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Prophylactic use of the probiotic strain Lactobacillus casei rhamnosus as part of a triple anti-infective regimen in very preterm infants during neonatal intensive care

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ABSTRACT

Background. Probiotics are increasingly used in neonatal intensive care and prove to reduce rates of necrotizing enterocolitis (NEC), sepsis and all-cause mortality by meta-analyses.
Objective. Aim of the study was to analyze the prophylactic use of the probiotic Lactobacillus casei rhamnosus (LCR) as part of a triple anti-infective treatment regimen in very preterm neonates in respect to complications and possible side effects.
Setting. This was a study on 1169 very preterm infants of 32 weeks of gestational age and less born between 2005 and 2015 who were admitted within the first 24 hours of life to the neonatal intensive care unit (NICU) and hospitalized for at least 7 days.
Design. Retrospective observational STROBE compliant single-center cohort study
Intervention. All infants received a standardized prophylactic anti-infective treatment regimen with enteral probiotics (LCR), antifungal agents, and oral gentamycin over the study time starting at the first day of life.
Outcome measures. Perinatal and neonatal data were collected for descriptive analysis. Complications possibly avoided by the anti-infective regimen included NEC, late-onset sepsis (LOS), late-onset multiple organ dysfunction syndrome (MODS), and ventilator associated pneumonia (VAP).
Main results. Eleven of 1169 infants 11 (0.9%) had diagnosis of NEC ≥ IIa, 141 (12.1%) exhibited at least one episode of LOS, 31 (2.7%) a VAP, and 44 (3.8%) a MODS. Those infants with complications were of younger gestational age ($p<0.001$), had lower birth weight ($p<0.001$), lower Apgar scores at 1/5/10 minutes ($p<0.001$), were more common SGA ($p=0.007$), had longer courses of mechanical ventilation and longer hospital stays and for longer time parenteral antibiotics (all $p<0.001$). Mortality rate was increased in infants having experienced complications (6.9 vs. 1.7%, $p<0.001$).
Conclusions. Over an 11-year period, the use of the probiotic LCR as part of an anti-infective regimen was safe and resulted in low rates of NEC, LOS, VAP, and MODS compared to the literature. Those infants with complications had higher mortality rates.

Key words: very preterm infant; probiotics; Lactobacillus casei rhamnosus; necrotizing enterocolitis; multiple organ dysfunction syndrome; neonatal intensive care; ventilator associated pneumonia; late-onset sepsis; antibiotic-associated diarrhea

INTRODUCTION

The development of intestinal microbiota in newborn infants, and its potential influence on the infants´ health has become an important issue. Moreover, intensive care in critically ill very preterm neonates potentially alters the intestinal microbiota and possibly enables the growth of pathogens. Hence, dysbiosis of the intestine, or imbalance of intestinal microbiota, increases the risk of diseases with inflammatory background such as late onset sepsis (LOS) and necrotizing enterocolitis (NEC) in neonates (1). Furthermore, these associated clinical conditions include antibiotic associated diarrhea (AAD), multiple organ dysfunction syndrome (MODS), and ventilator associated pneumonia (VAP), all of them resulting in prolonged hospital stays, increases in the cost of intensive care, and greater risks of mortality (1,2).
The risk of VAP generally increases as gestational age decreases (3-5) and has been reported to occur in up to 26% at a birth weight below 750 grams (6). Comparably, the risk of late-onset sepsis (LOS) increases by decreasing gestational age and varies geographically from 0.61% to 14.2% among hospitalized preterm infants (7). Rates of LOS up to 65% in infants with a birth weight below 500 grams have been reported (7).
Probiotics – as summarized in a recent systematic meta-analysis - showed significant benefits by lowering NEC rates and all-cause neonatal mortality (8). Probiotics increase the intestinal mucosal barrier to prevent the translocation of bacteria, exclude competitively potential pathogens, produce bacteriocins that kill pathogens, increase the amount of immunoglobulins by mucosal response, modulate host immune reactions to microbial products, and ameliorate enteral nutrition and gut maturation (9,10).
Early enteral trophic feeding as used in our neonatal intensive care unit (NICU) with human milk in all very low birth weight infants prevents atrophy of gastrointestinal mucosa, supports the establishment of healthy gut microflora, reduces the use of parenteral nutrition by facilitating full enteral feeding, and improves gut mucosal immunity (11-13).
Aim of the study was to analyze the prophylactic use of the probiotic Lactobacil-

lus casei rhamnosus (LCR) given as part of a multimodal anti-infective regimen in very preterm neonates in respect to complications and possible side effects. This regimen has been documented recently in critically ill term infants (14), and the study design used was a retrospective observational STROBE compliant single-center cohort study.

