), and thereafter twice a week, biopsies were collected from the rectal mucosa by experienced surgeons. These specimens were prepared within hours of collection for bacteriological analysis at Probi AB.

PAPER II

Cultures

Before starting the enteral feeding, rectal faecal samples were collected for culture of *C. difficile* and Lp299v, and such samples were subsequently taken twice a week as long as the patient stayed in the ICU. Defecation was infrequent in most of the patients, and the samples were often collected as rectal swabs. At the ICU in Lund also fresh faecal samples was collected from the patients, and those samples were analysed at Probi AB, besides for *L. Plantarum* 299v and total counts of lactobacilli, also *Enterobacteriaceae,* sulphite-reducing *Clostridia,* bifidobacteria, total counts of anaerobic bacteria, and Gram-negative anaerobic bacteria.

Gut permeability assessments

At the ICU in Lund, on the day of inclusion (before enteral feeding was started) the patients were given a mixture of non-metabolisable sugars (lactulose [5 g], L-rhamnose [1 g], D-xylose [0.5 g], and 3-O-metyl-D-glucose [0.2 g]) in 100 ml of water (240 mosm) for gut permeability assessment. On day 3 or 4, 13 of the patients (two had been discharged) did a second test using the same sugar solution. Metoclopramid 10 mg was given intravenously in conjunction with administration of the sugar solution. Urine or combined ultrafiltrate-dialysate (the latter from two patients [1 from each group] on continuous renal replacement therapy [CRRT])) was collected for five hours after the administration of the sugars. Vials of 10-ml samples from the respective bags were stored at –70 ^{o}C pending analysis and the samples from the patients on CRRT (2 or 3 bags per test) were analysed separately. Totals of the recovered amounts were used in further calculations.
The samples were analysed at the Department of Analytical Chemistry, Lund University, using a HPLC system with an HPAEC Carbopac PA10 column and pulsed amperometric detection (Dionex, Sunnyvale, CA,USA). To achieve good separation of the probes in samples supposed to be containing glucose, we modified a previously described method [123].

PAPER III

Oral care was performed twice a day. The control group was treated according to the department's standard protocol: dental prostheses were removed; secretions were removed by suction; teeth were brushed using toothpaste (Zendium, Opus Health Care, Malmö, Sweden); all mucosal surfaces were cleansed with swabs that had been moistened with a 1 mg/ml CHX solution (Hexident, Ipex, Solna, Sweden). In the group treated with *L. plantarum* 299 the initial mechanical steps were the same as in the control group, but the subsequent cleansing was instead done with gauze swabs soaked in carbonated bottled water, after which *L. plantarum* 299 was applied to the mucosal surface of the oral cavity. This was performed using two gauze swabs (one for each side of the oral cavity), which had been allowed to absorb 10 ml of a solution containing a total of 10^{10} CFU *L. plantarum* 299. Excess suspension was not removed. In both groups, when necessary between the oral care procedures, secretions were removed by suctioning, and gauze swabs moistened with carbonated bottled water were used to wipe off remaining secretions.

Cultures were taken from the oropharynx and from the trachea at inclusion. Sampling was repeated prior to the oral care procedures on days 2, 3, 5, 7, 10, 14, and 21 in patients that were still mechanically ventilated. If a patient was extubated on a non-culture day, cultures were taken before the extubation. One set of cultures was analysed according to normal routines at the Department of Clinical Microbiology, Lund University Hospital. Another set was sent blinded

to the research laboratory at Probi AB, Lund, Sweden for identification and quantification of total CFU of lactobacilli and identification of *L. plantarum* 299.

The patients were placed in a semi-recumbent position and were ventilated in pressure control or pressure support mode by a Servo^{i} ventilator (Maquet AB, Sweden) via a heat moisture exchange (HME) filter (Barrierbac "S", Mallinckrodt DAR, Mirandola, Italy). A closed suction system (TRACH-Care 72, Ballard Medical Products, Draper, UT, USA) was used. The patients inhaled 2.5 mg salbutamol (GlaxoSmithKline, Solna Sweden) and 0.5 mg ipratropium (Boehringer Ingelheim, Stockholm, Sweden) every six hours.

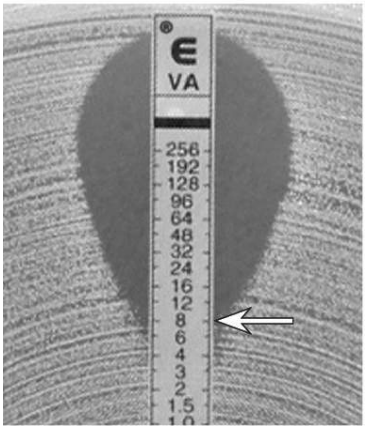
Chest radiographs were obtained after tracheal intubation and thereafter when clinically indicated. Lung function was assessed by use of the Lung Injury Score (LIS) [124]. Blood gases were obtained at least three times a day and were analysed at the ICU.

VAP was assessed although the study was not powered to detect differences in the frequency of the condition. The following criteria were used to identify VAP: (1) a new, persistent or progressive infiltrate on chest radiograph combined with at least three of the other four criteria; (2) a purulent tracheal aspirate; (3) positive culture of tracheal aspirates occurring after 48 hours of mechanical ventilation; (4) rectal or urine bladder temperature > 38.0°C or < 35.5°C; (5) WBC count > 12 or < 3 [108, 125].

PAPER IV

Re-Isolates of *L. plantarum* 299v from two of the clinical studies (Papers I and II) were analysed at Probi AB for susceptibility to several antibiotics. All strains had been stored at –80 ºC pending analysis. After reconditioning, the frozen strains were suspended in Brucella broth and then inoculated on Brucella agar plates (Oxoid). Etest strips of 22 different antibiotics (AB Biodisk, Solna, Sweden) were applied to the inoculated agar, and the plates were incubated anaerobically at 35 ºC for 72 hours. All tests were done in duplicate. The antibiotics that were tested against the Lp 299v isolates were mainly those that had been used clinically in the two studies, namely the following: ampicillin, piperacillin, cefepim, cefotaxime, ceftazidime, cefuroxime, imipenem, meropenem, erythromycin, clindamycin, chloramphenicol, levofloxacin, linezolid, quinupristin/dalfopristin, metronidazole, trimetoprim, gentamicin, kanamycin, netilmicin, streptomycin, tobramicyn, and vancomycin. Readings of minimum inhibitory concentrations (MICs) were made in half steps of dilution.

Fig 1
MIC determination with Etest



Arrow indicates MIC value=8µg/ml
It is not a *L plantarum spp* that is tested.

Statistics

The statistical analyses described in Papers II and III were performed after consultation with a biostatistician at the Region Skåne Clinical Competence Centre (RSKC), Lund, Sweden.

Student's t-test for two independent samples was used for comparisons of most parameters, particularly chemical analyses and counts of the main groups of bacteria. Fisher's exact test was employed to compare the findings of analyses of *C. difficile* colonisation (Paper II) and the results of microbiological cultures (Paper III). The Mann-Whitney test was considered to be more appropriate for the gut permeability analyses due to the limited sample size (Paper II). These analyses were performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA). In Paper I the proportions of conversion of bacterial adherence to the mucosa were analysed with the chi-square test (2 × 3 table) (Statview; SAS institute Inc., Cary, NC, USA).

Probability values < 0.05 were considered to be significant.

RESULTS

Patient characteristics

Table 3
Distribution of patients and patient characteristics

		Paper I	Paper II	Paper III
Patients included (**A/C**)		17 (9/8)	48 (25/23)	50 (25/25)
Patients completing study (**A/C**)		15 (8/7)	44 (22/22)	44 (23/21)
Age (years)	**A**	70.9 (38–85)	65.5 (29–89)	70 (20-87)
	C	57.5 (34–76)	64 (18–86)	70 (43-81)
APACHE II score	**A**	17 (13–29)	17 (7–29) /	22 (11–39)
	C	19 (14–36)	20 (11–38)	27 (9–37)
Sex M/F	**A**	3/5	13/9	13/10
	C	5/2	13/9	9/12
ICU stay in days	**A**	12 (4–-37)	5.5 (2.5–22.0)	7.7 (1.3–26.1)
	C	11 (4–49)	8.8(1.1–67)	6.6 (1.3–16.0)
Days on ventilator	**A**	12 (3–30)	4.4 (0–16.3)	5.8 (1.0–23.8)
	C	9 (2–42)	7.3 (0.9–20.5)	4.3(1.0–15.2)
ICU deaths	**A / C**	1 / 2	2 / 2	5 / 4
In-hospital deaths	**A/C**	1 / 0	1 / 0	5 / 6
Deaths within 6 months	**A/C**	0 / 0	0 / 4	0 / 0

A = patient given active treatment
C = patient given the respective control treatment

Admission diagnoses were similar for the active treatment and control patients in the respective studies. About half of the admissions were due to infections, and those admitted for non-infectious respiratory insufficiencies also constituted a large group. For details see Papers I-III.

Study products and nutrition

The study products were well tolerated and there were no differences in amounts distributed in either enteral formulas or study products (Papers I and II) With few exceptions there were only minor problems with the distribution of enteral nutrition. In one study (Paper II) two patients were excluded during the first 24 hours due to gastric retention problems.

Chemistry

In two of the three clinical studies (Papers I and II), many routine chemistry parameters were monitored but few showed differences between the groups. Only a small number of such parameters were assessed in the third investigation (Paper III). The test results that did differ are discussed in the respective papers.

Bowel function (Papers I and II)

No overall statistically significant differences in the frequency of bowel movements or consistency of faeces were found in either of these studies.

PAPER I

Biopsies from the rectal mucosa

In 1993 [53], it was shown that *L.* 299 and 299v could become established on the mucosa of the upper jejunum as well as in the rectum of healthy volunteers. These bacteria were identified in biopsies even 11 days after administration of the studied product was terminated. At the time the investigation reported in Paper I was initiated, products containing *L. plantarum* 299v (Lp299v) (i.e., the oatmeal drinks ProViva® and Havreblandning®), as well as other products containing probiotic bacteria, were frequently used in Swedish hospitals, including ICUs, without any attempts to document the effects and safety aspects. It was not known whether these bacteria survived and had a residual capacity to become established on the intestinal mucosa in antibiotic-treated patients.

Nine patients were randomised to be given a fermented oatmeal gruel containing Lp299v in addition to enteral feeding and eight subjects received only the enteral feeding (Table 3). No placebo product was available at this time and consequently the study was open. Since the main purpose was to investigate whether the Lp299v would adhere to the intestinal mucosa, rectal biopsies were taken from all patients at inclusion and then twice a week. Risks of bleeding and bacteraemia made inclusion somewhat tedious, because many screened patients had to be excluded. There was no significant bleeding or other side effects after the biopsies in any patient. The number of analyses of biopsies were as follows:

two in six patients (three patients in the treatment group and three patients in control group), three in four patients (2 vs. 2), four in two patients (1 vs. 1) and five in three patients (2 vs. 1). The groups differed (p = 0.029) with regard to bacterial conversion in the biopsies (Table 4). In the treatment group two patients converted to positive culture for *L. plantarum* 299v on the second biopsy and a third patient had done so at the third biopsy. Subsequent samples were also positive in these three patients.

Table 4
Findings of *L. plantarum* 299v in biopsies from the rectal mucosa, at admission and during the study period

	Lp299v patients	**Control patients**
Admission + / -	0 / 8	4/3
– → +	3	0
+ → –	**–**	4

Symbols: + culture positive; **–**culture negative

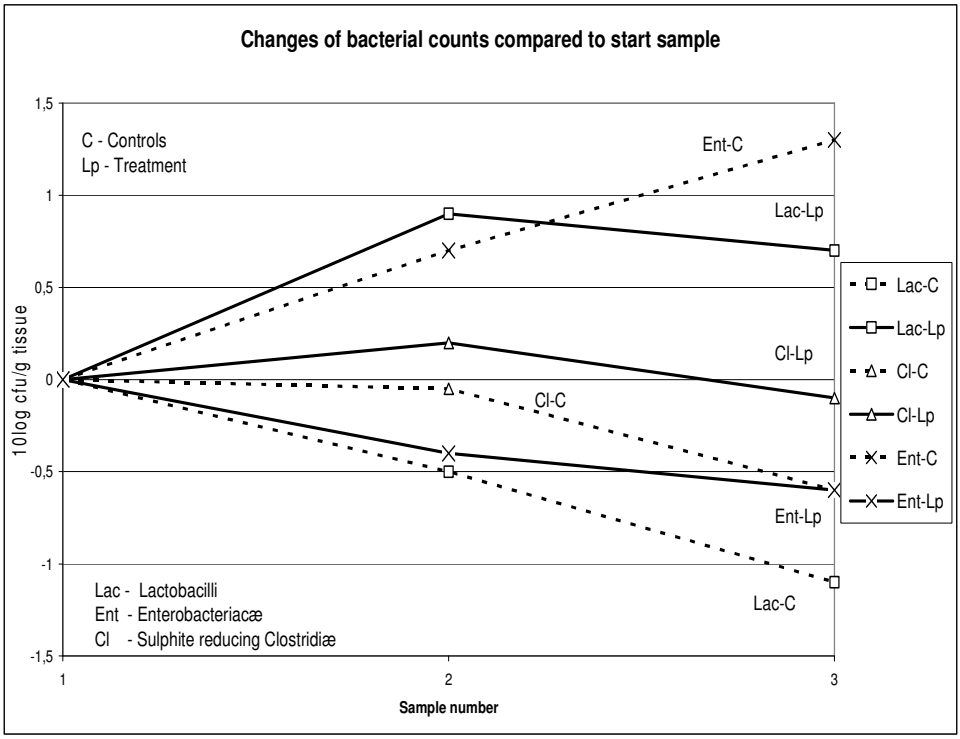
The first two patients in the treatment group were given 50-ml aliquots of bacteria product throughout their study periods. However, they showed distension of the colon, and thus the dose in the other six patients was adjusted to 50 ml every 6 hours for three days and then 25 ml every 6 hours throughout the rest of their stay in the ICU.

Fifteen patients, eight given Lp299v and seven controls, completed the study. The patients given active treatment were older, albeit not significantly (median ages 70.9 and 57.5, respectively), whereas other patient characteristics were similar between the two groups. White blood cell counts were lower in the *L. plantarum* 299v patients from day 6 (and significantly lower on day 6), but the other chemical analyses did not show any significant differences between the two groups.

Microbiology

The numbers of lactobacilli increased in the treated patients but showed a tendency for reduction in the controls (p = 0.061; samples from the second biopsies). No statistical differences between the groups were found regarding *Enterobacteriacæ* or sulphite-reducing *Clostridiæ* (Figure 2), although mean values of *Enterobacteriacae* increased in the control group and decreased in the treatment group (p = 0.27, comparison between initial and second sample).

Figure 2
Changes in bacterial counts in rectal biopsies (means) compared to initial sample



The Enterobacteriacæ species showed a 10 fold increase in mean values in the control group while lactobacilli decreased 10 fold. In contrast, in the treatment group Lactobacilli increased and Enterobacteriacæ decreased. Clostridiæ decreased in the control group.

From the 15 patients who completed the first study (Paper I), microbiological analysis was performed on a total of 240 samples over the days of investigation, from inclusion up to 36 hours after transfer to other units. Fifty-eight (24%) of those cultures were positive. In blood, five cultures (from three patients) out of 32 showed bacterial growth in the control group, whereas bacterial growth was not observed in any of the 30 cultures in the treatment group. The results were similar for samples from other locations. The species that were identified and the sampling locations are indicated in Table 5

Table 5
Species found at different locations

Location	Bacterium	*L. plantarum* 299v group	Control group
Blood	Coagulase-negative *Staphylococcus*	0	3
	Enterococcus faecalis	0	1
	Pseudomonas aeruginosa	0	1
Cather tips	Coagulase-negative *Staphylococcus*	3	3
	Enterobacter cloacae	0	1
	Enterococcus faecium	0	1
	Enterococcus faecalis	2	0
	Morganella morgani	1	0
Tra-cheal secre-tions	*Escherichia coli*	2	0
	Enterobacter cloacae	1	0
	Enterococcus faecium	1	0
	Enterococcus faecalis	0	2
	Pseudomonas aeruginosa	1	1
	Morganella morgani	1	0
	Klebsiella pneumoniae	0	3
	Candida albicans (scarce)	1	0
	Candida kefyr	1	0
Urine	*Enterococcus faecalis*	0	1
	Pseudomonas aeruginosa	0	1
	Candida albicans	2	0
	Candida tropicalis	2	0

No positive blood cultures were found in *L. plantarum* 299v group.
The growth of fungi in the treatment group (urine and tracheal secretions) might have been the result of better culture conditions for those specie due to the presence of fewer bacteria

PAPER II

Microbiology

The Lp299v and the control patients did not differ with regard to frequencies of positive cultures, with the exception of more positive results for *C. difficile* in the control group. Statistical analyses were performed on data representing the participating patients, not on separate cultures.

Clostridium difficile

For *C. difficile* diagnostics 71 samples were taken in the *L. plantarum* patients and 80 in the control patients. Thirty patients (15 in each group) had three samples, 19 (10 vs. nine) had four, and eight (three vs. five) had five or more samples. Emerging *C. difficile* infection was identified in samples from four control patients but none of the subjects in the group given *L. plantarum* 299v (p=0.0485) (Table 6). In all four cases, the first positive result was observed in the second sample (collected on day 3 or 4). One of the four patients had a positive culture, two had a positive culture and a positive toxin test, and the fourth had only a positive toxin test. A fifth patient was positive in the inclusion sample and according to the inclusion criteria that patient was excluded from analyses of emerging *C. difficile* cases.

Table 6
Number of samples for analysis of *C.difficile* and number of patients with positive tests

	Lp299v group	Control group
Samples taken	71	80
Positive samples	0	9
Patients with positive cultures	0	4 (5)*

*One of the patients was positive at the start of the study and was therefore excluded from calculations of emergence of *C. difficile*.

Antibiotics were given intravenously, and regimens were similar for the two groups. Three of the four patients who became positive for *C. difficile* received cefuroxime, and the fourth was given meropenem and levofloxacin. Also, three of the four positive patients had one or more days with loose or watery stools and for two of those three, cultures were found to be positive before the patients had any symptoms.

Lactobacillus plantarum 299v

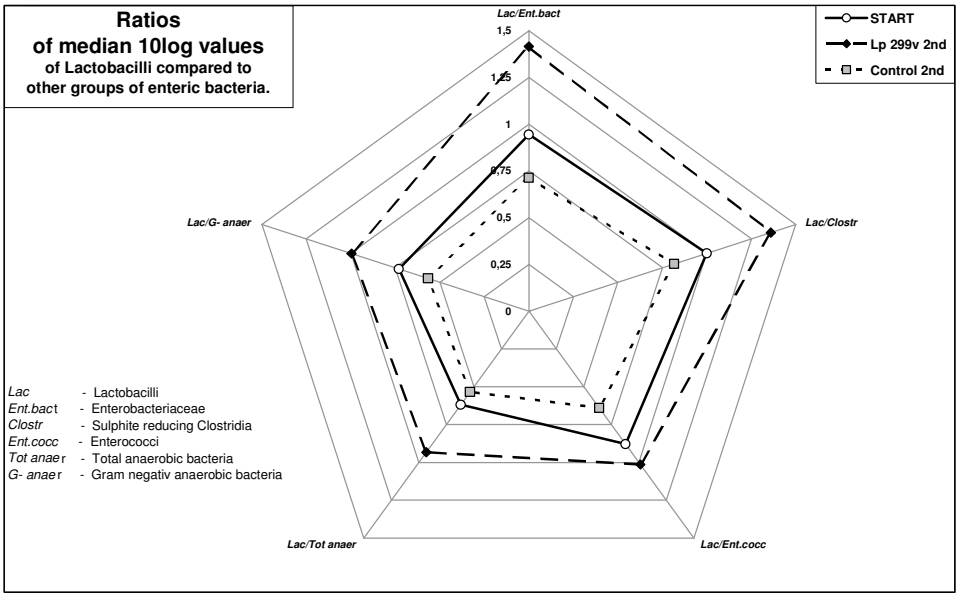
L. plantarum 299v was identified in the inclusion samples from four patients (two in each group), and it was found in one or more cultures of subsequent samples from 18 of 21 patients in the active treatment group and in three samples from control patients.

Enteric bacteria

For the 15 patients at the ICU in Lund, samples for analysis of enteric bacteria were collected at inclusion and then again three days later. Due to short ICU stays, samples for a third set of analyses could only be taken from two patients in the control group, and thus the results are presented only for series one and two. Most categories of bacteria increased in both patient groups. In the group given *L. plantarum* 299v, lactobacilli rose from 10^4 to 8 x 10^7 CFU/g of faeces,

and *L. plantarum* 299v represented the major part of that increase. All ratios of lactobacilli to other groups of bacteria increased in the active treatment group, but they decreased in the control group (Figure 3).

Figure 3



All ratios for initial samples were pooled to produce a common start value. Ratios of lactobacilli to all other groups of enteric bacteria decreased in the control group and, as expected, markedly increased in the Lp299v group.

Other cultures

After administration of study products, 83 cultures were taken in the Lp299v group and 151 in the control group. In the control patients the analyses of tracheal secretions showed a more varied spectrum of bacterial species, including several potential pathogens that were not found in the treatment group (e.g., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*). Fungal species were found only in the Lp299v group (in two patients; Table 7).

Bowel function

No overall differences in frequency of bowel movements or consistency of faeces were found. Eight patients in each group developed loose or watery stools (≥ 2/24 hours). Two patients in the Lp299v group and eight in the control group had no defecation during their ICU stay ($p = 0.07$).

Table 7 Species found at different locations

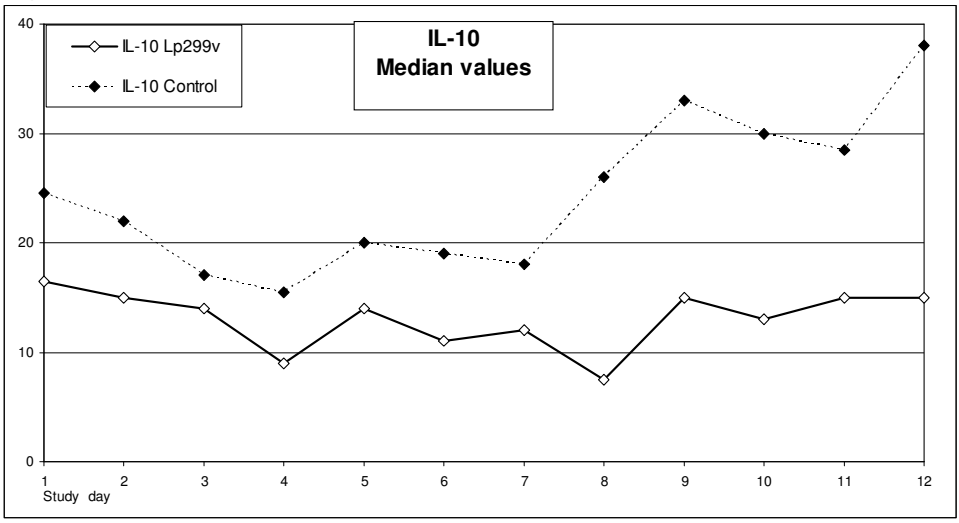
	Species	*L. plantarum* 299v group	Control group
Blood	Coagulase-negative *Staphylococcus*	0	3
	Staphylococcus aureus	1	0
	*Staphylococcus*sp.	0	2
	Enterobacter cloacae	3	0
	Enterococcus faecium	0	1
	Candida albicans	0	1
	Candida glabrata	0	1
	Pseudomonas aeruginosa	0	1
Catheter tips	Coagulase-negative *Staphylococcus*	0	5
	Enterobacter cloacae	1	0
	Candida albicans	0	3
Tracheal secretions	"Oropharyngeal" flora	0	2
	Alfa-streptococci	1	0
	Streptococcus sp.	0	1
	Haemofilus influenzae	1	3
	Moraxella catarrhalis	1	0
	Staphylococcus aureus	0	4
	Staphylococcus sp.	0	1
	Coagulase-negative *Staphylococcus*	1	0
	Escherichia coli	0	1
	Enterococcus	1	0
	Enterococcus faecium	0	1
	Enterobacter	0	2
	Enterobacter cloacae	1	0
	Pseudomonas aeruginosa	0	3
	Stenotrophomonas maltophilia	1	0
	Acinetobacter	1	0
	Klebsiella pneumoniae	0	2
	Klebsiella oxytoca	0	1
	Gram-positive rods (microscopy	0	1
	Candida albicans (scarce)	2	0
	Candida glabrata	1	0
	Candida tropicalis	1	0
Urine	*Enterococcus faecalis*	1	0
	Pseudomonas aeruginosa	0	1
	Escherichia coli ≥100 000/ml	3	0

In some cases more than one species was found in a sample. Tracheal secretions from the control group contained more *Staphylococcus* spp. and enteric bacteria (*Enterobacteriaceae*) but no fungal species. *Pseudomonas aeruginosa* and *Klebsiella* spp. were found only in the control group.

Infection and inflammation parameters

There were no significant differences between the two groups with regard to CRP, TNF-α, IL-1β, and IL-6. From the start, the median IL-10 value was higher (NS) in the control group, but the values declined somewhat faster, and the median values were almost parallel until day 6. Thereafter, the values for the Lp299v group rose again, and there was a significant difference ($p = 0.025$) on day 8 (Figure 4).

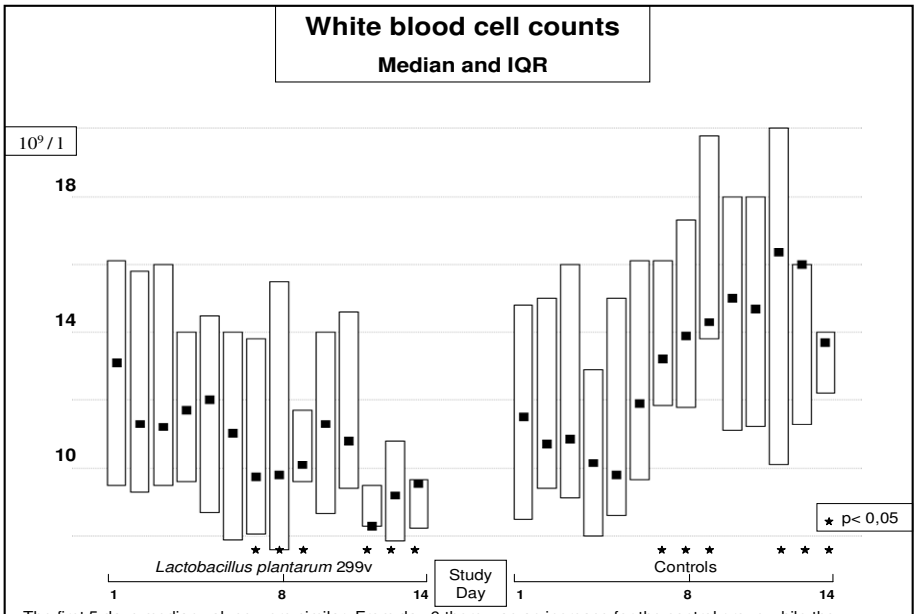
Figure 4



In contrast to what was expected, levels of IL-10 increased in the control patients after a week, but the median values for this protein were higher at all times. The significance of this difference is not known

White blood cell (WBC) counts were similar in the two groups during the first five study days. The counts in the *L. plantarum* 299v group stabilised around the upper normal limit, whereas WBC counts in the controls increased and stabilised at an elevated level during the subsequent week (Figure 5). Differences were significant on days 7–9 and 12–14.

Figure 5



The first 5 days median values were similar. From day 6 there was an increase for the control group while the treatment group stabilised around the upper normal limit.
This difference remained for the rest of the study period and was statistically significant on several days (7-9, 12-14).

Metabolic parameters

We found no significant differences in oxygenation index (P_aO_2/F_iO_2. Creatinine and urea levels were also similar during the first six days after admission, but both of those renal parameters subsequently showed increases in the controls compared to the *L. plantarum* 299v group. Four patients (two in each group) had chronic renal insufficiency, and one in each group wase on chronic dialysis. Since such treatment influences urea and creatinine, those four patients were excluded from further calculations. Nonetheless, the pattern of changes in these two variables was about the same with or without the dialysis patients. There were statistically significant differences in creatinine on days 8 and 9 and in urea on days 9 and 10 (Figure 6).

Lactate levels were above normal only on day 1. Values were lower in the *L. plantarum* 299v group than in the control patients from day 4 and onwards, and reached significance on days 6 and 9(Figure 7)

Figure 6

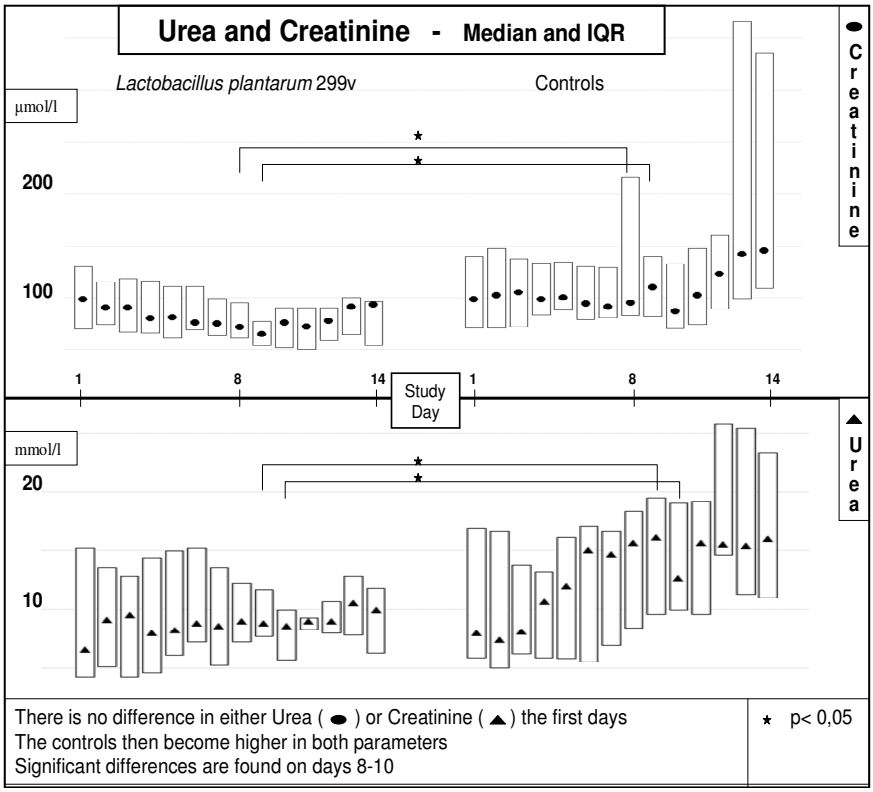
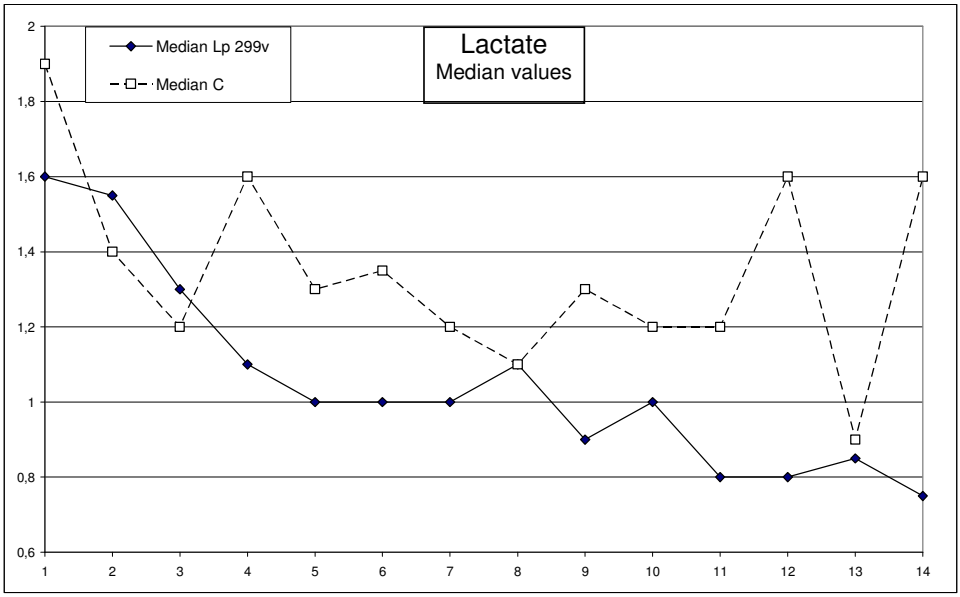


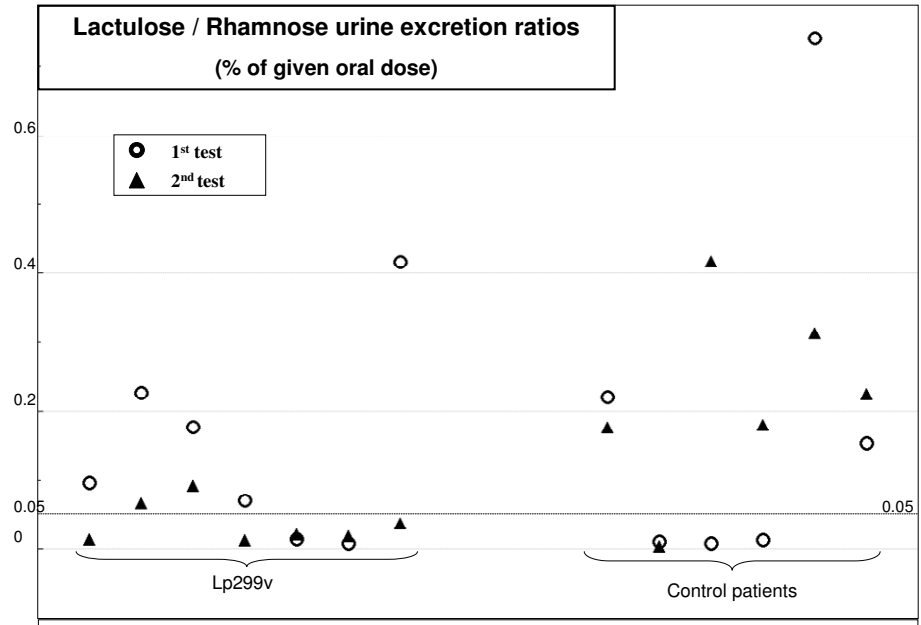
Figure 7



Gut permeability

The lactulose/rhamnose (L/Rh) excretion ratios at the initial test were similar for the Lp299v group (seven patients) and the controls (six patients), and two and three of the patients, respectively, had normal values (≤ 0.05). At the second test, the ratios had decreased (i.e. improved) in five of the seven Lp299v patients and remained normal in the other two; all values in that group were <0.10. Considering the six patients in the control group, permeability was increased in three patients, decreased in two, and unchanged in the sixth (the only value <0.10) (Figure 8). The L/Rh ratio in the second test was better for the *L. plantarum* 299v group than for the control group (P=0.0455).
Diuresis, creatinine values, bowel function, and medication were judged to be similar for the two tests for each variable and between groups. We saw no connection between high or low diuresis and the L/Rh ratios, nor were there any correlation between APACHE II score or SOFA score and the L/Rh ratio tests.

Figure 8



Excretion ratios improved in the patients given Lp299v. At the second test, five of seven ratios were within normal range (≤ 0.05) for the Lp299v group, whereas the ratios for the control patients indicated poorer results than at the start of the study, and only one of those patients had a normal value at the second test.

Values for both 3-O-metyl-D-glucose and D-xylose increased in a similar way in both study groups. No differences were found between active treatment and controls.

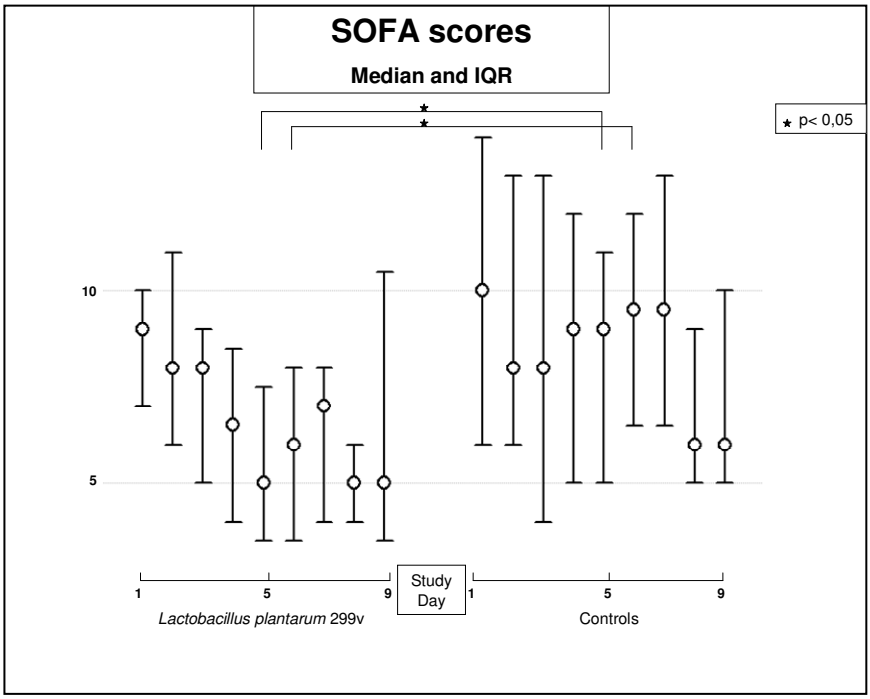
Medication

Nine patients in the Lp299v group and 15 in the control group received corticosteroids at some time during their ICU stay. Dosages were similar. The antibiotics used were mainly cephalosporines and carbapenems, and they were administered in similar ways. Fourteen patients in each group received H2-blockers or proton pump inhibitors (PPI). Three of the four patients who tested positive for *C. difficile* were given PPI.

PAPER III

There were no significant differences in age or gender between the groups (Table 3). Also, the admission diagnoses were similar in the two groups, as were the APACHE II scores. Some differences were found in the SOFA scores in favour of the *L. plantarum* 299 patients (Figure 9). The two groups did not differ significantly with regard to the number of ventilator days, LOS, or ICU or in-hospital mortality (Table 3). No deaths were caused by respiratory complications, and no additional deaths occurred within six months.

Figure 9



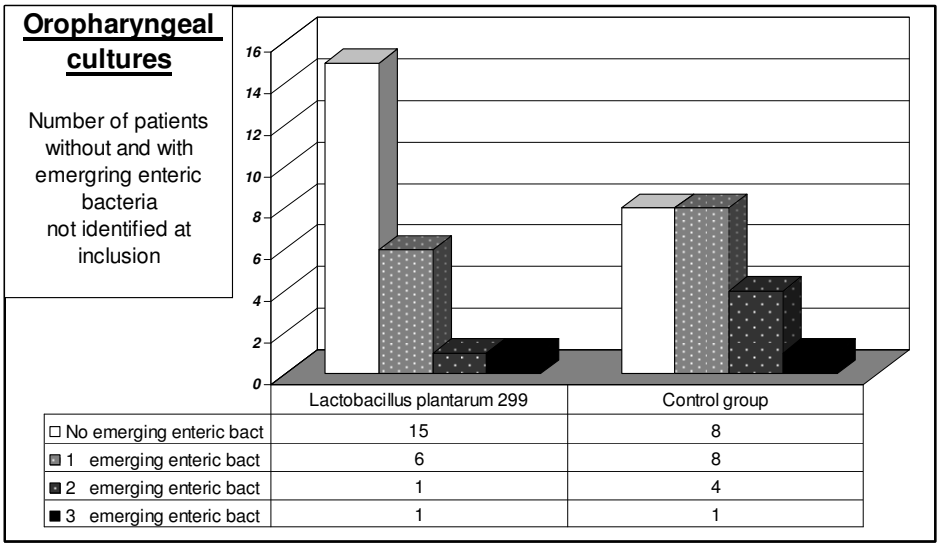
All patients were orotracheally intubated. Two in each group were reintubated, and two in the Lp group and one in the control group were tracheotomised (on days 3, 16, and 3, respectively

Cefuroxime was the most common antibiotic used in both groups, followed by imipenem Three patients in each group received piperacillin/tazobactam, and other antibiotics or combinations were administered to a few patients in each of the two groups. Three patients did not receive any antibiotics at admission, and one of those three was never treated with antibiotics during the stay in the ICU. Ten patients in each group received corticosteroids for one or more days. All patients received ezomprazol (Astra Zeneca, Södertälje, Sweden) iv as stress ulcer prophylaxis from admission until enteral nutrition was fully established (i.e., for 3–4 days).

Microbiological findings in the oropharyngeal and tracheal samples taken at inclusion did not differ significantly between the two groups. The same species were identified in samples from both the oropharynx and the trachea of six *L. plantarum* 299 patients and three controls. Subsequent oropharyngeal samples from eight *L. plantarum* 299 patients and from thirteen controls contained enteric species that had not been present in the inclusion samples from those subjects ($p = 0.13$) (Figure 10). Two or three emerging species (*Enterococci* and *Enterobacteriaceae*) were found in two patients in the Lp group and seven control patients (Figure 10). Culture analysis of the tracheal samples identified emerging species in seven Lp patients and nine controls (Figure 11). Other comparisons of the culture results were similar. Figure 12 shows the distribution of the positive cultures according to study day and sampling site.

L. plantarum 299 was found in the oropharyngeal samples from all of the patients in the Lp group (21/23 on day 2). In addition, Lp299 was identified in the tracheal secretion samples from 13 of the patients in the Lp group (56%), and enteric bacteria were also found in six of those subjects. Of the five patients in the *L. plantarum* 299 group that died in the ICU, *L. plantarum* 299 was identified in the tracheal samples from one, whereas no enteric bacteria were recovered from the trachea of any of those five patients.

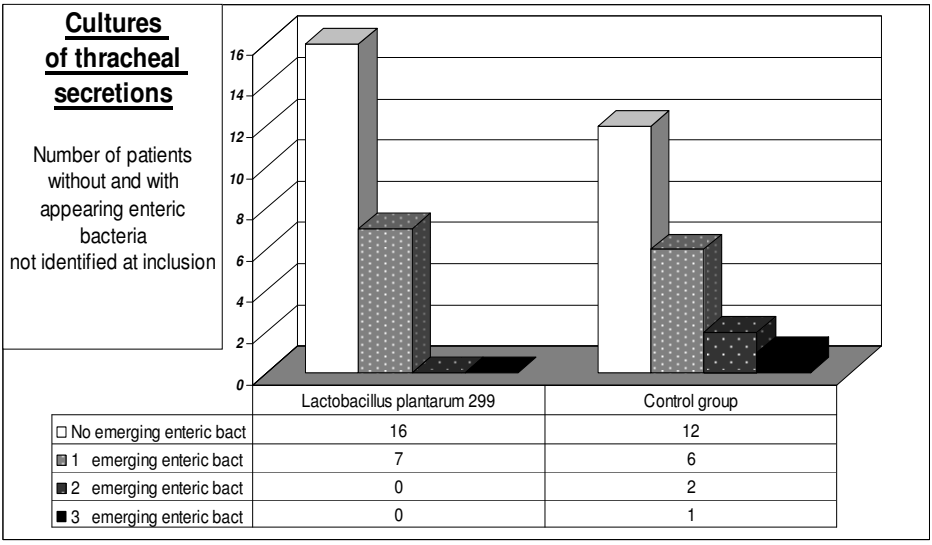
Figure 10



	Lactobacillus plantarum 299	Control group
No emerging enteric bact	15	8
1 emerging enteric bact	6	8
2 emerging enteric bact	1	4
3 emerging enteric bact	1	1

No new enteric species (i.e., taxa not found at inclusion) appeared in 65 % (15/23) of the patients in the Lp299 group compared to 38 % (8/21) in the control group.