METHODS

All very preterm infants admitted to the NICU of the Division of Neonatology of the Medical University of Graz between January 1, 2005 and December 31, 2015 were retrospectively adherent to the STROBE statement evaluated for analysis.
Inclusion criteria were as follows: admission within the first 24 hours of life, stay at the NICU for at least 7 days, and gestational age of 23 (+0) to 32 (+6) weeks. Exclusion criteria were as follows: admission to the NICU beyond the first day of life, stay at the NICU for less than 7 days, admission to and stay at the surgical department, or death before day 7. Further, neonates were excluded when data were missing or unavailable regarding outcome parameters.
All very preterm infants received the probiotic Lactobacillus casei rhamnosus (LCR) - Antibiophilus© 0.75 g [contains ≥ 0.75 x 108 colony forming units - CFU] per day divided in 2 doses- throughout the stay at the NICU. The multimodal anti-infective treatment additionally included enteral gentamycin (Refobacin© 40 mg Amp. 15 mg/kg/day divided in 2 doses) and enteral nystatin (Mycostatin© 1mL= 100.000 IU/kg per day divided in 4 doses).
The standardized enteral feeding regimen was applied to all preterm infants at the NICU and remained unchanged through the study time period. Enteral feeding of breast milk (expressed breast milk or pooled human milk) was initiated at day 1. A volume of 1-2 up to 5 mL was initiated every 4 hours when infants were stable. Daily enteral feeding was increased by 2-5 mL per feed and did not exceed 10 mL per feed and day. Total fluid intake was 90-100 mL/kg/d at day 1 with daily increases up to 160 (180) mL/kg/day at day 7 to 10. Enteral feeding was interrupted if there were signs of intolerance defined as the presence of gastric residuals exceeding 25% of the volume offered within the previous 8 hours, abdominal distension, or blood in the stool. Total or partial parenteral nutrition was routinely maintained until 120 - 150 mL/kg/day by enteral feeds were reached. All neonates in the study received concomitant therapy including antibiotics as considered appropriate by the attending physician.
Data were collected from the local electronic database openMedocs® for all infants regarding gender, gestational age (weeks), birth weight (grams), small for gestational age (birth weight below 10. Percentile), umbilical artery pH, length of stay (days), Apgar scores at 1, 5, 10 minutes, respectively, days on ventilator support (invasive mechanical plus continuous positive airway pressure – CPAP – ventilation), spontaneous birth, cesarean section, breast milk feeding, formula feeding, combined human milk and formula feeding, duration of antibiotic treatment (days), and main diagnosis for treatment at the NICU. Infectious events were always tested by blood culture for LCR.
Outcome parameters were checked for throughout the medical records of the neonates using the electronic patient data monitoring system (PDMS, Sanitas, Austria) at the NICU and the electronic documentation system called openMedocs (LKH University Hospital Graz, Austria). The following complications were looked for: Late-onset – NEC (onset day 7 or later), MODS, VAP, and LOS (day 7 and later).
NEC was defined according to the modified Bell's criteria as stage IIa or more (15,16). Signs and symptoms included abdominal pain, bloody stools, ileus (subileus), and pneumatosis intestinalis.
MODS was defined as the presence of at least 2 of 6 defined criteria for multiple organ dysfunction (17,18) that was mainly adapted from Goldberg et al. (17) not using the scoring system published recently (18):

- Cardiovascular dysfunction (any of the following): Despite isotonic intravenous bolus ≥40 mL/kg; systolic blood pressure <5% for age or need for vasoactive drugs; capillary refill time >3 seconds; urine output <0.5 ml/kg/h
- Respiratory dysfunction (any of the following): PaCO2 >65 torr or 20 mm Hg over baseline. Proven need for >50% FiO2 to maintain saturation ≥92%
- Neurologic dysfunction (any of the following): Seizures, irritability, and lethargy, any different from baseline neurologic function
- Thrombocytopenia: Platelets <80 000/μL or decline of 50% from highest value over past 3 days in neonates with baseline low platelets (< 150000/μL)
- Renal dysfunction: Creatinine ≥2 times upper limit for age or 2-fold increase in baseline creatinine in neonates with baseline elevations in creatinine.
- Hepatic dysfunction (any of the following): Total bilirubin not applicable to newborn. ALT (alanine transaminase) 2 times upper limit of normal for age or 2-fold increase in baseline abnormal ALT