Figure 11



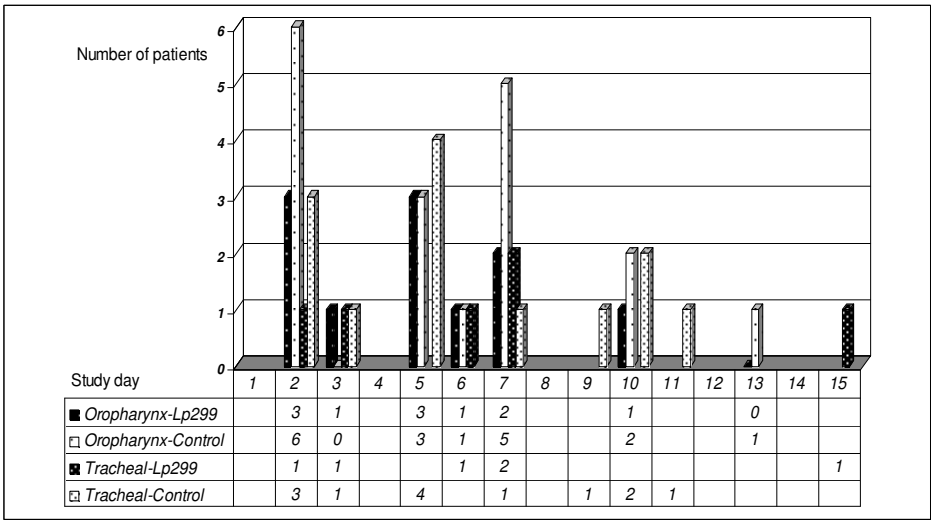
	Lactobacillus plantarum 299	Control group
No emerging enteric bact	16	12
1 emerging enteric bact	7	6
2 emerging enteric bact	0	2
3 emerging enteric bact	0	1

New enteric species appeared in a total of 30% (7/23) in the control group compared to 43% (9/21) in the control groupnew

Considering patients in both groups, a comparison of those with positive findings and those with negative findings in cultures of tracheal secretions (results reported by the microbiology laboratory) indicated a significantly lower number of ventilator days ($p < 0.001$) in the non-colonised subjects. VAP was identified in one patient in the Lp299 group and in three patients in the CHX group.

No differences in WBC counts were found between the groups. Furthermore, the groups did not differ with regard to changes in CRP, although the absolute values were higher for the controls on day 3.

Figure 12
Distribution of the findings of emerging enteric bacteria



Study day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Oropharynx-Lp299		3	1		3	1	2			1			0		
Oropharynx-Control		6	0		3	1	5			2			1		
Tracheal-Lp299		1	1			1	2								1
Tracheal-Control		3	1		4		1		1	2	1				

During the first days of ICU care, twice as many emerging enteric species were identified in the control patients. Despite a gradual decrease in the number of patients remaining in the study (similar in both groups), new cases of tracheal infection appeared on the later days of investigation, primarily in the control group.

PAPER IV

Forty-two *L. plantarum* 299v RAPD-type isolates from two of our clinical studies (Papers I and II) had been retrieved and were analysed together with the original strain and the genomically closely related strain *L. plantarum* 299. Six isolates (three from each of the two clinical studies) originated from samples collected at inclusion, and 24 (seven and 17) came from samples taken over the period of investigation. From three patients in the control group in the second study (not given *L. plantarum* 299v; Paper II), 12 samples had been taken after the actual end of study participation, when those patients had been given the *L. plantarum* 299v-containing fruit drink ProViva® during ongoing antibiotic therapy.

The MIC values determined for *L. plantarum* 299v and *L. plantarum* 299 were equivalent, or the differences found for some of the antibiotics tested were ≤ 1one step of dilution (Table 8).

To be able to compare the MIC values for the harvested isolates with those obtained for the original strain, the isolates were divided into four groups in accordance with their exposure to antibiotics and administration of *L. plantarum* 299v, as follows: (1) isolates from both studies, found in samples taken at inclusion; (2) isolates from rectal mucosa biopsies (Paper I); (3) isolates from faecal samples (Paper II); (4) isolates from faeces from control patients who received fruit drink containing *L. plantarum* 299v after participation in the study was concluded.

Both *L. plantarum* 299v and *L. plantarum* 299 are inherently resistant to aminoglycoside antibiotics, vancomycin, and metronidazole, and we also found high and stable MIC values for levofloxacin. Ratios of MICs of the remaining 13 antibiotics to corresponding MICs for the original Lp299v strain are shown in Figures 13 a-d. We found no significant changes in susceptibility to the antibiotics tested. For ampicillin, there were several isolates with increases in MIC values corresponding to up to two steps of dilution, and MIC increases of more than one step but less than two steps were found for some other antibiotics in some scattered isolates.

Table 8
MIC values (mg/l) determined by Etests

Antibioticum	*L. plantarum* 299	*L. plantarum* 299v
Ampicillin	0.094	0.094
Piperacillin	0.5	0.75
Cefepim	0.047	0.047
Cefotaxime	0.094	0.094
Ceftazidime	0.5	0.75
Cefuroxime	0.25	0.5
Imipenem	0.064	0.064
Meropenem	0.064	0.064
Erythromycin	0.75	1
Clindamycin	3	2
Chloramphenicol	2	2
Levofloxacin	32	32
Linezolid	1	0.75
Quinupri/Dalfopri	0.5	0.5
Metronidazole	>256	>256
Trimethoprim	0.125	0.125
Gentamicin	32	32
Kanamycin	>256	>256
Netilmicin	48	32
Streptomycin	>256	>256
Tobramycin	>256	>256
Vancomycin	>256	>256

Figure 13 a
Re-isolates of *L. plantarum* 299v found on inclusion samples

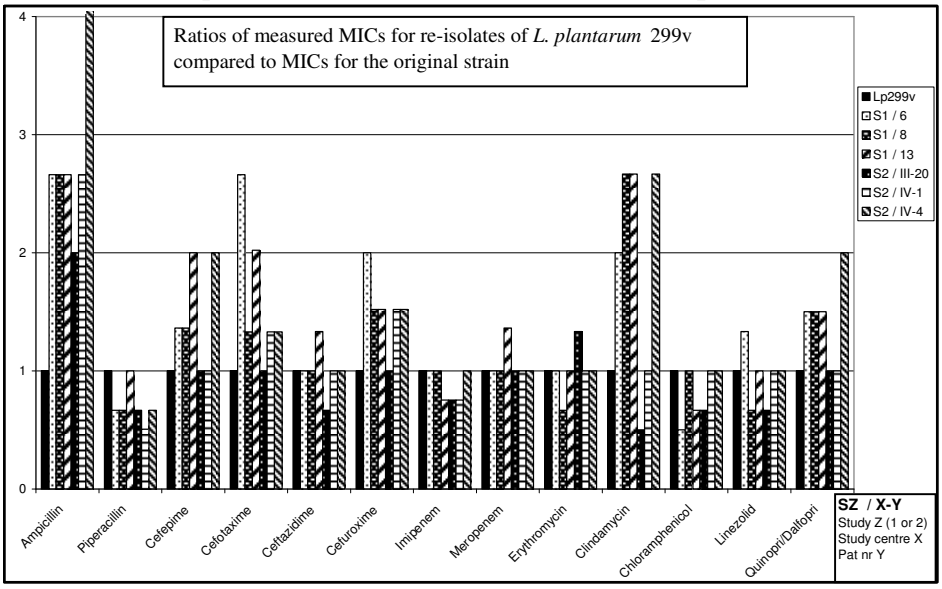


Figure 13 b
Re-isolates of *L. plantarum 299v* collected during the first clinical study (Paper I).

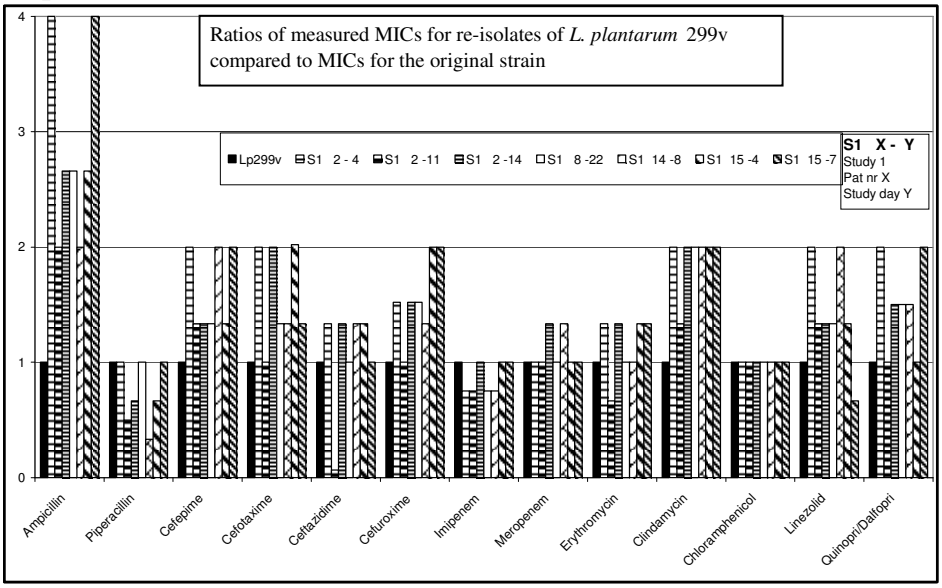


Figure 13 c
Re-isolates of *L. plantarum 299v* collected during the second clinical study (Paper II).

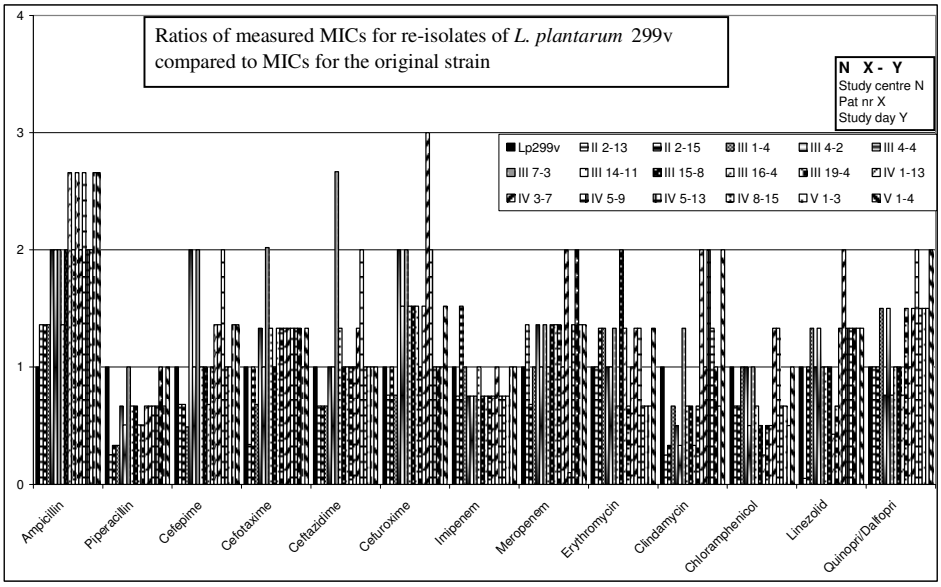
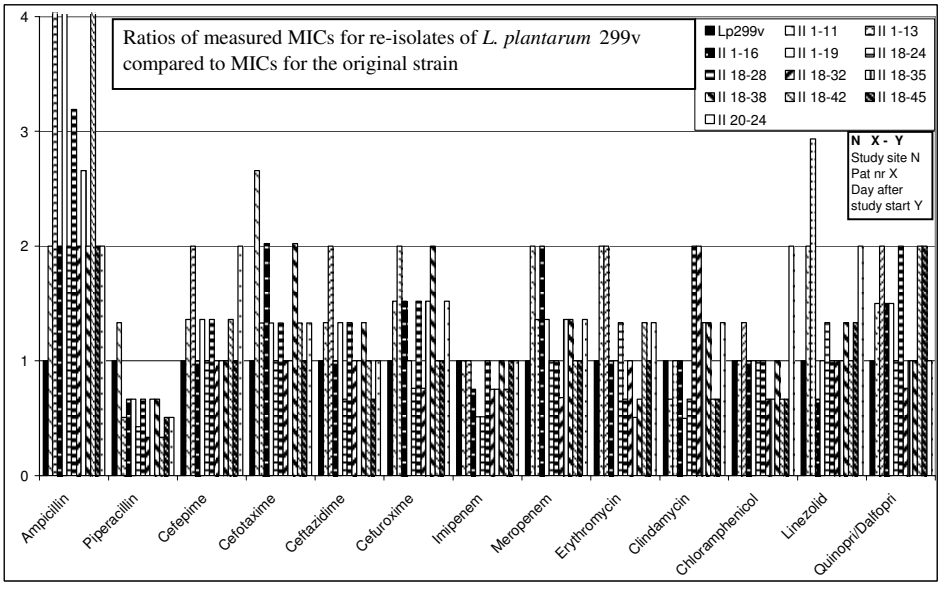


Figure 13 d
Re-isolates of *L. plantarum 299v* collected after end of the second clinical study (Paper II)



Discussion

In this thesis results from investigations and application of the use of the probiotic strains *Lactobacillus plantarum* 299 and 299v in the intensive care setting are presented and discussed.

Establishment of *L. plantarum* 299 and 299v

The best way for added microorganisms to interact with a host is to still be alive when they reach their optimal site of growth in the GIT, and to be viable enough to become established at that location. Nonetheless, some investigations have shown that heat-killed [77], sonicated, or otherwise non-viable bacteria, or even DNA [82], can provide results similar to those obtained with the corresponding live microorganisms. In the first of the present clinical studies (Paper I), we found that orally administered *L. plantarum* 299v was able to survive and establish on the rectal mucosa to the same extent in patients treated with different broad-spectrum antibiotics as in healthy volunteers [53]. Furthermore, in our second study (Paper II), *L. plantarum* 299v was identified in faeces from almost all of the patients in the active treatment group, which confirms that this probiotic bacterium can survive passage through the GIT, even in patients on antibiotics.

The patients who were positive at inclusion (hospitalised at three different ICUs) had probably ingested the Lp299v in commercial food products either before hospital admission or in a ward within the hospital. The positive samples in the control groups were most likely the result of protocol violations. Notably, some control patients that had been given ProViva in hospital after they had ended study participation (Paper II) were culture-positive for Lp299v in subsequent samples.

In the study described in Paper III, *L. plantarum* 299 was found in oropharyngeal samples from all the patients that were treated with the probiotic oral care. That procedure was performed at approximately 12-h intervals, and all the patients in the active treatment group were also on antibiotics. Biopsies were not taken, but, considering that the counts of *L. plantarum* 299 were rather high in most of the samples (10^4–10^6 CFU/g), it is reasonable to conclude that the applied bacteria did become established in the oral cavity, despite the concomitant use of antibiotics. Inasmuch as *L. plantarum* 299v has been shown to adhere to the tonsillar surface [63], it is probable that the bacterium was also able to become established on the mucosa in the antibiotic-treated critically ill patients we studied.

Clostridium difficile and *Lactobacillus plantarum* 299v

In the second clinical study (Paper II), we assessed the ability of *L. plantarum* 299v to counteract colonisation of *Clostridium difficile*. None of the patients

given *L. plantarum* 299v had tests positive for *C. difficile*, whereas 19% (4/21) of the control patients were positive for that pathogenic species, and that proportion agrees with rates reported in the literature for antibiotic-treated hospitalised patients [90, 106]. In an article published in the *British Medical Journal* in 2007 [106], Hickson et al. described a study in which hospitalised, non-ICU patients were given a product containing *L. casei, L. bulgaris*, and *Streptococcus thermophilus*, and *C. difficile* was not found in the active treatment group, whereas 17% of the subjects in the control group were positive for that species. That particular investigation has been criticised for the selection of elderly patients (mean age 74 years) and also for several of the exclusion criteria that were used. However, *C. difficile* infection is more common in elderly people, and that age group is the most vulnerable and deaths are not rare. In the United Kingdom alone, more than 8,000 deaths per year are caused by or associated with *C. difficile* [101], and most of those cases are patients over 65. Thus, it is reasonable to assume that Hickson et al. [106] did choose an appropriate population for their study, and also that their results support the findings presented in Paper I showing that administration of probiotics to antibiotic-treated patients can reduce colonisation with *C. difficile*. Based on the data in Paper I, we suggested that, in cases of critical illness, *L. plantarum* 299v is prophylactic against *C. difficile* colonisation.

The observation that the patients that became colonised with *C. difficile* had converted on their second sample emphasises that probiotics should be started in conjunction with antibiotic therapy. Regardless of whether the mentioned patients started out as asymptomatic carriers of *C. difficile* or were exposed in the hospital environment, administration of Lp 299v does appear to offer protection against overgrowth of *C. difficile*.

When patients are treated in the ICU, their vital parameters and gut function are monitored continuously. Consequently, the caregivers are constantly alert to the appearance of diarrhoea, and tests for *C. difficile* and enteral medication with metronidazole are initiated on wide indications. However, most patients stay only a few days in the ICU, and most of them have been on antibiotics in that facility and continue to receive such therapy after being transferred to a regular ward, where they often share a room and toilet with other patients being treated with antibiotics. After leaving the ICU, attention to bowel movements is reduced to a minimum, and delay of diagnosis and treatment of a *C. difficile* infection, which is actually the result of a chain of circumstances related to the hospitalisation, may cause a number of secondary cases. A prophylactic approach to the issue of *C. difficile* infections and CDAD should be able to reduce patient suffering and also lower the number of patients that need prolonged hospital care due to diarrhoea, and thereby also diminish the costs of medical care.

In our clinical investigation (Paper II), length of ICU stay and length of treatment with antibiotics were not factors that increased the risk of *C. difficile* colonisation, which agrees with other studies [127]. In critically ill patients, as well as other vulnerable patient groups, probiotics given routinely in

conjunction with treatment with antibiotics (especially those substances more likely to induce CDAD) [85-88] represents a more ecologically correct approach than to treat emerging CDAD with a second set of antibiotics. In our patients, *L. plantarum* 299v proved to have the properties necessary for a suitable prophylactic against colonisation with *C. difficile*. Loose or watery stools are not equivalent to CDAD, and testing for *C. difficile* in antibiotic-treated hospitalised patients on a regular basis may be one way to achieve early detection of *C. difficile* infections and to reduce the risks of spreading to caregivers and fellow patients, and within and between wards.

Lactobacillus plantarum 299v and the intestinal microflora

In the first study (Paper I), there were no positive blood cultures in the treatment group, and there was evidence that *L. plantarum* 299v was able to reduce secondary systemic infections, although that could not be verified in the subsequent investigation (Paper II). When we combined the results of those two studies, we detected a slight trend towards more patients with positive PPM findings in tracheal secretions in the control groups. This seems to support the more pronounced findings in the oropharyngeal and tracheal samples collected in our third study (Paper III). It is reasonable to anticipate that, when probiotics are administered enterally, the changes between species that occur in faecal samples will also take place in the upper GIT. Furthermore, the gastric contents will contain fewer PPMs, and when regurgitation occurs the risk of respiratory complications will be reduced.

Dose – Response of probiotics

In the two studies using *L. plantarum* 299v (Papers I and II), the daily dose of the bacteria was given enterally and contained 8×10^{10} to 1.6×10^{11} CFU. The results showed that *L. plantarum* 299v adhered to the rectal mucosa (Paper I), and it was identified in almost all of the patients that had received the active study product and was associated with reduced *C. difficle* colonisation (Paper II). Although the patients were treated with antibiotics, those observations imply that the dose of probiotic given was sufficient. In a meta-analysis conducted by McFarland [127], it was concluded that a level of 10^{10} CFU probiotics/day was associated with a significant reduction in AAD, and our results confirm that the intake of microorganisms must be relatively high in order to be able to detect positive (or negative) effects, at least when the subjects are on antibiotics. Indeed, in two other studies that used *L. plantarum* 299v [80, 128], differences in favour of the probiotic group were not statistically significant, and the investigators themselves mentioned a low intake of bacteria as a contributing factor in that context. No investigations thus far have considered dosage titration, but it stands to reason that optimal doses of various species and strains, and combinations of strains, will be different and that a dose reduction should be considered if the stomach is bypassed.

Bowel function

L. plantarum 299v did not prevent loose stools or diarrhoea. Despite that, it did seem to have the beneficial effect of preventing constipation (Paper II), a condition that is not unusual in the critical care setting and can constitute a serious and annoying problem for both the patients and the staff. Other investigations have also demonstrated the positive effects of probiotics on constipation in patients suffering from gastrointestinal diseases [46, 47].

Impact of *Lactobacillus plantarum* 299 and 299v on inflammatory and infectious parameters

In our clinical studies of *L. plantarum* 299v (Papers I and II), WBC counts were lower from approximately day 6 and onward (with significant differences some days) in the patients given their respective active treatments as compared to the control patients. A positive impact of this strain on the gut mucosa in the form of improvement in the intestinal permeability ought to reduce the influx of inflammatory material from the gut lumen. Other researchers [80] have studied critically ill patients given *L. plantarum* 299v and found that levels of IL-6 were significantly lower on day 15 in the active treatment group compared to a control group, and our findings of lower WBC counts also indicate a late attenuation of the inflammatory response. It is estimated that it takes a few days for added probiotics to become established in the GIT and to become a factor strong enough to influence the metabolism in the mucosa. However, our cytokine analyses (Paper II) did not reveal any differences in the pro-inflammatory parameters (TNF-α, IL-1β, and IL-6), and levels of the anti-inflammatory protein IL-10 were higher in the control group. Although those values remained elevated throughout the study, it is still difficult to explain why there were lower WBC counts in the group given *L. plantarum* 299v (indicating an attenuation of infection and inflammation), when it would have been more logical if the results for IL-10 had been reversed (i.e., the levels had been higher in the active treatment group), considering that probiotics have been shown to stimulate the production of IL-10.[129, 130], A possible explanation for this apparent incongruity is that, even though IL-10 is an anti-inflammatory cytokine, its synthesis is stimulated by lipopolysaccharide (LPS) [131, 132],, which might pass through a compromised gut barrier more easily, as indicated by the gut permeability tests.

In one of the studies performed by the MacFie group [80], the level of IgM anti-endotoxin core antibody (IgM EndoCAb) was higher in the group that received *L. plantarum* 299v than in the control group, which indicates less pronounced exposure to endotoxins. In the same investigation, it was also found that intestinal permeability had improved in the active treatment group.

Gut barrier assessment

It is sometimes asserted that the success of using mono- and disaccharides to assess the gut barrier function is decided by whether the correct parameter is being measured. It is correct to say that the number of bacteria in the GIT (i.e., those against which the gut barrier is intended to protect) is highest in the colon and that the "sugar test" is primarily aimed at determining the degree of preservation of the barrier in the small intestine. However, such tests have been used and validated as non-invasive surrogate techniques for assessment of gut barrier functionality, although some investigators have questioned their suitability for evaluation of critically ill patients [133, 134]. An improved method that includes sucralose and also gives an indication of the barrier function in the colon [135, 136] was introduced after the conclusion of our first study (Paper I).

Although we tested only a limited number of patients, the results of the second permeability assessment were better for those in the *L. plantarum* 299v group than for the controls (statistically significant). Compared to the results obtained at inclusion, the values in the second test had improved or remained normal for all the patients who received *L. plantarum* 299v, whereas they had deteriorated for half of the control patients. Such a difference was also observed in a somewhat larger study performed by the Scarborough group [80], in which it was found that the second test results were improved in a group given the same probiotic, whereas the median value was essentially unchanged in the control group (with a very wide interquartile range). In an investigation of critically ill patients treated with a multi-strain probiotic preparation (VSL#3), Alberda et al. [82] found that intestinal permeability was improved in a group given live bacteria, as well as a group that received a filtered sonicate (verified DNA content) of the same probiotics.

Our results, as well as the findings of those other two investigations [80, 82], are unambiguous and indicate that probiotics can interact with the gut mucosa to improve the barrier function.Our observations suggest that *L. plantarum* 299v has a positive impact on restoration of the paracellular permeability of the gut barrier after the initial phase of critical illness 137.

Metabolic parameters

In the second clinical study (Paper II), creatinine and urea showed interesting differences between the groups (in an unexpected and puzzling way), and since both parameters exhibited the same trend,it must be assumed that there was an actual impairment in renal function in the control group. It is not possible to determine whether this was the result of some gut-related factor. Lactobacilli stimulate the growth of bifidobacteria, and the latter microbes utilise urea as a source of nitrogen [138-140], but that cannot explain the lower creatinine values. Bifidobacteria counts were not done. Stimulation of the intestine by the probiotic bacteria might improve reduced blood flow through the splanchnic area that has been caused by a critical illness, and such restoration of perfusion

would also improve liver function, and hence a lower level of serum lactate could be expected.

Probiotics used in oral care

The participants in the third study (Paper III) were intubated, critically ill patients on mechanical ventilation, and the aim was to test an alternative procedure using *L. plantarum* 299 for oral care in comparison with a method using the antiseptic chlorhexidine that is recognised as being effective [115, 116]. To my knowledge, the approach of using probiotics for oral care in intubated patients has not been investigated by other researchers.

Compared to the control group, there were fewer patients in the active treatment group who had emerging enteric bacteria in both their oropharyngeal and their tracheal secretion samples, although these differences were not significant. Pathogenic enteric bacteria appeared in 35% of the patients in the *L. plantarum* 299 group compared to 62% in the CHX group, which indicates that *L. plantarum* 299 may be able to lower the rate of infections with such harmful microbes and thereby lead to fewer cases of VAP. Despite the lack of statistically significant differences, we regard the findings as very interesting. Our hypothesis was that a probiotic could be just as efficient as the established CHX-based routine in counteracting PPMs in the oropharynx of intubated patients, but our results suggest that the alternative treatment is even better. To verify the trend observed in this pilot study, it will be necessary to perform a larger investigation, and we are planning a multi-centre study that will hopefully provide enough material to demonstrate a significant reduction in cases of VAP. Our pilot study was not powered or intended to find differences in incidence of VAP, but was instead meant to be a screening of the feasibility and safety of the use of probiotics in a new application.

It was assumed that aspiration of the administered *L. plantarum* 299 would occur in some cases, but the risk of complicating events following an aspiration was judged to be low based on the results of an earlier animal study on *L. plantarum* 229v bacteraemia [141] and the lack of evidence indicating that lactobacilli are likely to cause pneumonia [142]. We found no connection between identification of *L. plantarum* 229 in tracheal secretions and the development of infiltrates on chest radiographs, and there were no indications of bacteraemia associated with the administered probiotic. The topic of safety is discussed further in the following section.

CHX has some common side effects, including discolouration of the teeth (due to dead bacteria), a burning sensation on the tongue, and irritation of the oral mucosa [143, 144]. Serious allergic reactions are rare. Gram-negative bacteria appear in the oropharynx in most severely ill patients [21], and those microorganisms constitute the potential threat of complicating infections, and

unfortunately CHX shows little activity against those PPMs [145]. CHX is inactivated and diluted by saliva [146], and more long-acting pastes containing CHX have been shown to ensure that the same degree of reduction of complications as is accomplished with other preparations [CJJ]. The frequency of oral care procedures varies in praxis and in research investigations [106]. Our established protocol for use of CHX in oral care is associated with an incidence of VAP of about 10%, which is approximately the same rate that is observed with other CHX concentrations, preparations, and frequency of care [106], and is considered to be acceptable, and thus we considered it suitable as a reference for testing our alternative procedure using probiotic bacteria that are known to adhere to the intestinal mucosa. To my knowledge, it has not been determined whether the CHX concentration remains high enough to be inhibitory throughout the day, even when using a slow-release preparation such as a paste. Repeated mechanical cleansing may be more important than a short exposure to an antiseptic agent for reducing the numbers of pathogenic (and non-pathogenic) bacteria. An established non-virulent bacterium such as *L. plantarum 299* (Paper III) can exert inhibitory effects on PPM around the clock, and it offers a microbiologically attractive alternative to the use of chemical agents like CHX. Also, there is a risk of selection of bacteria strains resistant to CHX when the concentrations of CHX are low and inadequate, as must be the situation in between oral care treatments. What is even more alarming is that bacteria strains that are not susceptible to common antibiotics (e.g., methicillin-resistant *Staphylococcus aureus*, MRSA) also often carry genes for resistance to CHX [148]. *L. plantarum* strains are genetically stabile and generally regarded as safe (GRAS), and are therefore not likely to impose resistance to antibiotics in other strains.

In conclusion, if the promising findings of Study III can be verified in the enlarged study that is planned, it will be feasible to use specific probiotics as a means of reducing colonisation with potentially pathogenic Gram-negative species.

Safety of probiotic use in critically ill patients

Clearly, it may seem contradictory to administer live bacteria to patients when the main goal is to minimise the risks of primary and secondary infections. In patients that require intensive care, the intestinal microbiological balance is ultimately deranged in almost all cases, and consequently there are more pathogenic and potentially pathogenic bacteria. Furthermore, there is an increased risk that bacteria with induced virulence will translocate through the more or less disturbed or deteriorated gut barrier. Accordingly, an added probiotic bacterium should also be able to pass such a leaking barrier and cause *Lactobacillus* bacteraemia.

Although no proper safety screening for bacteraemia was performed in our three clinical studies, blood cultures (and other non-protocol cultures) were taken on clinical indications, representing an average of one blood culture per three days. In the previously mentioned investigation using an animal model of intravenously administered *L. plantarum* 299v [141], all injected bacteria had been cleared upon analyses, indicating that they had been removed by the immune defence. Analyses of blood cultures done on clinical suspicion of infection are positive in 10–20% of the cases, and we estimated that, with each of our study protocols, it would have been necessary to perform very frequent sampling, probably more than once a day, in order to obtain a single positive blood culture. In addition, the fact that almost all of our patients were concomitantly treated with antibiotics would have made it even more difficult to trace any bacteraemia caused by the two *Lactobacllus* strains we studied. No *Lactobacllus* spp. were found in the blood cultures that were taken.

In the studies using *L. plantarum* 299v (Papers I and II), the bacteria were given in an oatmeal-based preparation. In the initial investigation, the first two patients developed distension of the large intestine, and hence their participation was terminated. We subsequently changed the protocol for administration and added a third propulsive agent, and thereafter the problem did not reappear. It is very probable that the explanation for the gut problem is multifaceted. We do not believe that the bacteria per se were the reason, but rather the combination of a more prebiotic substrate (the fermented oatmeal gruel), an intestine that was slowed by opiates and sedative drugs, and, of course, the gas produced by intestinal bacteria. After our first study was performed (Paper I), sedation protocols have been changed in most ICUs so that patients are now given much smaller amounts of sedatives. Accordingly, we believe that the risk of bowel distension will be minimal, if the regimen outlined in Paper II is followed.

The only adverse effects that we observed upon administration of the present study products were distension of the colon in the first two patients in the initial investigation (Paper I) and patients that described a somewhat peculiar taste of the suspension in the third study using *L. plantarum* 299 (Paper III). The results of most investigations of probiotics in critically ill patients have also been encouraging, although sometimes inconclusive. Reported adverse events have been few and infrequent. When Besselink et al. [64] published their results obtained in the PROPATRIA study in the *Lancet* early in 2008, the world of probiotics was awakened from a state of confidence in which the worst scenario involved investigations that did not give results favouring the use of probiotics. The observations of Besselink and colleagues confronted us with a dramatic negative outcome in a group of patients with acute pancreatitis that had been given a six-strain probiotic preparation designated Ecologic 641. Increased morbidity and mortality were seen in the group that received Ecologic 641, and nine patients with bowel ischaemia (eight of whom died) constituted the most conspicuous finding. As mentioned in the introduction, ongoing studies with

probiotics were stopped as a consequence of the results with Ecologic 641, and, in an invited editorial in the journal *Clinical Nutrition* [150], Professor Soeters concluded that "at present probiotics should not be used in critically ill patients." However, other researchers took a less radical standpoint [151-152], and several things in the original protocol have been questioned. Two of the aspects that have been debated include the strategy of adding the probiotics to the enteral feeding, which was administered through a nasojejunal tube, and the low number of reasonably healthy subjects in whom the probiotic mixture had been tested before the study was initiated.

In further discussion of the paper published by Besselink et al. [64], it can be noted that the authors did not state in what condition the patients were at the time administration of the probiotics was started. During the first period of a pancreatitis that is predicted to become severe, patients tend to be hypovolemic and hypoperfused in the splanchnic region, and peristalsis in the jejunum is often impaired. When the stomach is bypassed and the nutrition and the bacteria are delivered directly to the intestine, there will be no dilution of the administered formula and no reduction in the bacterial counts, which will result in high numbers of bacteria and a concentrated substrate that may act osmotically to further dehydrate the upper part of the jejunum. Due to the impaired peristalsis a locally increased metabolic demand may induce low oxygen saturation in the gut wall. The large active biomass may also produce large quantities of gases (as we observed in two of our patients, Paper I) that are not cleared due to the paralytic state of the gut. There may also be other factors that, independently or in combination with those already mentioned, can be deleterious and eventually cause necrosis and peritonitis

Precautionary measures should be taken when administering probiotics to critically ill patients. The circulation and respiration must be stabilised before the start of enteral nutrition that includes a probiotic preparation. Use of a nasogastric tube for the feeding can help to avoid the above-mentioned risks of bypassing the stomach and the possibility of measuring gastric retention gives the treating physician a tool to monitor gut function. If the gut does not accept enteral feeding even though propagating agents are given iv, something must be wrong. It might be the result of a paralytic ileus due to the severity of the illness, or perhaps a condition of greater pathological significance, caused by the probiotics or by some other factor.

Susceptibility to antibiotics

Strains of resistant bacteria are found in most people, but illnesses are not caused by the insensitivity of those microbes to antibiotics but rather by their virulence. Exchange of genetic material between microorganisms is an ongoing process, and the GIT is a suitable milieu in which that can occur. Despite the use of broad-spectrum antibiotics and an anticipated increase in the population

of resistant bacteria in the GIT of patients, we could not detect any clinically significant changes in the susceptibility to antimicrobial agents exhibited by the re-isolated strains of *L. plantarum* 299v that we investigated. This finding contributes valuable information to the documentation of a safety profile for these probiotic bacteria. A tendency to acquire resistance genes implies a risk of establishment of multi-resistant strains that can be difficult to deal with if they become involved in infections. Requirements outlined by regulatory authorities stipulate that strains used in probiotic preparations must be susceptible to at least two groups of antibiotics, and if the used strains are prone to exchange genetic material with other organisms, it may result in an unacceptable situation.

Conclusions

The results of the studies presented in this thesis have led to the following conclusions:

-- Enterally administered *Lactobacillus plantarum* 299v is safe even for use in critically ill patients.

-- *L. plantarum* 299 and *L. plantarum* 299v become established in the gastrointestinal tract of antibiotic-treated critically ill patients in the same manner as in healthy volunteers.

-- *L. plantarum* 299v given enterally in the form of a fermented oatmeal gruel to antibiotic-treated critically ill patients reduces colonisation with *C. difficile*.

-- *L. plantarum* 299v improves intestinal permeability.

-- *L. plantarum* 299v does not attenuate cytokine production in the type of patients included in our studies.

-- Limited changes in susceptibility to antibiotics were seen in strains of *L. plantarum* 299v that were re-isolated after previous collection from critically ill patients that had been treated with that bacterial strain as a probiotic.

-- *L. plantarum* 299 seems to be as effective as chlorhexidine in reducing colonisation with pathogenic bacteria in the oropharynx of intubated ICU patients.

-- *Lactobacillus plantarum* 299 can be safely used in the future in larger studies of oral care in intubated, mechanically ventilated, intensive care patients.

Summery in Swedish
Populärvetenskaplig sammanfattning på svenska

Ordet Probiotika kommer av grekiskans probios, som betyder "för livet". Probiotika är levande mikroorganismer som när de ges i tillräcklig mängd utövar positiva hälsoeffekter hos den som intagit dem.

I denna avhandling ingår tre kliniska undersökningar vilka genomförts på patienter som vårdats på intensivvårdsavdelning (IVA), samt en laboratorieundersökning av antibiotikakänslighet hos bakteriestammar som isolerats från patienter som deltagit i två av undersökningarna.

När vi föds är tarmen steril, men redan efter förlossningen exponeras barnet för en värld i vilken det finns en mängd olika typer av bakterier varav de flesta inte är sjukdomsalstrande. Under de första levnadsåren får den enskilda individen en unik uppsättning (ett slags ”inre fingeravtryck” av mikroorganismer (fr.a. bakterier och jästsvampar) i sin magtarmkanal, som inte påverkas mycket förrän i högre ålder. Det finns i tarmen ca 10 gånger fler bakterier (ca 1 kg) än det finns celler hos en vuxen person. Det finns också mycket bakterier på huden (200 g), o mindre mängder i lungor, näsa, mun och svalg.
I magtarmkanalen finns hos den friske individen en välfungerande balans mellan olika typer av microorganismer. De flesta är även bärare av bakterier vilka kan orsaka sjukdomstillstånd, men dessa är för det mesta undertryckta av det stora flertalet av ”goda”, icke-sjukdomsalstrande mikroorganismer. Utöver konkurrens om utrymme och näringsämnen, utsöndrar många bakterier ämnen som verkar tillväxthämmande på andra bakterier, såväl liknande som bakterier av andra typer.
Även t.ex. stress och sjukdomar som inte är infektionsutlösta, men fr.a. antibiotika kan rubba balansen mellan olika mikroorganismer. En del bakteriegrupper minskar eller slåss ut, och det ges då utrymme för andra, vilka är naturligt eller förvärvat resistenta mot det använda preparatet att tillväxa. Det vanligaste symtomet på obalans i det mikrobiologiska systemet är lös avföring eller diarré.
En frisk fullt fungerande tarmvägg har förmågan att hålla innehållet i tarmen med upp till 1000 miljarder bakterier per ml åtskild från en nästan steril miljö i underliggande vävnader. Denna barriärfunktion försämras vid allvarliga sjukdomstillstånd, såsom svåra infektioner och tillstånd som medför nedsatt genomblödning till tarmen. Bakterier och bakteriedelar (endotoxiner) får lättare att ta sig genom denna defekta barriär och när de kommer ut i cirkulationen drabbas patienten av ”blodförgiftning” (sepsis), vilket kan ge mycket svåra sjukdomssymtom med hög feber och cirkulationssvikt (septisk chock) och kan i värsta fall leda till döden.

Probiotiska microorganismer har visats kunna minska magbesvär i samband med antibiotikaanvändning och även minska risken för återinsjuknande vid svårare fall av diarresjukdom orsakad av *Clostridium diffiicle*. *C. difficile* infektioner har nästan alltid samband med antibiotikaanvändning vilket medfört att balansen i tarmfloran rubbats och då kan denna bakterie orsaka allt från lös avföring till mycket svår tjocktarmsinflammation som kan vara ett hot mot överlevnad.

Laktobaciller eller mjölksyrebakterier omvandlar kolhydrater till mjölksyra, och finns i alla miljöer.
Under årtusenden har människan använt sig av mjölksyrajäsning för att bl.a. bevara livsmedel, och laktobaciller finner vi idag bl.a i form av surdeg, surkål, inlagda oliver, men även i charkuterivaror och drycker såsom fil och yoghurt.
Lactobacillus plantarum (Lpl) är en grupp av bakterier som förekommer rikligt såväl på växter som i tarmen på djur och människor och *Lactobacillus plantarum* 299 och 299v tillhör denna grupp. Dessa båda bakterier har förmåga att fästa sig på slemhinnan i hela magtarmkanalen och blir även kvar flera dagar efter att man slutat att inta dem. Detta är visat genom att små vävnadsprover tagits från slemhinna från friska frivilliga försökspersoner. Lpl 299 och 299v har använts i många undersökningar på såväl djur som människor och har bl.a. visats kunna dämpa inflammation i tarmen och motverka att sjukdomsalstrande bakterier förorsakar att tarmbarriären försämras. Lpl 299v är den bakterie som används i bl.a. fruktdrycken ProViva.

I

I den första undersökningen (Arbete I) togs små vävnadsbitar (biopsier) från ändtarmsslemhinnan från 15 intensivvårdpatienter före och under behandling med eller utan Lpl 299v-tillförsel.
Hos patienter som hade bakterien vid starten (positivt prov), men som inte fick bakterien tillförd kunde Lpl 299v inte påvisas på fler prover (negativt prov). Hos de som varnegativa från början kunde vi påvisa att Lpl 299v fastnat hos dessa antibiotikabehandlade svårt sjuka patienter i samma omfattning som hos friska frivilliga.

II

I den andra undersökningen som utfördes på fem intensivvårdsavdelningar fick hälften av patienterna en havrebaserad lösning innehållande Lpl 299v och de andra bara havrelösningen. Prover för analys av *C. difficile* togs vid undersökningens början och sedan två gånger per vecka.
I den grupp som fick Lpl 299v kunde inga fall av *C. difficile* påvisas, men av patienterna i den andra gruppen identifierades *C. difficile* hos 19 %. Antalet patienter var relativt få (totalt 44 patienter fullföljde) varför resultatet vilar på lite svag statistisk grund.
Tretton patienter som behandlades på IVA i Lund genomförde också undersökningar av tarmbarriärfunktionen vid inkomst och efter ett par dagar. De patienter som fått Lpl 299v förbättrades alla eller behöll sina normala värden,

medan värdena för hälften av kontrollpatienterna hade försämrats vid den andra undersökningen.

III

Som svårt sjuk behöver man ofta hjälp med sin andning. En respirator blåser in syrgasberikad luft (kan vara 100 % syrgas), oftast genom ett plaströr (på sjukvårdspråk ”tub”) som via munnen går ner i luftröret (trachea). Denna tub har en liten ballong (”kuff”) som blåses upp för att täta mot luftrörsväggen så att den inblåsta gasen kan komma längre ut i luftvägen och inte läcker tillbaka sidan om tuben. Trots detta kan små mängder slem (som vanligen vid svår sjukdom innehåller sjukdomsalstrande bakterier) från svalget komma ner i lungorna och orsaka lunginflammation. Sådan lunginflammation (kallad ventilator-associated pneumonia - VAP) är en relativt vanlig komplikation till respiratorbehandling. Genom att flera gånger dagligen rengöra munhålan minskas mängd bakterier och därmed minskas riskerna för VAP. Användning av klorhexidinlösning vid sådana munhygienska åtgärder har visats kunna minska risken för VAP. Klorhexidins inverkan på de mera farliga bakterierna är inte så bra och en del upplever obehag i munnen när det används.

I det tredje arbetet gjordes därför en jämförande undersökning mellan den etablerade metoden och en alternativ modell där *Lactobacillus plantarum*299 (snarlik ProViva-bakterien) provades som hämmare av de sjukdomsalstrande bakterierna i munnen. Vi fann då att, utöver de bakterier som fanns vid undersökningens början, färre nya bakterietyper hittades i odlingar hos de patienter som behandlades med den nya metoden jämfört med den andra gruppen patienter. Hos 15 av 23 patienter (65%) hittades inga nya bakterier i den patientgrupp som behandlats med Lpl 299 jämfört med endast 8 av 21 (38%) i andra gruppen. Skillnaden är dock inte säkerställd statistiskt och undersökningen var inte heller tillräckligt stor för att kunna avgöra om denna alternativa behandling innebär färre fall av VAP. En sådan större undersökning är under planering.

IV

I det fjärde arbetet som presenteras har antibiotikakänsligheten för bakteriestammar som vid analyser identifierats som *Lactobacillus plantarum* 299v i prover tagna i de två första arbetena.

En del bakterier är inte känsliga för vissa sorters antibiotika, och denna resistens kan höra till bakterietypen eller resistensen kan vara förvärvad från andra bakterier. Eftersom utbyte av genetiskt material sker och kanske inte minst i tarmen, gjordes denna undersökning av de bakteriestammar som varit utsatta för antibiotika, och därför också för ett förmodat ökat antal resistenta bakterier (som inte slagits ut av antibiotika) som skulle kunna ge våra tillförda laktobaciller resistensgener.

Vid jämförelsen med den ursprungliga bakteriestammen sågs inga säkra förändringar i resistensmönster, men för ett antibiotikum – ampicillin – fanns en tendens till minskad känslighet

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Research

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Adhesion of the probiotic bacterium *Lactobacillus plantarum* 299v onto the gut mucosa in critically ill patients: a randomised open trial

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Abstract

Introduction To achieve any possible positive effect on the intestinal mucosa cells it is important that probiotics adhere tightly onto the intestinal mucosa. It has been shown in healthy volunteers that *Lactobacillus plantarum* 299v (Lp 299v) (DSM 9843), a probiotic bacterium, given orally in a fermented oatmeal formula adheres onto the intestinal mucosa, but whether this also occurs in critically ill patients is unknown.

Methods After randomisation, nine enterally fed, critically ill patients treated with broad-spectrum antibiotics received an oatmeal formula fermented with Lp 299v throughout their stay in the intensive care unit; eight patients served as controls. Biopsies of the rectal mucosa were made at admission and then twice a week, and the biopsies were analysed blindly.

Results Four patients in the control group were colonised with Lp 299v at admission but thereafter all their biopsies were negative (Lp 299v is an ingredient in a common functional food, ProViva®, in Sweden). Of the treated patients none was colonised at admission but three patients had Lp 299v adhered on the mucosa from the second or third biopsy and in the following samples.

Conclusion This study shows that Lp 299v could survive the passage from the stomach to the rectum and was able adhere onto the rectal mucosa also in critically ill, antibiotic-treated patients.

Introduction

In critical illness, the intestine has been indicted as a source of pathogens sustaining the inflammatory response initiating or maintaining multiple organ failure. Various interventions have therefore been proposed to limit the growth of putatively causative pathogens in the gut; for example, selective intraluminal eradication of facultative aerobic Gram-negative bacteria – selective digestive decontamination. Indeed, selective digestive decontamination reduces the infection rate, especially in the respiratory tract [1]. Although a meta-analysis [2] and a recent study in critically ill patients [3] suggest a decreased mortality using selective digestive decontamination, there is a risk of emergence of multiresistant bacteria by the high antibiotic load.

Another method, potentially more beneficial for the microbiological environment, to reduce growth of pathogens in the gut is the administration of probiotics – lactobacilli and bifidobacteria [4]. Intestinal permeability is increased during critical illness, particularly after burns, major trauma and sepsis [5-7], and bacterial translocation has been demonstrated in patients with bowel obstruction [8,9]. The administration of probiotic *Lactobacillus* strains in animal experiments has been associated with reduced bacterial translocation and intestinal inflammation [10,11].

The strain *Lactobacillus plantarum* 299v (Lp 299v) has excellent adherence characteristics using the mannose binding sites on the mucosal cells [12]. In fact, in healthy volunteers

ICU = intensive care unit; Lp 299v = *Lactobaccilus plantarum* 299v.

oral administration of Lp 299v produced adherence onto and colonisation of the rectal mucosa and remained viable, verified by biopsies, for more than 11 days after end of administration [13]. The positive effects might be due to the lactobacilli fermenting nutritional carbohydrates and fibres to the preferred substrates for enterocytes – the short chain fatty acids. However, the mannose binding adhesion of Lp 299v [12] and the ability for Lp 299v to adhere to the intestinal mucosa are a possible basis for exclusion of other bacteria from adhering, thus preventing translocation. Furthermore, Lp 299v has been shown to stimulate the mucin-production in HT-29 cells [14,15]. To have beneficial effects, however, the lactobacilli should survive and adhere to the gut wall in sufficient numbers. Lp 299v is sensitive to several of the commonly used antibiotics (e.g. ampicillin, erythromycin, clindamycin, and trimethoprim/sulphamethoxaxol). In addition, the decreased gut motility often seen in critical illness might influence the transport of Lp 299v down to the lower gastrointestinal tract. Whether Lp 299v survives and adheres to the mucosa in the lower gastrointestinal tract in critically ill patients is therefore uncertain.

The primary aim of this pilot study was to examine this survival and adherence by obtaining rectal biopsies from critically ill, antibiotic-treated patients given Lp 299v enterally. The secondary aims were to evaluate the influence on the main groups of bacteria in the gut and explore the side effects of the treatment and to evaluate how the given product was tolerated when given to critically ill patients.

Materials and methods

The present study was approved by the Human Ethics Committee at Lund University and was performed in compliance with the Helsinki Declaration. Informed consent was obtained from the patient or from the next of kin. The study was performed in the general intensive care unit (ICU) (nine beds) at Lund University Hospital.

The inclusion criteria were that the patient should be 18 years or older, should be critically ill (defined by a presumed need of intensive care for 3 days or more), should tolerate enteral feeding, should have no significant coagulation disorder or thrombocytopenia, and should have an indication for broad-spectrum antibiotics.

After inclusion (which was made within 12 hours after admission), randomisation was performed with sealed envelopes. Enteral nutrition was started within 24 hours after admission to the ICU. Nine patients (treatment group) were given the test solution in addition to the enteral formula, and eight patients (controls) received the enteral formula alone (Nutrodrip Standard, Nutrodrip Fiber, or Impact; Novartis AG, Basel, Switzerland)

The test solution consisted of a fermented oatmeal formula containing 10^9 colony-forming units/ml Lp 299v (Probi AB, Lund, Sweden and Skånemejerier AB, Malmö, Sweden). The formula was given through a nasogastric catheter every 6 hours. The two first patients in the treatment group were given 50 ml portions throughout their study period but, due to bowel distension, the dose was adjusted in the other six patients to 50 ml test solution every 6 hours for 3 days and then 25 ml every 6 hours throughout the rest of their stay in the ICU.

All patients received prokinetic agents – metoclopramid (Primperan; Sanofi, Paris, France), cisapride (Prepulsid; Janssen-Cilag, Beerse, Belgium and sodium picosulphate (Laxoberal; Boehringer Ingelheim, Ingelheim, Germany).

Biopsies from the rectal mucosa were taken in both groups on the admission day and thereafter twice a week. The first biopsy from patients in the treatment group was taken before the administration of bacteria. Administration of enteral nutrition was started as soon as the patients' circulatory and respiratory functions had been stabilised and in all patients before 24 hours after admission. Biopsies were sent blinded for analysis to the laboratory.

Analysis of the biopsies

The pieces of tissue were washed three times in a solution (0.9% NaCl, 0.1% peptone, 0.1% Tween, and 0.02% cysteine) before dilution and inoculation. Viable counts were obtained from Rogosa agar (Oxoid; Basingstoke, Hampshire, England) incubated anaerobically at 37°C for 3 days for the enumeration of lactobacilli, from Violet Red Bile Glucose agar (Oxoid) incubated aerobically at 37°C for 24 hours for the enumeration of *Enterobacteriaceae*, and from perfringens agar base (Oxoid) + TSC selective supplement (Oxoid) incubated anaerobically at 37°C for 3 days (sulphite reducing clostridia). Colonies suspected to be Lp 299v on the Rogosa agar plates (large, creamy, white–yellowish and somewhat irregular) were counted. Representative colonies were picked, purified on Rogosa agar and were identified by Randomly Amplified Polymorphic DNA typing [16].

Clinical routine cultures

Specimens from blood, urine and tracheal secretion, from wounds and from other relevant locations were sent for culture weekly or when clinically indicated. Tips from central venous catheters and occasionally, on suspicion of infection, arterial lines were sent for culture at removal.

The specimens were cultured and analysed at the Department of Clinical Microbiology, Lund University Hospital, according to clinical routines.

Chemistry

Blood gases were analysed in the ICU and other routine experiments were performed at the Clinical Chemistry Laboratory, Lund University Hospital.

Table 1

Patient characteristics

Patient	Age (years), gender	Diagnosis at admission	APACHE II score	Length of stay in ICU (days)
Treatment group				
2	38, female	Pneumonia	13	14
4	63, male	Gun shot wound	15	10
5	52, female	Respiratory insufficiency	15	15
10	69, female	Pancreatitis	17	37
12	84, male	Pneumonia	24	4
14*	84, female	Pneumonia	23	10
15†	72, male	Respiratory insufficiency	29	20
17	77, female	Sepsis	17	4
Control group				
1	33, male	Multi-trauma	14	5
3	57, female	Pancreatitis	19	20
6	57, male	Pneumonia	15	11
8†	61, male	Septic arthritis	24	49
11†	60, male	Retropharyngeal abscess	19	19
13	76, male	Respiratory insufficiency	36	4
16	56, female	Sepsis	16	7

APACHE, Acute Pathophysiology and Chronic Health Evaluation. *Died in the hospital after the intensive care unit (ICU). †Died in the ICU.

Statistics

The proportions of conversion of bacterial adherence to the mucosa were analysed with the chi-square test (2 × 3 table) (Statview; SAS institute Inc., Cary, NC, USA). Differences in chemistry and bacterial counts of the main groups of bacteria were analysed with the Student *t* test (Statistica 6.0; Statsoft, Tulsa, OK, USA). $P < 0.05$ was considered significant. The results are presented as the median and range unless otherwise indicated.

Results

All patients tolerated total or partial enteral feeding, and from day 2 the patients received at least 25% of the calculated daily nutritional needs via the enteral route. Supplementary nutrition was given parenterally.

Patients in the treatment group were older than the controls (median 70.9 [38–85] years versus 57.5 [34–76] years). There were no differences in the Acute Pathophysiology and Chronic Health Evaluation II score (17 [13–29] and 19 [14–36] for the treatment and control groups, respectively) in the days on a ventilator, in the median length of stay in the ICU (12 [4–37] days versus 11 [4–49] days), in hospital mortality (two patients died in each group) or in 6-month mortality (all patients discharged from the hospital survived) between the groups (Table 1).

All the patients were treated with broad-spectrum antibiotics, mainly imipenem and cefuroxime (Table 2), in consensus with a consultant physician from the Department of Infectious Diseases and according to results from previous cultures. In two patients, one from each group (patients 7 and 9), only one biopsy (before the start of the treatment) was obtained due to short stay; hence, these patients were excluded from the study. The calculations are thus based on eight patients in the treatment group and seven patients in the control group.

C-reactive protein was similar in the two groups throughout the study. The leukocyte count tended initially to be higher in the treatment group, but after day 5 the leukocyte count was lower in the treatment group ($P = 0.036$ on day 6). There was no difference in the other routine chemistry.

After the adjustment of the dose of the test solution the enteral solutions were well tolerated. There was no difference in the incidence of diarrhoea or gas bloating between the two groups.

Cultures of biopsies and colonisation of Lp 299v

There was no significant bleeding or other side-effects after the biopsies in any patient.

The number of analyses of biopsies in the treatment group and in the control group were two analyses in six patients (three

Table 2

Identification of *Lactobacillus plantarum* 299v (Lp 299v) from biopsies and the antibiotics used

Patient	Lp 299v, first biopsy	Lp 299v, later biopsies	Antibiotics prior to ICU admission (≤ 12 days if not specified)	Antibiotics in ICU before first biopsy	Antibiotics in ICU (during biopsy period)
Treatment group					
2	No	Yes	Erythromycin	Erythromycin + imipenem	Erythromycin + imipenem
4	No	No	Cefuroxime	Cefuroxime	1 Imipenem, 2 +metronidazol
5	No	No	Cefadroxile, 10 days	Cefadroxile	1 Cefuroxime, 2 meropenem
10	No	No	Cefuroxime, 3 days	Imipenem	1 Imipenem, 2 +metronidazol
12	No	No	No antibiotics	Imipenem	Imipenem
14	No	Yes	1 Metronidazol + cefotaxime/ cefuroxime, 2 -metronidazol, 3 - cefotaxime/cefuroxime; + imipenem; 12 days in total	Imipenem	Imipenem
15	No	Yes	Ciprofloxacin + two doses metronidazol (rectally)	Ceftazidime	1 Ceftazidime, 2 +metronidazol
17	No	No	Cefuroxime	Imipenem	Imipenem
Control group					
1	No	No	Cloxacillin	1 Cloxacillin, 2 cefuroxime	1 Cefuroxime, 2 +metronidazol
3	Yes	No	Imipenem	Imipenem	1 Imipenem, 2 +metronidazol
6	Yes	No	1 Penicillin G, 2 erythromycin, 3 +netilmicin, 4 cefotaxime (- netilmicin, -erythromycin), 5 erythromycin, 6 imipenem; 3 weeks in total	Imipenem	Imipenem
8	Yes	No	Penicillin G	Imipenem	1 Imipenem, 2 +clindamycin, 3 - clindamycin, +metronidazol, 4 vancomycin+ ciprofloxacin
11	No	No	Metronidazol and cefuroxime	Metronidazol and cefuroxime	1 metronidazol + cefuroxime, 2 +isoniazid, 3 +rifampicin, 4 -(1, 2, 3), +imipenem
13	Yes	No	1 PenicillinV, 2 cefuroxime; 6 days in total	Cefuroxime	Cefuroxime
16	No	No	Cefuroxime	Cefuroxime	1 Cefuroxime, 2 penicillin G

Figures indicate the order in which antibiotics were been given (and changed). +, added medication; -, withdrawn medication. ICU, intensive care unit.

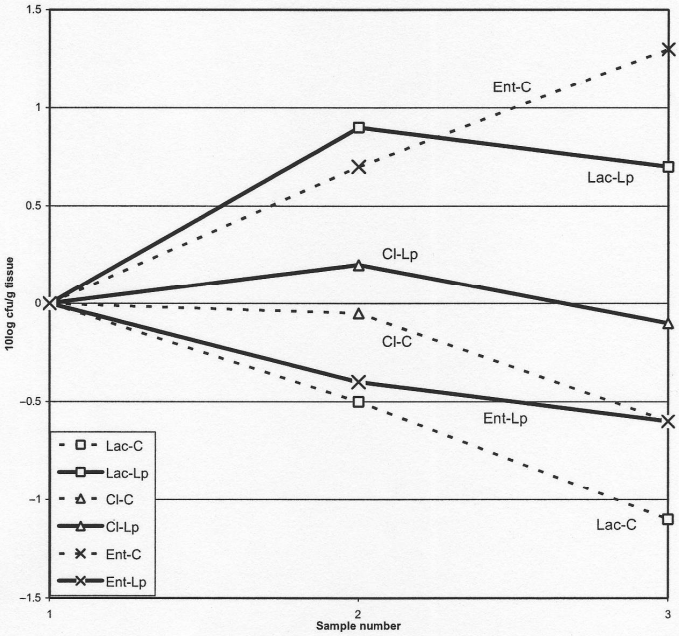
patients and three patients, respectively), three analyses in four patients (two patients and two patients, respectively), four analyses in two patients (one patient and one patient, respectively) and five analyses in three patients (two patients and one patient, respectively). There was a difference ($P = 0.029$) of bacterial conversion in the biopsies between the groups. At the start of the study, four out of seven control patients were positive for Lp 299v on the first biopsy but Lp 299v was not detectable in subsequent biopsies. In the treatment group, no patient was positive at admission, but two patients converted to positive culture for Lp 299v on the second biopsy and a third patient converted from the third biopsy. The successive tests remained positive in these three patients.

All patients received two or more doses of antibiotics before inclusion and the first biopsy. Five patients had been treated with antibiotics for more than 24 hours (3 days–3 weeks) before ICU admission. The antibiotics used before and during the study and the findings of Lp 299v from the biopsies are depicted in Table 2.

The numbers of *Lactobacillus* increased in treated patients while there was a tendency for a reduction in the controls ($P = 0.061$) (samples from the second biopsies). We could not discern any statistical differences between the groups regarding *Enterobacteriaceae* or sulphite reducing clostridia (Fig. 1), although the mean values of *Enterobacteriaceae* increased in the control group and decreased in the treatment group ($P = 0.27$ comparing samples from the second round of samples).

From the 15 patients who completed the study, 240 cultures were performed from inclusion until 36 hours after transfer to other units. Fifty-eight (24%) of these cultures were positive (Table 3). In blood, five out of 32 cultures showed bacterial growth in the control group whereas none of 30 cultures in the treatment group had bacterial growth. In the treatment group

Figure 1



Changes of bacterial counts from rectal biopsies (means): comparisons with the initial sample. The *Enterobacteriaceae* (Ent) species show a 10-fold increase in mean values in the control (-C) group while *Lactobacillus* (Lac) decrease 10-fold. In contrast, in the treatment group (-Lp) *Lactobacillus* (Lac) increase and *Enterobacteriaceae* decrease. Sulphite reducing clostridia (Cl) decrease in the control group. cfu, colony-forming units.

Table 3

Number of cultures

Type of culture	Control group			Treatment group			Fisher's exact test
	n	Positive *n*	Number of patients with positive cultures	*n*	Positive *n*	Number of patients with positive cultures	
All	122	25	5/7	118	33	6/8	NS
Blood	32	5	3/7 (3/5)	30	0	0/8 (0/5)	NS
Catheter tips	22	4	3/7 (3/4)	22	4	3/8 (3/6)	NS
Tracheal secretions	14	6	2/7 (2/6)	15	6	5/8 (5/6)	NS
Urine	19	1	1/7 (1/7)	18	4	2/8 2(/6)	NS

Figures in parentheses show the number of patients with positive cultures in relation to the number of patients from whom the respective type of culture were taken. In the treated group, five cultures were positive in the control group while no positive cultures were found in the treatment group. Due to the small numbers of patients (we performed statistics as participating patients and not as independent cultures), a significant difference was not reached (NS, not significant).

Table 4

Species found at different locations

Location	Control group	*Lactobacillus plantarum* 299v group
Blood	Coagulase-negative *Staphylococcus*, 3	None
	Enterococcus faecalis, 1	
	Pseudomonas aeruginosa, 1	
Catheter tips	Coagulase-negative *Staphylococcus*, 3	Coagulase-negative *Staphylococcus*, 3
	Enterococcus faecium, 1	*Morganella morgani*, 1
	Enterobacter cloacae, 1	*Enterococcus faecalis*, 2 (1 scarce)
Tracheal secretions	*Klebsiella pneumoniae*, 3	*Escherichia coli*, 2
	Pseudomonas aeruginosa, 1	*Morganella morgani*, 1
	Enterococcus faecalis, 2	*Pseudomonas aeruginosa*, 1
		Enterococcus faecium, 1
		Enterobacter cloacae, 1
		Candida albicans (scarce), 1
		Candida kefyr, 1
Urine	*Pseudomonas aeruginosa*, 1	*Candida albicans* (scarce), 2
	Enterococcus faecalis, 1	*Candida tropicalis* (samples from one patient, same day but separated in time), 2

Main differences between the treatment and control groups are, besides no positive blood cultures, the more abundant findings of fungi. The growth of fungi in the treatment group (urine and tracheal secretions) might be due to less bacteria giving better conditions for the culturing of fungi.

blood cultures were taken from five out of the eight patients, and blood cultures were taken from five of seven patients in the control group. The positive cultures came from three patients. In patient 3 we found two different strains of coagulase-negative *Staphylococcus*. The samples were taken the same day but at different occasions. In patient 8 different enteric bacteria were found on two occasions, days apart. The fifth finding was a coagulase-negative *Staphylococcus* from patient 11. Findings were more equal in cultures from other sites.

The species found from the blood, the catheter tips, the tracheal secretions, and the urine results are presented in Table 4.

Discussion

This pilot study shows that Lp 299v administered to critically ill, antibiotic-treated patients can survive and colonise the gut mucosa, and that repeated administration of the bacteria is necessary to obtain this effect.

The commercial market for probiotics today is worth about €6 billion, and the European Union has invested more than €15 million in studies of probiotics, but very few results have so far emerged [17]. Probiotics have been proposed to be beneficial for the gut as well as to decrease the risk of superinfections and the development of gastrointestinal malignancies, and to have positive effects on the immune system. However, although animal experiments have shown some beneficial effects [10,11,18], very little is proven in humans. One reason for this could be that some of the proposed probiotics have no effect; even if the bacterium is 'friendly' or harmless but it does not adhere closely to the intestinal mucosa, it is probably not beneficial for the mucosal cells.

Manipulation of the gut flora by stimulating certain species, as opposed to the prevalent therapy today of suppression with antibiotics, may be a possible measure to prevent or reduce the frequency of secondary infections in severely ill patients.

Lactobacillus is an important component of the mucosa-associated flora in humans, but it is not the predominating genus on the colonic mucosa. Other genera are present at the same level or at higher levels [18-20]. Lactobacilli have been claimed to have several therapeutic functions; for example, to prevent diarrhoea, to reduce translocation and to exert immune modulation. Lp 299v is obtained from human colonic mucosa, and this particular strain possesses an excellent ability to establish itself and to adhere to the mucosa [12,13,21]. This is the first time it has been shown that a bacteria like this can be established on the gastrointestinal tract mucosa in critically ill patients.

We have previously shown that Lp 299v does adhere to the mucosa in about 40% of healthy volunteers [13]. In a study on healthy volunteers where 19 different strains of *Lactobacillus* were given in fermented oatmeal soup, only five strains were retrieved from any of the 13 participants either from jejunal or

rectal mucosal biopsies [13]. Biopsies were taken before administration and on day 1 and day 11 after administration had ended. On day 1 post treatment, Lp 299v or *Lactobacillus plantarum* 299 (similar to Lp 299v and hence analysed as the pair) was found on rectal biopsies from four of the 13 volunteers and, remarkably, on biopsies from six participants on day 11 post treatment. By comparing this with our results where three out of eight treated patients turned from negative to positive on these cultures for Lp 299v, we conclude that the frequency of establishment is about the same as in healthy non-antibiotic-treated volunteers. Why all volunteers or patients did not convert to detectable levels (2×10^3/g tissue) probably has multifactorial explanations, including genetic factors and original microbiotic flora.

In the present pilot study on critically ill patients, however, antibiotics did not seem to be an important factor in preventing survival and mucosal adherence of Lp 299v when distributed enterally.

Our study was not powered to analyse gastrointestinal or systemic effects but there is a demand for such studies because probiotics are now routinely used in many ICUs without any strong scientific proof of beneficial effects. There are, however, some small studies indicating positive effects. In a study by Oláh and colleagues, 22 patients with acute pancreatitis were given *Lactobacillus plantarum* 299 and 23 patients were given only the oatmeal formula (with heat-inactivated bacteria) [22]. The authors found a significant decrease in episodes of sepsis and pancreatic abscesses in the treated patients.

Rayes and colleagues randomised 95 liver transplantation recipients into three groups, all feed enterally [23]. One group received standard enteral formula plus selective bowel decontamination, a second group received fibre-containing formula plus *Lactobacillus plantarum* 299, and the third group received the same regimen as the second group but the lactobacilli had been heat-killed. The infection rate was reduced by 35% in the group given active bacilli compared with the group given standard formula or heat-killed bacteria. On the other hand, in another study by the same research group there was no difference in the infection rate between surgical patients that received active *Lactobacillus plantarum* 299 and patients who received heat-killed lactobacilli [24].

In addition, two studies by McNaught and colleagues have not shown any positive effect of probiotics in patients undergoing major surgery [25,26]. It should be pointed out, however, that the amount of bacteria administered in the three latter studies was probably inadequate; the daily doses of bacteria were only 5–10% of the daily dose administered in our study. Which dose is sufficient and whether probiotics have any positive effects in critically ill patients are thus still inconclusive factors.

The increase of lactobacilli on the rectal mucosa is most probably due to the administration of relatively large numbers of the study bacteria. All other changes that occurred in the amount of bacteria were not statistically significant. It is possible, however, that this is only due to the low power of the study and does not indicate a biological fact. Mean values of *Enterobacteriaceae* showed dispersing values for treated patients and control patients, and this might imply that the enterally added *Lactobacillus* changes the gut milieu so that the growth of pathogenic bacteria is inhibited.

Interestingly, the result from other cultures showed no growth of bacteria in blood cultures from the treated patients in contrast to the control group showing 15% positive cultures. This could indicate an effect of Lp 299v on the mucosal barrier, or on the immune system, as shown in the studies on *Lactobacillus plantarum* 299 on pancreatitis transplant patients and liver transplant patients [23,24].

Our study has several limitations. First, only a few patients were included. We wanted to study as low a number of patients as possible, due to the inherent risks with rectal biopsies, but still wanted to be able to assess whether adherence of Lp 299v could occur in critical illness. An experienced surgeon performed the biopsies and we used very strict inclusion criteria in order to increase the safety of the procedure and to prevent harmful side-effects. Indeed, we had no complications.

Second, four patients in the control group already had growth of Lp 299v on rectal biopsies when entering the study. This is most probably due to the fact that this bacteria is commercially available as part of a probiotic fruit beverage (made from the same base as our study product) in Sweden and is widely consumed by the population. In addition, since the organism used was originally harvested from human mucosa [27], our findings might be explained by the natural occurrence of the bacteria. The bacteria, however, were not identified on the subsequent biopsies in these patients, suggesting that regular administration is necessary to maintain the adhesion onto the mucosa.

Third, the statistics used could be questioned. Nevertheless, there is no reasonable explanation for the conversion from no adherence to adherence of the Lp 299v onto the mucosa other than the enteral administration of this strain *per se*.

Finally, in the patients in whom we did not find any bacterial adhesion on the rectal mucosa, we cannot exclude that that the bacteria adhered onto the mucosa at other parts of the gastrointestinal tract.

Conclusion

In conclusion, this pilot study shows that enteral administration of Lp 299v is feasible in the intensive care setting. The study also shows that this bacterium can survive transport in the gas-

trointestinal tract and seems to colonise the gut mucosa, as assessed from rectal biopsies, in critically ill patients treated with broad-spectrum antibiotics.

Key messages

- The probiotic bacteria *Lactobacillus plantarum 299v*, given enterally to critically ill patients on antibiotic therapy survives the passage through the gastrointestinal tract and has the ability to colonize the rectal mucosa
- It is necessary to administer Lp 299v daily when patients are on antibiotic therapy.
- We saw no adverse effects and the study product containing oatmeal soup was well tolerated.
- Administration increases the number of lactobacilli and reduces the number of *Enterobacteriaceae*.
- The absence of positive cultures in the treatment group indicates that Lp 299v may have an effect on the mucosal barrier or even have a positive impact on the immune system.

Competing interests

BJ, GM and M-LJ are shareholders in Probi AB. Probi AB provided the study product.

Authors' contributions

BK, the primary investigator, was active in study planning, performed all beside work apart from the biopsies, handled the primary data and some of the statistical work, and prepared and finalised the manuscript together with GM, AL and BJ. M-LJ was active in the planning and practical performance of the study, and performed some of the statistical analysis. AL was involved in the study layout, performed some of the statistical analysis and was active in preparing the manuscript. GM contributed to analyses of the results from the bacterial cultures and to finalising the manuscript. BJ participated actively in the planning of the study and in the preparation of the manuscript.

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Lactobacillus plantarum 299v reduces colonisation of *Clostridium difficile* in critically ill patients treated with antibiotics

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Background: The incidence of *Clostridium difficile*-associated disease (CDAD) in hospitalised patients is increasing. Critically ill patients are often treated with antibiotics and are at a high risk of developing CDAD. *Lactobacillus plantarum* 299v (Lp299v) has been found to reduce recurrence of CDAD. We investigated intensive care unit (ICU) patients with respect to the impact of Lp299v on *C. difficile* colonisation and on gut permeability and parameters of inflammation and infection in that context.
Methods: Twenty-two ICU patients were given a fermented oatmeal gruel containing Lp299v, and 22 received an equivalent product without the bacteria. Faecal samples for analyses of *C. difficile* and Lp299v were taken at inclusion and then twice a week during the ICU stay. Other cultures were performed on clinical indication. Infection and inflammation parameters were analysed daily. Gut permeability was assessed using a sugar probe technique.
Results: Colonisation with *C. difficile* was detected in 19% (4/21) of controls but in none of the Lp299v-treated patients ($P < 0.05$).
Conclusions: Enteral administration of the probiotic bacterium Lp299v to critically ill patients treated with antibiotics reduced colonisation with *C. difficile*.

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THE use of antibiotics, especially broad-spectrum agents, constitutes the most important risk factor for developing antibiotic-associated diarrhoea (AAD) and *Clostridium difficile*-associated disease (CDAD).[1–4] The incidence of CDAD in the general population is increasing,[1,5–7] as is the number of deaths reported to be due to *C. difficile* infection.* It has been found that 3–29% of hospitalised patients treated with antibiotics have AAD,[8] and *C. difficile* accounts for 20–60% of those cases.[9] Furthermore, the severity of this problem has been amplified by the emergence of a highly virulent strain of these bacteria [polymerase chain reaction (PCR) ribotype 027], which causes even greater morbidity and mortality.[1,10–12] Case reports[13–15] and retrospective investigations[16,17] have been published, but, to our knowledge, there have been no studies of the incidence of *C. difficile* infections in intensive care units (ICUs), even though this rate is probably higher in ICU patients than in the general hospital population.

CDAD is usually treated with oral metronidazole or vancomycin, and this strategy, in combination with probiotics (*Saccharomyces boulardii* or *Lactobacillus* spp.), has been found to reduce the recurrence rates.[18,19] *Lactobacillus plantarum* 299 v (Lp299v) (DSM9843) when given orally colonises the gastrointestinal mucosa in healthy volunteers as well as in critically ill patients treated with antibiotics.[20,21] Both Lp299v and the genomically closely related *L. plantarum* 299 (DSM 6595) have a high safety profile: no important side effects have been found in studies of critically ill patients given those probiotics,[21–24] and in a model of endocarditis, intravenously injected Lp299v could not be retrieved.[25]

The primary objective of the present study was to determine whether enteral administration of

**Statistics from UK Government, http://www.statistics.gov.uk/cci/nugget.asp?id=1735.*

Lp299v could decrease the incidence of *C. difficile* infection in critically ill patients treated with antibiotics.

Methods

This study was approved by the Human Ethics Committees at Lund University and Gothenburg University, and by the Swedish Medical Products Agency. GCP/ICH was applied, and the work was performed in compliance with the Helsinki Declaration. The study was carried out at five Swedish ICUs. Informed consent was obtained from the patients or their next of kin.

Inclusion was done within 24 h of admission to the ICU and the patients included fulfilled the following criteria: (1) age $\geq$ 18 years; (2) presumed need for intensive care for 3 days or longer; (3) no known positive test for *C. difficile* during the week before enrolment; (4) anticipated to tolerate enteral feeding started within 24 h of admission to ICU; (5) no allergy to any of the components in the study product; and (6) not moribund. Patients were excluded if enteral feeding could not be started within 24 h of admission. Furthermore, those who were positive for *C. difficile* in the inclusion sample were excluded from the analyses of *C. difficile* cultures. Randomisation was blinded to the investigators, the ward staff, and the sponsor (Probi AB, Lund, Sweden). Packages of the active and control study products came from an independent company (Trensums Food AB, Tingsryd, Sweden).

The active study product consisted of a fermented oatmeal gruel containing 8×10^8 colony-forming units (CFU)/ml of Lp299v (Probi AB). As a control, the same gruel without Lp299v bacteria but with lactic acid added to achieve the same pH was used. The products were given as bolus doses: initially, six 100-ml doses of gruel at 12-h intervals and thereafter 50 ml twice a day as long as the patient stayed in the ICU. Enteral feeding was carried out according to local protocols and the participants received metoclopramid (Primperan, Sanofi, Paris, France) and picosulphate (Laxoberal, Boehringer Ingelheim, Ingelheim, Germany, or Cilaxoral, Ferring, Malmö, Sweden). All feeding was recorded and the energy intake was calculated.

Samples for chemical and cytokine analyses were taken daily. Routine chemical analyses were performed at point of care or at the hospital clinical chemistry laboratories. Human ELISA sets (BD Biosciences, San Diego, CA) were used to analyse cytokines.

Microbiology

Before starting the enteral feeding, rectal faecal samples were collected for culturing of *C. difficile* and Lp299v, and these samples were subsequently taken twice a week as long as the patient stayed in the ICU. Defecation was infrequent in most of the patients, and the samples were often collected as rectal swabs.

Identification of *C. difficile* and testing for toxins were performed at the clinical microbiology departments at the hospitals. Lp299v was analysed in blinded samples according to an established method,[26] and this was done at the research laboratory of Probi AB. Furthermore, at the Lund University Hospital ICU, a second set of rectal swabs was collected on sampling days and sent blinded to Probi AB for analyses of lactobacilli, *Enterobacteriaceae*, sulphite-reducing clostridia, enterococci, and total viable count of anaerobes and Gram-negative bacteria.

Specimens of blood, urine, and tracheal secretions, and samples from wounds or other relevant locations were sent for culture weekly or when clinically indicated. All tips of removed central venous catheters were sent for microbiological analysis.

Gut permeability

Gut permeability assessment was performed on the 15 patients at the ICU in Lund. On the day of inclusion, these patients received a mixture of non-metabolisable sugars containing 5 g of lactulose, 1 g of L-rhamnose, 0.5 g of D-xylose, and 0.2 g of 3-*O*-methyl-D-glucose in 100 ml of water (240 mosm). In 13 of the patients (two had been discharged), a second test was performed on day 3 or 4. Metoclopramid (10 mg) was given intravenously in conjunction with administration of the sugar solution. Urine or combined ultrafiltrate-dialysate [the latter from two patients (1 and 1) on continuous renal replacement therapy] was collected for 5 h after giving the sugars, and vials of 10-ml samples from the respective bags were stored at -70 °C pending analysis.

The samples were analysed using an HPLC system with an HPAEC Carbopac PA10 column and pulsed amperometric detection (Dionex, Sunnyvale, CA). To achieve good separation of the probes in the samples that were assumed to contain glucose, a modified version of a previously described method[27] was used.

Statistics

Statistical analyses were performed after consultation with a biostatistician. To our knowledge, this is the first investigation of the incidence rate of *C. difficile* in critically ill patients, and thus an accurate power calculation could not be carried out. Nevertheless, the goal was to randomise 100 patients. With a power of 80% and an α-level of 5%, 43 patients were required in each group, assuming 30% incidence in the control group and 5% in the treatment group. This was based on the conjecture that the frequency of CDAD would be higher than in a general hospital material[5,6] and the ability of Lp299v to inhibit colonisation with *C. difficile* would be greater than its ability to reduce recurrence of CDAD.[19]

Student's *t*-test for two independent samples was performed for most variables. However, the Mann–Whitney test was considered to be more appropriate for the gut-permeable analyses due to the limited sample size. Fisher's exact test was used for comparison of colonisation with *C difficile*. Probability values <0.05 were considered to be significant and calculations were carried out using Statistica 6.0 (StatSoft, Tulsa, OK).

Results

Forty-eight patients were included according to the protocol. Two patients declined participation, and two were excluded because the enteral feeding and the tested product were not given as instructed in the protocol. Thus, a total of 44 patients completed the study; 22 were given the active treatment and 22 received the control product.

Age, gender, APACHE II scores, Sequential Organ Failure Assessment scores, length of stay at ICU, and number of days on ventilator were similar for the two study groups (Table 1). ICU and in-hospital deaths were also similar, and, additionally, four control patients died from their concomitant diseases within 6 months of inclusion in the study (Table 1). Admission diagnoses were equally distributed between the Lp299v and the control patients (Table 2).

Microbiology

C. difficile. Seventy-one and 80 faecal samples were analysed in the Lp299v group and the control group, respectively. For *C. difficile* diagnostics, 30 patients (15 in each group) had three samples, 19 (10 vs. nine) had four, and eight (three vs. five) had five or more samples. Samples that were positive for *C. difficile* were found for five patients in the control group (one was the inclusion sample and consequently this patient was excluded from analyses of emerging cases of *C. difficile*) but for none of the patients in the Lp299v group ($P = 0.0485$). In the four cases of emerging *C. difficile* infection, the

Table 1

Patient characteristics.

	Lp299v group	Control group
APACHE II score	17 (7–29)	20 (11–38)
Age	65.5 (29–89)	64 (18–86)
Sex M/F	13/9	13/9
ICU stay in days	5.5 (2.5–22.0)	8.8 (1.1–67)
Days on ventilator	4.4 (0–16.3)	7.3 (0.9–20.5)
BMI	28.1 (18.5–47.7)	24.9 (14.7–39.9)
ICU deaths	2	2
In-hospital deaths	1	0
Deaths within 6 months	0	4

Results presented as median (range) except for sex and death rates. Differences are not significant.

Table 2

Admission diagnoses.

Diagnosis at admission	Lp299v group	Control group	Lund ICU, Lp299v group*	Lund ICU, control group*
Sepsis, septicaemia	6	4	4	4(1)†
Pneumonia, respiratory insufficiency, or laryngeal oedema	8	9	3	2
Intracranial processes	3	5	0	1
Meningitis, encephalitis	2	3	0	1†
Burns	1	1	0	0
Trauma	1	0	0	0
Other	1	0	0	0

*Only patients from the Lund ICU, underwent analyses for enteric bacteria and gut permeability tests.
†Only one gut permeability test was performed on these patients.
The Lund patients are also included in the total values for the two groups (given to the left in the table).
ICU, intensive care unit.

Table 3

Number of cultures in total and those with positive results and the number of patients with positive cultures from the respective analyses.

	Lp299v group			Control group		
Type of culture	*n*	Positive *n*	Patients with positive cultures *n*	*n*	Positive *n*	Patients with positive cultures *n*
Clostridium difficile	71	0	0	80	9	(5) 4*
Other except C diff (total)	83†	22	11	151†	43	12
Blood	29	4	2	52	5	3
Catheter tips	5	1	1	18	8	3
Tracheal secretions	19	9	7	32	17	9
Urine	10	4	2	21	1	1

*One of the patients was positive at the start of the study and was therefore excluded from calculations of emergence of *Clostridium difficile*.
†Two patients in the control group had very long ICU stays and consequently many cultures were taken.
The Lp299v and the control patients did not differ with regard to frequencies of positive cultures, with the exception of more positive results for *C. difficile* in the control group. Statistical analyses were performed on participating patients, not on independent cultures.

first positive result was observed in the second sample (collected on day 3 or 4).

L. plantarum 299v. Lp299v was identified in the inclusion samples from four patients (two in each group), and it was found in one or more cultures of subsequent samples from 18 of 21 patients in the active treatment group and in three samples from control patients.

Enteric bacteria. Most categories of bacteria increased in both patient groups. All ratios of lactobacilli to other groups of bacteria increased in the active treatment group, but they decreased in the control group.

Other cultures. After administration of study products, 83 cultures were taken in the Lp299v group and 151 in the control group (Table 3). In the control patients, there was a more varied spectrum of bacterial species (data not shown), including several potential pathogens that were not found in the treatment group (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Bacillus cereus*).

Bowel function

No overall differences in the frequency of bowel movements or consistency of faeces were found.

Infection and inflammation parameters

There were no significant differences between the two groups with regard to C-reactive protein, tumour necrosis factor-α, interleukin (IL)-1β, or IL-6. IL-10 was higher in the control group, with a significant difference ($P = 0.025$) on day 8. White blood cell (WBC) counts were significantly higher in the control group on days 7–9 and 12–14.

Metabolic parameters

Lactate levels were lower in the Lp299v group than in the control patients from day 4 and onwards, and reached significance on days 6 and 9.

Gut permeability

The lactulose/rhamnose (L/Rh) excretion ratios at the initial test were similar for the Lp299v group (seven patients) and the controls (six patients), and two and three of the patients, respectively, had normal values (≤ 0.05). At the second test, the ratios had decreased (i.e. improved) in five of the seven Lp299v patients and remained normal in the other two; all values in that group were <0.10. Considering the six patients in the control group, permeability was increased in three patients, decreased in two, and unchanged in the sixth (the only value <0.10). The L/Rh ratio in the second test was better for the Lp299v group than for the control group ($P = 0.0455$).

Nutrition and medication

The study product was well tolerated in both groups.

Nine patients in the Lp299v group and 15 in the control group received corticosteroids at some time during their ICU stay. The antibiotics used were mainly cephalosporines and carbapenems, and they were administered in similar ways. Fourteen patients in each group received H_2-blockers or proton pump inhibitors (PPI). Three of the four patients who tested positive for *C. difficile* were given PPI.

Discussion

We found that the incidence of colonisation with *C. difficile* was lower in critically ill patients given Lp299v than in those given placebo. To our knowledge, this study is the first to consider the incidence rate of *C. difficile* in critically ill patients.

C. difficile tests were positive for 19% (4/21) of the controls, which agrees with the rates reported in the literature for hospitalised patients treated with antibiotics.[9,28] None of the patients in our Lp299v group had a positive *C. difficile* test, which indicates that the probiotic Lp299v might counteract or even prevent colonisation with *C. difficile* in critically ill patients receiving antibiotics. Our results agree with a recently published investigation,[28] in which antibiotic-treated hospitalised non-ICU patients were given a product containing *Lactobacillus casei*, *L. bulgaris*, and *Streptococcus thermophilus*. Hickson and co-workers found no *C. difficile* in their active treatment group, whereas 17% of the subjects in the control group were positive for that species. Thus, it is probable that *C. difficile* colonisation in antibiotic-treated patients can be reduced by administering probiotics together with the antibiotics. Our data suggest that, in cases of critical illness, Lp299v is prophylactic against *C. difficile* colonisation.

The rate of recovery of the administered Lp299v in our active treatment group was high even though these patients were on antibiotics, which confirms that Lp299v remains viable throughout the gastrointestinal tract and that the amount given was sufficient. In a meta-analysis conducted by McFarland,[29] it was concluded that treatment with probiotics in doses of $\geq 10^{10}$ CFU/day was associated with significant efficacy against AAD. In our study, the patients in the active treatment group were given $\geq 8 \times 10^{10}$ CFU/day, which provided beneficial effects. This confirms that a relatively high intake of probiotics is required to achieve efficacy in patients on antibiotics. The subjects in our investigation who were positive for Lp299v at inclusion (hospitalised in three different ICUs) had probably ingested the bacteria in commercial food products. The three Lp299v-positive samples in the control group were most likely the result of protocol violations, i.e. because a ProViva fruit drink had mistakenly been given by hospital staff; none of these three patients were among those who later became positive for *C. difficile*. Notably, some control patients who had been given ProViva in hospital after concluding participation in our study were culture positive for Lp299v in subsequent samples.

The number of lactobacilli in the rectal samples increased in an expected manner in the Lp299v-treated patients but showed a relative decrease in the control group. The overall frequency of positive cultures was similar in the two groups. We were unable to verify our earlier observation that Lp299v could reduce the number of secondary systemic infections.[21] Tracheal secretions from control patients contained more potentially pathogenic enteric bacteria compared with the Lp299v group. However, many species were identified and statistical analysis was not possible, and thus these results should be considered merely as trends that require further investigation.

There were no significant differences in the pro-inflammatory cytokines. Nevertheless, we did observe that WBC counts in the Lp299v group stabilised at a level close to normal after 5 days, whereas counts in the control group increased and remained elevated during the following week. The clinical relevance of this observation is dubious, although it might serve as an indicator of a decreased inflammatory influx from the GI tract.

The use of acid-reducing medication has been shown to constitute a risk factor for *C. difficile* infection[30–32] and this finding is not contradicted by our results.

We found no adverse impact of the given probiotic preparation. However, it is essential to be particularly cautious about possible side effects in studies involving administration of live organisms to vulnerable patients. This is illustrated by a recently published randomised study based on thorough, systematic (albeit mainly *in vitro*) research,[33,34] in which a multispecies probiotic preparation (no *L. plantarum* strains included) was given to patients with pancreatitis, and several cases of bowel ischaemia as well as increased mortality were found in the group given the probiotics.[35] Even if different kinds of bacteria can be collectively denominated as probiotics, it is important to emphasise that each strain has individual properties. Indeed, this should be taken into consideration when analysing research results, because it means that findings from a single study cannot be generalised. Probiotics, as well as other microorganisms, interact with one another and also with the species serving as a host, and, as evidenced by the findings published by Besselink et al.,[35] outcomes of treatments may be unexpected. Notably, Lp299v and *L. plantarum* 299 have been used in

many clinical studies, one of which focused on patients with pancreatitis,[24] and those strains have also been given to hospitalised patients for many years, but there have been no reports of associated bacteraemia or sepsis or any other adverse events. Subsequent findings concerning the pancreatitis patients[35] highlighted the importance of close monitoring for potential detrimental side effects.

Although the gut permeability test has some drawbacks, especially when applied to critically ill patients,[36,37] in our study it did indicate an improvement in the lactulose/rhamnose ratio for the Lp group. In half of the control patients, the results of the second test had deteriorated compared with the first test. Moreover, despite the limited number of participants in our study, the results of the second permeability test were better for the Lp299v group than for the control patients (findings statistically significant). Interestingly, McFie et al.[22] have also observed such a difference. Our observations suggest that Lp299v has a positive impact on restoration of the paracellular permeability of the gut barrier after the initial phase of critical illness.[38]

The number of participants in our investigation was low and consequently the results have to be interpreted with caution. Furthermore, the study was ended prematurely due to the low inclusion rate and decreased funding. However, the reduction in *C. difficile* cases we observed agrees well with the findings of another recent investigation of hospitalised patients given probiotics,[28] and hence it is plausible that such treatment can be equally or more beneficial for ICU patients than for other patient groups.

Conclusions

In conclusion, we found that colonisation with *C. difficile* in critically ill patients being treated with antibiotics was reduced by enteral administration of an oatmeal product containing *L. plantarum* 299v.

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Trial registration: Current Controlled Trials ISRCTN85655545.

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III

Use of the probiotic *Lactobacillus plantarum* 299 to reduce pathogenic bacteria in the oropharynx of intubated patients: a randomised controlled open pilot study

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ABSTRACT

Introduction: Ventilator-associated pneumonia (VAP) is usually caused by aspiration of pathogenic bacteria from the oropharynx, and hence oral decontamination with antiseptics such as chlorhexidine (CHX) or antibiotics has been used as prophylaxis against this complication. We hypothesised that the probiotic bacterium *Lactobacillus plantarum* 299 (Lp299) would be just as efficient as CHX in reducing the pathogenic bacterial load in the oropharynx of tracheally intubated, mechanically ventilated, critically ill patients.

Methods: Fifty critically ill patients on mechanical ventilation were randomised to either oral mechanical cleansing followed by washing with 0.1% CHX solution or to the same cleansing procedure followed by oral application of an emulsion of Lp299. Samples for microbiological analyses were taken from the oropharynx and from the trachea at inclusion and thereafter at defined intervals.

Results: Potentially pathogenic bacteria that were not present at inclusion were identified in oropharyngeal samples from eight of the patients treated with Lp299 and thirteen of those treated with CHX ($p = 0.13$). Analysis of tracheal samples yielded similar results. Lp299 was recovered from the oropharynx of all patients in the Lp299 group.

Conclusions: In this pilot study, we found no difference between Lp299 and CHX used in oral care procedures, when we examined the effects of those agents on colonisation of potentially pathogenic bacteria in the oropharynx of intubated, mechanically ventilated patients.

Keywords: probiotics, *Lactobacillus plantarum* 299, chlorhexidine, critical illness, ICU
oral care, VAP
Trial registration: Current Controlled Trials ISRCTN00472141

INTRODUCTION

Ventilator-associated pneumonia (VAP) is a common complication in intubated, mechanically ventilated patients in intensive care units (ICUs). VAP is connected with longer ICU and hospital stays, additional costs, and high mortality, and the risk of developing this condition increases by 1% with each additional day of mechanical ventilation [1,2].

The major cause of VAP is aspiration of either microorganisms from the oropharynx or fragments of biofilms from the endotracheal tube. Formation of such biofilms can be delayed, but not prevented, by the use of tubes with special coatings [3]. Selective decontamination using antibiotics in the oral cavity alone [4–6], or throughout the gastrointestinal (GI) tract [7,8], has been shown to lower the incidence of VAP and reduce mortality. However, the use of such procedures is limited due to the risk of bacteria developing resistance to antibiotics [9–10]. In recent meta-analyses, it was concluded that oral decontamination with chlorhexidine (CHX) can prevent VAP [11], but that strategy does not reduce the time on ventilator, the length of stay (LOS) in the ICU, or mortality [12]. Thus, there is a need for alternative approaches to lower the oropharyngeal load of pathogenic microorganisms as a means of decreasing the risk of VAP.

For decades, probiotics have been given enterally to improve the microbiotic flora in the GI tract. However, in recent years orally administered probiotics have also been shown to reduce bacteria and yeasts in biofilms on vocal prostheses [13,14]. Therefore, we hypothesised that swabbing the oral mucosa with probiotics would be an effective (and microbiologically attractive) method of reducing pathogenic oral microorganisms in tracheally intubated, mechanically ventilated, critically ill patients. Accordingly, the primary aim of

the present pilot study was to evaluate the feasibility and safety of an oral care procedure using the probiotic *Lactobacillus plantarum* 299 (Lp299) (DSM 6595) in this patient category.Like the genomically closely related strain *L. plantarum* 299v (DSM 9843), it has been shown that Lp299 can adhere to the mucosa throughout the GI tract [15–17]. Another objective of this preliminary investigation was to obtain an estimate of the number of patients needed for a definitive study examining the effectiveness of oral Lp299 in reducing the incidence of VAP.

MATERIALS AND METHODS

The study was approved by the Human Ethics Committee of Lund University and was performed in compliance with the Helsinki Declaration. GCP/ICH was applied and the investigation was carried out in the ICU of the Department of Anaesthesiology and Intensive Care, University Hospital, Lund, Sweden. Informed consent was obtained from the patients or their next of kin. Consent was not obtained from patients as they had recovered, as this was not required by the Human Ethics Committee.

The patients were randomised in groups of ten to receive either the department's standard oral treatment (the control group) or the study treatment with Lp299 (the Lp group). The day of inclusion was designated day 1. To be included in the study, patients had to fulfil the following criteria: (1) age $\geq$ 18 years; (2) critically ill with an anticipated need for mechanical ventilation of at least 24 hours; (3) not moribund; (4) not suffering from pneumonia at admission; (5) no fractures in the facial skeleton or the base of the skull; 6) no oral ulcers; (7) not immune deficient; (8) not a carrier of HIV or viral hepatitis.

After screening, patients were included when ventilation and circulation had been stabilised and before the first oral care procedure. Oral care was performed twice a day. The control group was treated according to the department's standard protocol: dental prostheses were removed; secretions were removed by suction; teeth were brushed using toothpaste (Zendium, Opus Health Care, Malmö, Sweden); all mucosal surfaces were cleansed with swabs that had been moistened with a 1 mg/ml CHX solution (Hexident, Ipex, Solna, Sweden). In the Lp group the initial mechanical steps were the same as in the control group, but the subsequent cleansing was instead done with gauze swabs soaked in carbonated bottled water, after which Lp299 was applied to the mucosal surface of the oral cavity. This was performed using two gauze swabs (one for each side of the oral cavity), which had been allowed to absorb 10 ml of a solution containing a total of 10^{10} colony-forming units (CFU) of Lp299. Excess suspension was not removed. In both groups, when necessary between the oral care procedures, secretions were removed by suctioning, and gauze swabs moistened with carbonated bottled water were used to wipe off remaining secretions.

Cultures were taken from the oropharynx and from the trachea at inclusion. Sampling was repeated prior to the oral care procedures on days 2, 3, 5, 7, 10, 14, and 21 in patients that were still mechanically ventilated. If a patient was extubated on a non-culture day, cultures were taken before the extubation. One set of cultures was analysed according to normal routines at the Department of Clinical Microbiology, University Hospital. Another set was sent blinded to the research laboratory at Probi AB, Lund, Sweden for identification and quantification of total CFU of lactobacilli and identification of Lp299. Viable counts of all lactobacilli were done on Rogosa agar (Oxoid, Basingstoke, Hampshire, England) incubated anaerobically at 37°C for three days. Colonies suspected to be Lp299 (large, creamy white-yellowish, and somewhat irregular in shape) were selected and identified by randomly amplified polymorphic DNA typing (RAPD) [18].

The patients were placed in a semi-recumbent position and were ventilated in pressure control or pressure support mode by a Servo*i* ventilator (Maquet AB, Sweden) via a heat moisture exchange (HME) filter (Barrierbac "S", Mallinckrodt DAR, Mirandola, Italy). A closed suction system (TRACH-Care 72, Ballard Medical Products, Draper, UT, USA) was used. The patients inhaled 2.5 mg salbutamol (GlaxoSmithKline, Solna Sweden) and 0.5 mg ipratropium (Boehringer Ingelheim, Stockholm, Sweden) every six hours.

Chest radiographs were obtained after tracheal intubation and thereafter when clinically indicated. Lung function was assessed by use of the Lung Injury Score (LIS) [19]. Blood gases were obtained at least three times a day and were analysed at the ICU. Samples for white blood cell (WBC) counts and C-reactive protein (CRP) were collected daily and analysed at the hospital clinical chemistry laboratory.

Enteral nutrition (EN) was started and increased according to the department's protocol. The amount of enteral formula given and the total volume of other enterally administered fluids were recorded. All patients received ezomprazol (Astra Zeneca, Södertälje, Sweden) iv as stress ulcer prophylaxis from admission until enteral nutrition was fully established (i.e., for 3–4 days).

The study was neither intended nor powered for assessment of differences in the frequency of VAP. However, it was aimed at obtaining a basis for estimating the number of patients needed for a larger investigation in which VAP also constitutes a parameter. The following criteria were used to identify VAP: (1) a new, persistent or progressive infiltrate on chest radiograph combined with at least three of the other four criteria; (2) a purulent tracheal aspirate; (3) positive culture of tracheal aspirates occurring after 48 hours of mechanical ventilation; (4) rectal or urine bladder temperature > 38.0°C or < 35.5°C; (5) WBC count > 12 or < 3 [4,20].

Statistics
Because no previous investigation has examined the effect of probiotics in this context, we estimated that 20 patients in each group would be sufficient to assess the safety, important positive effects, and possible side effects, and to give an indication of the number of patients that would be needed in a definitive study. Statistical methods were chosen after consulting a biostatistician, and the statistical analyses were performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Student's t-test was used for the daily comparisons (days1–9) of the parameters. Fisher's exact test was employed to compare the results of microbiological cultures . $P < 0.05$ was considered significant.

RESULTS
After screening, 50 patients were included. Consent was withdrawn for two patients, and another three were transferred to other ICUs shortly after inclusion. For one patient in the control group, samples were obtained only at inclusion. Altogether, 23 patients in the Lp group and 21 in the control group completed the study.

All patients were orotracheally intubated. Two in each group were reintubated, and two in the Lp group and one in the control group were tracheotomised (on days 3, 16, and 3, respectively). The proportion of patients receiving EN and the volumes given were similar in the two groups. The patients in both groups were treated with antibiotics at the discretion of the attending physician, and changes were made in compliance with culture results. Cefuroxime was the most common antibiotic used in both groups, followed by imipenem. Three patients in each group received piperacillin/tazobactam, and other antibiotics or combinations were administered to a few patients in each of the two groups. Three patients did not receive any antibiotics at admission, and one of those three was never treated with antibiotics during the stay in the ICU. Ten patients in each group received corticosteroids for one or more days.

As indicated in Table 1, there were no significant differences in age or gender between the groups. Also, the admission diagnoses were similar in the two groups, as were the APACHE II scores. Some differences were found in the SOFA scores in favour of the Lp patients (data not shown). The two groups did not differ significantly with regard to the number of ventilator days, LOS, or ICU or in-hospital mortality (Table 1). No deaths were caused by respiratory complications, and no additional deaths occurred within six months.

No differences in WBC counts were found between the groups. Furthermore, the groups did not differ with regard to changes in CRP, although the absolute values were higher for the controls on day 3.

No significant differences between the two groups were found when considering microbiological findings of the oropharyngeal and tracheal samples

taken at inclusion. The same species were identified in samples from both the oropharynx and the trachea of six Lp patients and three controls. Subsequent oropharyngeal samples from eight Lp patients and from thirteen controls contained enteric species that had not been present in the inclusion samples from those subjects ($p = 0.13$) (Table 2). Two or three emerging species (*Enterococci* and *Enterobacteriaceae*) were found in two patients in the Lp group and seven control patients (Figure 1). Culture analysis of the tracheal samples identified emerging species in seven Lp patients and nine controls. Other comparisons of the culture results were similar. Figure 2 shows the distribution of the positive cultures according to study day and sampling site.

Lp299 was found in the oropharyngeal samples from all of the patients in the Lp group (21/23 on day 2). In addition, Lp299 was identified in the tracheal secretion samples from 13 of the patients in the Lp group (56%), and enteric bacteria were also found in six of those subjects. Five patients in the Lp group died in the ICU, and Lp299 was identified in the tracheal samples from one of those individuals, whereas no enteric bacteria were recovered from the trachea of any of those five patients.

Considering patients in both groups, a comparison of those with positive findings and those with negative findings in cultures of tracheal secretions (results reported by the microbiology laboratory) indicated a significantly lower number of ventilator days ($p < 0.001$) in the non-colonised subjects. VAP was identified in one patient in the Lp299 group and in three patients in the CHX group.

DISCUSSION

This pilot study shows that it is feasible and safe to use Lp299 as an adjunct in oral care of intubated patients. When we compared patients subjected to an Lp299-based oral care procedure with those who underwent the standard CHX-based oral treatment used at the department, we did not find any significant difference in the incidence of emerging, potentially pathogenic bacteria in the oropharynx or trachea. The emerging bacteria were, as expected, mainly Gram-negative species.

The use of CHX in oral care procedures is considered to be an effective method to reduce pathogens in the oropharynx and to prevent VAP [11,12]. Aspiration of pathogenic bacteria constitutes the main cause of VAP, and thus reducing the occurrence of such microorganisms in the oropharynx should lower the rate of VAP. In our study, pathogenic enteric bacteria appeared in fewer of the patients in the Lp299 group (38%) than in the CHX group (65%), which indicates that Lp299 might be able to lower the rate of infection with such harmful microbes and in turn lead to fewer cases of VAP. As anticipated, the difference in the incidence of VAP between the treatment groups in our study (one case in the Lp299 group and three in the CHX group) was inconclusive. It should also be

mentioned that there are some common side effects associated with CHX used in oral care, including discoloration of the teeth, a burning sensation on the tongue, and irritation of the mucosa [21,22]. More serious but rare adverse effects are local allergic reactions in the mouth and throat. Of particular importance is that CHX shows little activity against Gram-negative bacteria [23]. Moreover, it is diluted and inactivated by saliva [24], and since bacteria can be resistant to CHX, a low concentration (which will regularly occur between oral care treatments) represents an additional risk of selection and emergence of resistant strains. What is even more alarming is that bacteria strains that are not susceptible to common antibiotics, (e.g., methicillin-resistant *Staphylococcus aureus*, MRSA) also often carry genes for resistance to CHX [25]. *Lactobacillus plantarum* strains are genetically stabile and they are not likely to incorporate resistance genes or plasmids or to transfer genetic material, characteristics that are related to their inherent resistance to certain antibiotics and to other species. Consequently *L. plantarum* does not contribute to the development of antibiotic-resistant strains. In humans, lactobacilli colonise the oropharynx soon after birth, and thereafter constitute part of the normal oropharyngeal flora. Accordingly, these bacteria will enter the lower respiratory tract whenever an aspiration occurs, but, to our knowledge, they have never been implicated as a cause of pneumonia. However, other strains of lactobacillus found in immunocompromised patients have been associated with severe infections such as endocarditis [26-28]. A limitation of our study is that we did not perform surveillance blood cultures, although the Lp299 aspirated did not produce any detectable infiltrates indicating pneumonia or bacteraemia. Furthermore, aspiration of Lp299 alone did not influence the oxygenation index, LOS, or days of mechanical ventilation. Notably, the genomically closely related *L. plantarum* 299v, has been found to be safe in an animal model of endocarditis [29]. In the cited study, *L. plantarum* 299v could not be detected in the blood or heart of the laboratory animals, nor on implanted catheters 96 hours after intravenous injection of the bacteria. Both Lp299 and *L. plantarum* 299v have also been proven safe for enteral use in the ICU setting [16, 30–34]. Furthermore, except for the calculated risk of aspiration, so far we have not seen any side effects of using Lp299 as an alternative in oral care. It may be more effective to add other probiotic bacteria to the treatment suspension, but at present we do not consider that approach to be safe, since it was recently found that enteral administration of a mixture of six strains of probiotics (none of them *L. plantarum*) was associated with increased mortality in patients with severe pancreatitis [35]. In contrast to those results, studies of *L. plantarum* 299 and 299v given enterally to critically ill patients have not revealed any adverse effects of those strains [16, 30-34]. Also, since we did not remove excess Lp299 suspension after the oral care procedure, some of the bacteria must have reached the GI tract, where they probably had a positive influence on the microflora. A combination of enteral and oral treatments would probably have a greater impact on the oral flora, because if any gastric content is regurgitated, it is likely to have a lower content of potentially pathogenic bacteria.

The oral care procedure in our study was performed twice a day, which seems to correspond to the protocols in use in many ICUs [11], although it is plausible that even better results can be obtained by treating more frequently, as done by Koeman et al. [36]. According to most of the relevant studies in the literature, as well as a meta-analysis [11,12] different preparations and concentrations of CHX have been effective in reducing the incidence of VAP.

Lactobacillus spp can be detected in interdental spaces, plaques, and carious lesions [37], but we have found no data in the literature that seem to suggest a link between lactobacilli and initiation of caries. On the contrary, two Finnish studies have shown improved dental status and lowered counts of *Streptococcus mutans* in school children who consumed milk or cheese containing *L. rhamnosus* GG [38, 39]. Furthermore, in an investigation of different species of *Lactobacillus*, it was observed that *L. plantarum* strains had the most pronounced antimicrobial effect on *S. mutans*, and they were also highly efficacious against other pathogens that are frequently found in periodontal disease [40].

The present results indicate that Lp299 might be used as a component of oral care in intubated ICU patients. Besides offering a promising alternative to antiseptics like CHX, a probiotic that adheres to the oral mucosa will be able to counteract potentially pathogenic bacteria around the clock, which is superior to the fairly short-term effect of orally applied chemical agents.

Clearly, it is also important to point out that the findings of this pilot study must be interpreted with great caution, and the trends indicated by the data must and will be further examined in a larger investigation. Nevertheless, our main objectives have been met, because we found that Lp299 did become established in the oral cavity, it had no apparent adverse effects, and the results provide a basis for calculating the number of patients needed to test the trends observed in the planned definitive study.

CONCLUSIONS

Based on the results of this pilot study, we conclude that the probiotic bacterium *Lactobacillus plantarum* 299 constitutes a feasible and safe agent for oral care. Also, it seems that *L. plantarum* 299 is as effective as chlorhexidine in mitigating colonisation with pathogenic bacteria in the oropharynx of intubated ICU patients.

Key messages

Lactobacillus plantarum 299 might be as effective as chlorhexidine in reducing the incidence of emerging potentially pathogenic bacteria in the oropharynx of intubated, mechanically ventilated, critically ill patients.

We did not observe any adverse effects of the oral care procedure involving use of the probiotic bacterium *Lactobacillus plantarum* 299.

Abbreviations

APACHE II	Acute Pathophysiology and Chronic Health Evaluation.
CFU	colony forming unit
CHX	chlorhexidine
CRP	C reactive protein
EN	enteral nutrition
ICU	intensive care unit
LOS	length of stay
LIS	Lung Injury Score
Lp299	*Lactobacillus plantarum* 299
SOFA	Sequential Organ Failure Assessment

Competing interests

Probi AB provided the study product as an unconditional grant and performed bacterial analyses. Probi AB has also done the same in earlier studies performed by BK. BJ and GM are shareholders in Probi AB, and GM resigned as a board member in 2005. Probi AB holds the patent for the investigated bacterium, but there is no patent regarding the studied application. Other financially related matters regarding GM´s position as Professor at Lund University is regulated in a central and official agreement between Lund University and Probi AB.

AUTHORS' CONTRIBUTIONS

BK was the prime investigator and did most of the planning and performance of the study. BK handled the primary data and did most of the statistical work, and also collaborated with GM, BJ, and AL to prepare and finalise the manuscript. GM contributed substantially to the analysis of the results of the bacterial cultures and completion of the manuscript. BJ took part in planning of the study and in finalising the manuscript. AL helped plan the study and was very active in preparing and competing the manuscript.

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Figure legends

Figure 1

Bottom

No new enteric species (i.e., taxa not found at inclusion) appeared in 65 % (15/23) of the patients in the Lp299 group compared to 38 % (8/21) in the control group.

Figure 2

Top **Distribution of the findings of emerging enteric bacteria**

Bottom On the first days of ICU care, identified emerging enteric species were twice as many in the control patients.

Despite a gradual decrease in the number of patients remaining in the study (similar in both groups), new cases of tracheal infection appeared in the latter part of the study period, primarily in the control group.

Table 1

Patient characteristics and admission diagnosis

	Lp299 group	**Control group**
Age	70 (20–87)	70 (43–81)
Sex M/F	13/10	9/12
APACHE II score	22 (11–39)	27(9–37)
ICU mortality	5/23	4/21
In-hospital mortality	5/23	6/21
ICU stay (days)	7.7 (1.3–26.1)	6.6 (1.3–16.0)
Ventilator days	5.8 (1.0–23.8)	4.3 (1.0–15.2
Diagnosis at admission	**Lp299 group**	**Control group**
Sepsis, septicaemia	6	5
Other infections	2	1
Cardiological: arrests and insufficiencies	5	4
Respiratory insufficiencies	3	5
Abdominal	1	2
Vascular (emergency aneurysms)	0	3
Trauma	3	0
Other	3	1

Data are presented as median (range) except for sex and death rates.
Differences are not significant.

Table 2 Number of positive findings of bacteria species at inclusion and in subsequent samples

Species	Throat samples				Tracheal secretions			
	Inclusion		Subsequent		Inclusion		Subsequent	
	Lp	C	Lp	C	Lp	C	Lp	C
1 *Haemophilus infl*	1	0	0	0	1	2	1	0
2 *Moraxella catarrhal*	0	0	0	0	0	1	1	0
3 *Beta-Strepts grp G*	1	0	1	0	0	0	0	0
4 *Strept pneumoniae*	1	0	1	0	2	0	0	0
5 *Strept pyogenes*	0	1	0	0	0	1	0	0
1–5 Airway bacteria	3	1	2	0	3	4	2	0
6 *Staphy aureus*	6	2	1	0	3	0	2	0
7 *Citrobacter* sp	0	0	1	0	0	0	1	0
8 *Escherichia coli*	1	2	1	2	1	1	0	1
9 *Enterob aerogenes*	1	2	0	0	0	0	0	1
10 *Enterobact cloacae*	1	1	0	2	1	1	0	1
11 *Hafnia alvei*	0	0	0	1	0	0	0	0
12 *Klebsiella oxytoca*	0	0	1	0	0	1	0	1
13 *Morgan morgani*	0	0	0	1	0	0	0	0
14 *Proteus mirabilis*	0	0	1	1	1	0	0	1
15 *Proteus vulgaris*	0	0	1	0	0	0	1	0
16 *Pseud aeruginosa*	0	0	1	2	2	1	0	1
17 *Pseudomonas* sp	0	0	1	1	0	0	2	0
18 *Serr marcescens*	0	0	0	0	0	0	1	0
19 *Serratia* sp	0	0	0	0	1	0	0	0
20 *Stenotr maltophilia*	1	0	0	2	0	0	1	1
21 *Strept agalactiae*	1	1	0	0	0	0	0	1
22 *Enterococ faecalis*	0	0	3	3	0	0	1	2
23*Enterococ faecium*	1	0	1	2	0	0	0	2
7–23 Enteric bacteria	6	6	11	17	6	4	7	12
24 *Candida albicans*	5	4	5	9	3	7	5	5
25 *Cand parapsilosis*	0	0	1	0	0	0	1	0
26 Candida tropicalis	0	0	0	0	0	1	0	0
24–26 Fungi	5	4	6	9	3	8	6	5

Abbreviations: Lp, patients treated with Lp299; C, control patients treated with chlorhexidine. Only the first sample in which the species was identified is included in the presented data.
All the isolated *Staphylococcus aureus* strains were non-*MRSA*.
No significant differences were found between the two groups.

Figure 1

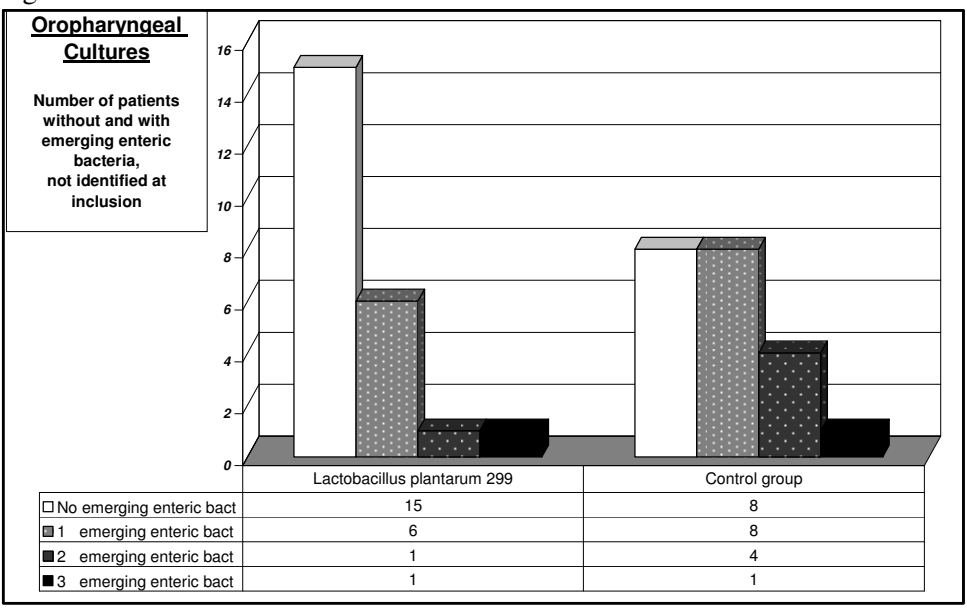
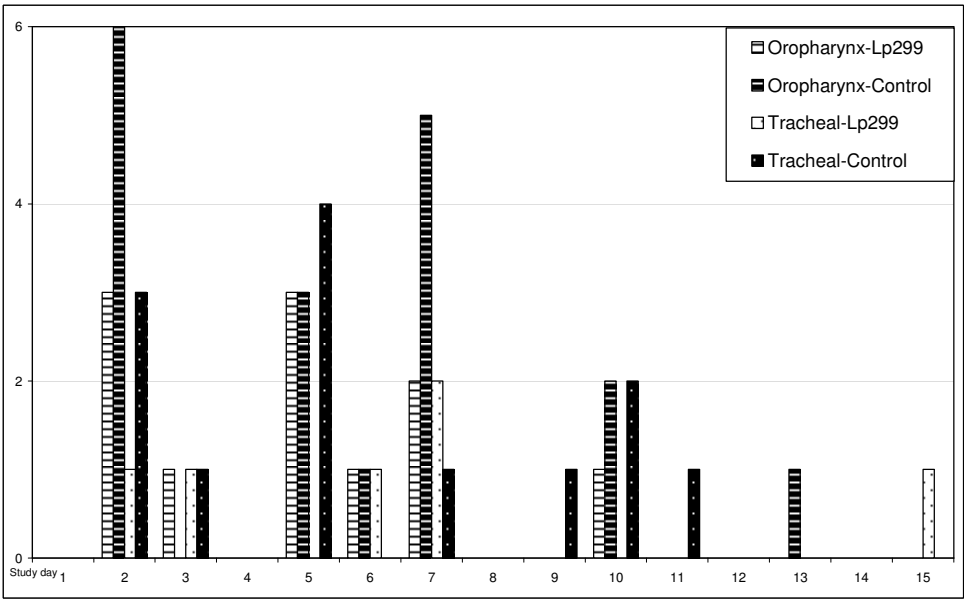


Figure 2



IV

Susceptibility to antibiotics for isolates of *Lactobacillus plantarum* RAPD-type Lp299v, harvested from critically ill patients after administration as probiotic

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ABSTRACT

Background

Antibiotics are used frequently in intensive care unit (ICU) patients and, imply significant risk of selection of resistant bacterial strains. In particular, genetic transfer of antibiotic resistance to the resident gastro intestinal flora, as well as administered probiotics, may be increased in this setting. The aim of the present study was committed to detect possible changes in antimicrobial susceptibility in re-isolates of the probiotic strain *Lactobacillus plantarum* 299v (Lp299v) given to antibiotic treated critically ill patients.

MethodsTo (antal?) patients in intensive care units (ICUs), receiving a variety of antibiotics.
the probiotic strain *Lactobacillus plantarum* 299v (Lp299v) was enterally administrated. Isolates of the strain (confirmed by RAPD-typing) were retrieved in order for antibiotic susceptibility to be monitored by Etests,

Results

Forty-two isolates were tested against 22 different antibiotics and decreased susceptibility was not found for any isolate.

Conclusion

The susceptibility to antibiotics for probiotic strain Lp299v, isolated from the rectal mucosa and faeces respectively, was not detectably changed in critically ill patients in spite of treatment with broadspectrum antibiotics.

Introduction
Probiotics are being widely used in Society for health promotion. In addition, probiotics are often used medically, i.a. with the intention to prevent side effects of antibiotics. Interestingly, a number of studies have suggested beneficial effects of probiotics in critically ill patients. However, some important issues in this context, such as the possible impact of probiotics on the normal microbiological flora [1-2], and, of given drugs on the antibiotic susceptibility of the probiotic, are largely unknown.
Critically ill patients are often receiving antibiotics, mostly broad-spectrum drugs, as an important part of their treatment. Therefore, in the intensive care unit (ICU) environment with a high antibiotic pressure, ongoing selection of bacteria with resistance to antibiotics may be significant. Probiotics are used in many ICUs mainly in order to reduce occurrence of antibiotic associated diarrhoea. Meanwhile, the possible transfer of antibiotic resistance to probiotics should also be taken into account in this context.

Lactobacillus plantarum 299 v (Lp299v; = DSM9843) is the probiotic component of several products commercially available for more than 15 years in Sweden. In addition, it has been used in many Swedish hospitals, including ICUs, as a fruit drink prophylactic remedy (ProViva®),. In several ICU studies on strain Lp299v, and the genomically closely related *L. plantarum* 299 (= DSM 6595) [1-5] no important side effects have been noted to date.

Lactobacillus plantarum as species is considered to be genomically stabile. Furthermore, regular tests of in vitro antimicrobial susceptibility of Lp 299v to a number of agents have failed to reveal any changes over the years. (Guidelines for interpretive breakpoints of MIC values for some antibiotics to different *Lactobacillus plantarum* strains, have been published by the European Food Safety Agency (EFSA) [6], but has to our knowledge not been approved or varified by others.

The purpose of the present study was to examine whether probiotically used Lp299v isolates recovered from the GI tract of antibiotic treated, critically ill patients had undergone any changes with regard to antibiotic susceptibility profile.

Methods: Within two separate controlled studies, probiotic strain Lp299v was given enterally twice a day to critically ill patients in an ICU environment. Isolates of Lp299v were then retrieved from washed biopsies of the rectal mucosa [1] (study 1) or faecal samples [2] (study 2). In both studies samples were taken at inclusion and thereafter twice a week. The patients were treated with different kinds of antibiotics, initially empirically and then in accordance with clinical findings and results from microbiological cultures. All patients had received one or more doses of antibiotics before inclusion and the first faecal sample or biopsy.

Viable counts of all lactobacilli were performed using Rogosa agar (Oxoid, Basingstoke, Hampshire, England) incubated anaerobically at 37 °C for three days. Colonies suspected to be Lp299v (large, creamy white-yellowish, and somewhat irregular in shape) were isolated and further identified by randomly amplified polymorphic DNA typing (RAPD) [7].All strains were stored at - 80º C pending analysis. After reconditioning of the frozen strains, Brucella broth suspensions of the respective strain, were inoculated on Brucella agar plates (Oxoid). E-test strips of 22 different antibiotics (AB Biodisk, Solna, Sweden) were applied to the inoculated agar plates and incubated anaerobically in 35º C for 72 hours. All analyses were done in duplicate

For comparisons of MIC values of the harvested isolates to the original strain, the isolates were divided into four groups in accordance with their exposure to antibiotics and administration of Lp299v: (1) isolates, from both studies, found in samples taken at inclusion ; (2) isolates from rectal mucosa biopsies (study 1) [1]; (3) isolates from faecal samples (study 2) [2]; (4) isolates from faeces from control patients who received fruit drink containing Lp299v after participation in the study [2] was completed.

The antibiotics that were tested against the Lp 299v isolates were mainly those that had been used clinically in the two studies, namely: ampicillin; piperacillin; cefepim; cefotaxime; ceftazidime; cefuroxime; imipenem; meropenem; erythromycin; clindamycin; chloramphenicol; levofloxacin; linezolid; quinupristin/dalfopristin; metronidazole; trimetoprim; gentamicin; kanamycin; netilmicin; streptomycin; tobramicyn; vancomycin.

Results

Forty-two retrieved study-isolates the Lp229v were analysed together with the original strain and the genomically closely related strain *Lactobacillus plantarum* 299. Six isolates (3 from study 1 and 3 from study 2) were from samples taken at study inclusion, 24 (7 and 17, respectively) from samples taken during the studies. From three patients in the control group (not given Lp299v) in study 2, 12 samples were obtained after the actual study participation was ended, and patients had been given Lp299v contained in the fruit drink ProViva®, during ongoing antibiotic therapy.

The MIC values determined for Lp299v and *L. plantarum* 299 were within one step of dilution (Table 2) and thus did not differ measurably. Both Lp299v and *L. plantarum* 299 show inherent resistance to aminoglycosides, vancomycin, and metronidazole (ref?), and also displayed high MIC values for levofloxacin. Ratios of MICs for the remaining 13 antibiotics to MICs of the Lp299v original strain are shown in Figures 1-4. We found no significant changes of susceptibility to most of the drugs tested, MIC levels differenes were within one dilution step except for ampicillin, where several isolates showed MIC increase of 2 dilution steps.

The different isolates were exposed to different antibiotics and combinations of antibiotics, and the environment in the GI tract, for varying periods of time. In almost all cases the drugs were given intravenously. Cephalosporines and

karbapenems were used most frequently. For patients with several subsequent isolates, we found no gradual change in susceptibility. Four of six patients with isolates from the inclusion sample, had a period of one to 20 days of pre-study treatment with antibiotics

Discussion

The present study was designed to find possible changes in antibiotic susceptibility of a probiotic Lactobacillus strain given to intensive care patients. Since these patients received heavy parenteral antibiotic therapy, major residual flora changes, in particular selection of antibiotic resistant bacterial species or clones, could be anticipated. Conceivably, this situation would favour dissemination of antibiotic resistance to include the probiotic strain under study. However, from two cohorts of patients, we were unable to detect any changes of susceptibility in the probiotic strain to a number of antibiotics, most of which had been used clinically at our intensive care unit.
The Lp 299v and the *L. plantarum* 299 have been used in several clinical studies without any reports of infections with species with extended antimicrobial resistance with possible origin from the given probioticum and now we have performed a post-exposure survey.

The GI tract is estimated to harbour 400 to 600 bacterial species, many of which show inherent or acquired resistance to various antibiotics. Inevitably, such species or strains to a variable degree may be positively selected during antibiotic treatment, as exemplified by the occurrence of antibiotic-associated diarrhoea, mostly caused by the opportunistic pathogen *Clostridium difficile* [8]. For pharyngeal streptococci, even short periods of treatment with macrolides (three days in the case of azitromycin) in healthy volunteers, was sufficient to increase the proportion of macrolide-resistant strains from 26 to 86 %; these ecological changes persisted for at least six months [12]. In a study on patients admitted for thoracic surgical procedures and given cefazolin for various periods of time, a significant increase in the prevalence of resistant *Escherichia coli* at discharge compared to admission was shown [13]. These figures may not be representative for all species and locations, but displays dramatic changes in selection of resistant strains within a few days of antibiotic treatment. Many of the retrieved isolates presented in this report came from patients with long periods of antibiotic load, where the probability of either an induced resistance or a selection of resistant bacteria would be considered high.

In the human (and animal) GI tract, and especially in the colon, conditions are suitable for genetic exchange between species [9], some being more prone than others to act as donors and/or recipients. Also from animal studies there is overwhelming evidence for in vivo trans-bacterial transfer of resistance genes [10,11]. It seems likely that also bacteria transiently colonizing the intestine, e.g. probiotics,, can take part in the exchange of resistance genes. Therefore, probiotics in preparations marketed to the healthy public, as well as for prophylactic use in hospitals – as part of their safety profile - should not be

prone to antibiotic resistance development, which should be documented by available in vitro and in vivo methodology..

In the determinations of MIC values for the harvested isolates of Lp299v, from a milieu where a high pressure from antibiotics increased the risk of selection of resistant bacteria, we found no evidence for the Lp299v to be prone to acquire genetic material coding for antimicrobial resistance.

Conclusions

From the findings in this study we conclude that *Lactobacillus plantarum* 299v is stable in the context of susceptibility to antimicrobial agents also in a clinical setting with high antimicrobial pressure.

Keywords: Antibiotic resistance, antibiotic susceptibility, *Lactobacillus plantarum* 299, *Lactobacillus plantarum* 299v, ICU, critical illness

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Abbreviations

ICU	Intensive care unit
Lp 299v	*Lactobacillus plantarum* 299v

Table 1
MIC values (mg/l) determined with E-tests

Antibiotic	*L. plantarum* 299	*L. plantarum* 299v
Ampicillin	0.094	0.094
Piperacillin	0.5	0.75
Cefepim	0.047	0.047
Cefotaxime	0.094	0.094
Ceftazidime	0.5	0.75
Cefuroxime	0.25	0.5
Imipenem	0.064	0.064
Meropenem	0.064	0.064
Erythromycin	0.75	1
Clindamycin	3	2
Chloramphenicol	2	2
Levofloxacin	32	32
Linezolid	1	0.75
Quinupri/Dalfopri	0.5	0.5
Metronidazole	>256	>256
Trimethoprim	0.125	0.125
Gentamicin	32	32
Kanamycin	>256	>256
Netilmicin	48	32
Streptomycin	>256	>256
Tobramycin	>256	>256
Vancomycin	>256	>256

Fig 1

Re-isolates of *L. plantarum* 299v found on inclusion samples

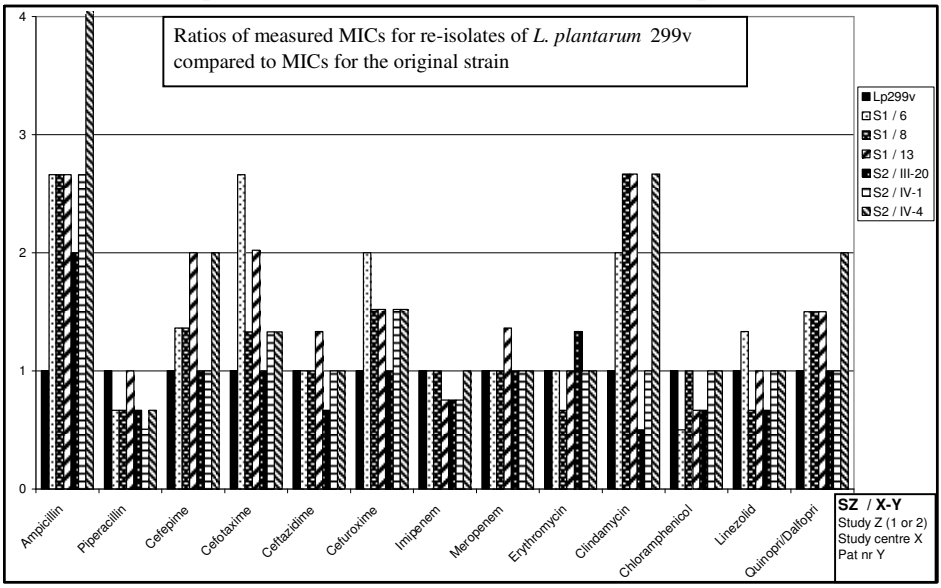
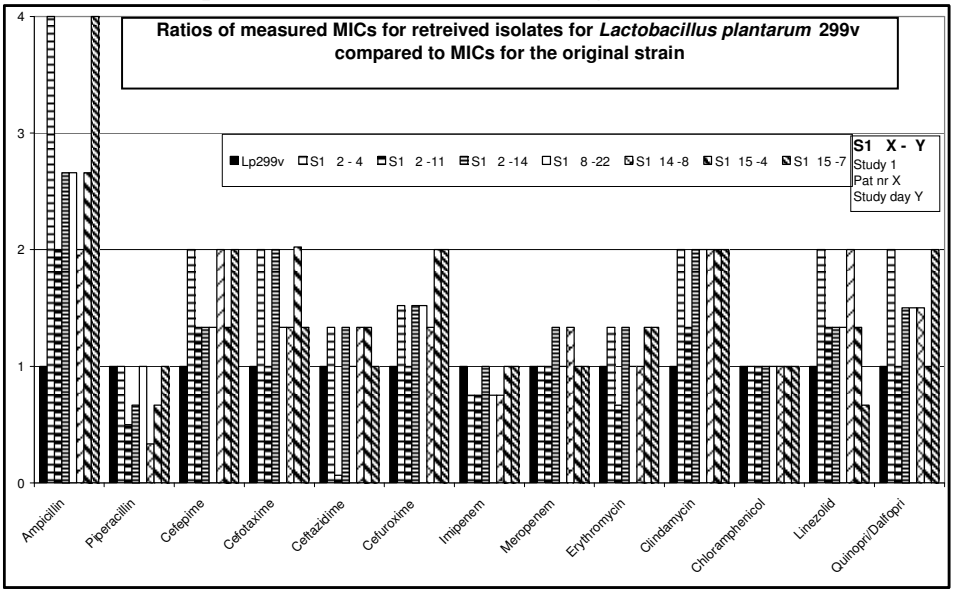
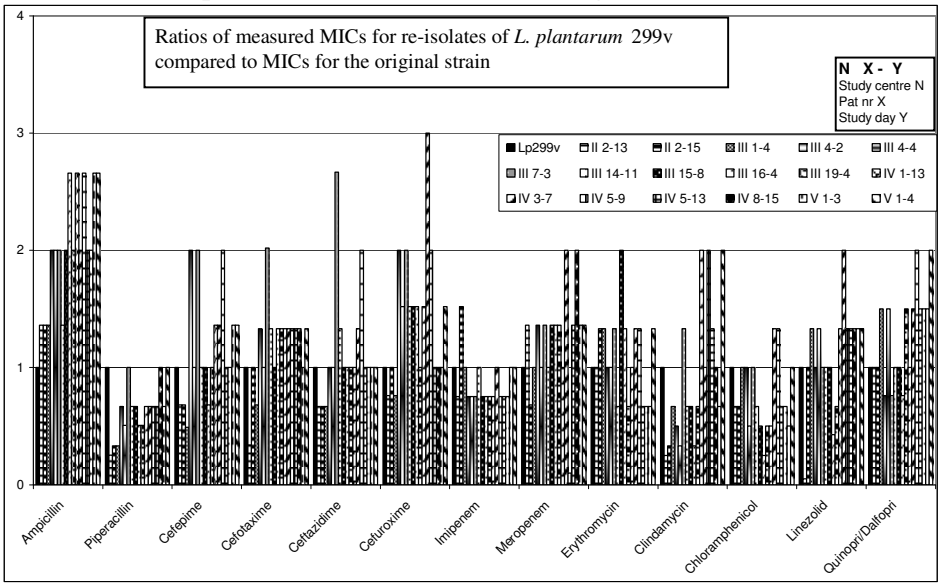


Fig 2

Re-isolates of *L. plantarum 299v* collected during the first clinical study



Re-isolates of *L. plantarum 299v* collected during the second clinical study



Re-isolates of *L. plantarum 299v* collected after end of the second clinical study