For the diagnosis of VAP, the patient was required to have received mechanical ventilation for at least 48 hours and to have developed new and persistent radiographic evidence of focal infiltrates lasting for a minimum of 48 hours after the initiation of mechanical ventilation. In addition, these patients had to receive antibiotics for treatment of VAP for at least 7 days (2).
LOS was defined as a nosocomial bacterial infection not present or incubating at the time of NICU admission and occurring at day 7 or later. We did not differentiate between culture proven and clinical sepsis (clinical symptoms + 2 or more abnormal values including white blood cell count, neutrophils, immature to total neutrophil ratio >0.2 and C-reactive protein >8mg/L) (19).
Ethical aspects: Due to the design of the study informed consent was not available, thus, patient data were pseudonymized for study purposes and only BR and KL had access to the original data. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethic committee of the Medical University of Graz (number 28-332 ex 15/16 in March 2016) and the study started in 2017.
Statistical analyses were performed using the t-test and Wilcoxon test for numerical data and the chi-square-test using Yates´ correction and Fisher's exact test as appropriate for categorical data. For all statistical tests, a level of significance of 0.05 was used. Descriptive statistics were done using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA, 2007), further analyses were done with SPSS version 17 (SPSS Inc., Chicago, IL, USA, 2008).

RESULTS

Between January 1, 2005 and December 31, 2015, a total of 1287 very preterm infants were born. One hundred eighteen infants had to be excluded (figure 1), thus, the study population comprised 1169 infants with a gestational age of 23+0 to 32+6 weeks.
Perinatal data are given in table 1. Mean length of stay was 53 days (range 7 to 431). Respiratory support was given in 970 infants (83%). Mean duration of respiratory support was 19.5 days (range 1 – 264). Di-

Table 1: Perinatal data of 1169 preterm infants with gestational age of 23 to 32 weeks from Graz, Austria, born between 2005 and 2015

Parameter	Data
Gestational age (weeks)	29.8 ± 2.4 (30.3; 23 – 32)
Birth weight (grams)	1330 ± 440 (355 – 3380)
Male gender	622 (53)
Small for gestational age	209 (18)
Cesarean section	984 (84)
Umbilical artery pH	7.29 ± 0.09 (7.31; 6.68 – 7.55)
Apgar score at 1 minute	6.9 ± 1.9 (8; 0-9)
Apgar score at 5 minutes	8.4 ± 1.3 (9; 0-10)
Apgar score at 10 minutes	8.9 ± 0.9 (9; 4-10)

Data are given as mean ± SD (median; range) or n (%)

Table 2: Outcome of 1169 preterm infants with gestational age of 23 to 32 weeks born between 2005 and 2015 regarding complications of neonatal intensive care

Gestational age (weeks)	Number of infants	Number of Infants per week of GA with complication(s)
	n (%)	n (%)
23	18 (1.5)	13 (72.2)
24	41 (3.5)	23 (56.1)
25	50 (4.3)	20 (40.0)
26	69 (5.9)	28 (40.6)
27	98 (8.4)	27 (27.6)
28	112 (9.6)	18 (16.1)
29	130 (11.1)	11 (8.5)
30	159 (13.6)	15 (9.4)
31	226 (19.3)	10 (4.4)
32	266 (22.8)	8 (3.0)
Sum	1169 (100)	173 (14.8)

GA = gestational age; n = number

Table 3: Comparison of perinatal parameters of very preterm infants with and without complications including LOS, VAP, MODS or NEC

	Infants with complications N=173	Infants without complications N=996	p-Wert
Gestational age (weeks)	27 (23 -32)	30 (23 - 32)	< 0.001
Birth weight (grams)	900 (355- 2200)	1400 (410 – 3380)	<0.001
Small for GA	44 (25)	165 (17)	0.007
Male gender	94 (54)	528 (53)	0.805
Caesarean section	23 (13)	162 (16)	0.367
Umbilical artery pH	7.30 (6.73 – 7.49)	7.31 (6.68 – 7.55)	0.140
Apgar score at 1 minute	6 (0-9)	8 (0-9)	<0.001
Apgar score at 5 minutes	8 (0-10)	9 (1-10)	<0.001
Apgar score at 10 minutes	9 (4-10)	9 (4-10)	<0.001
Human milk: : Formula both	46 (26): 41 (24): 86 (50)	358 (36): 184 (18): 454 (46)	0.041
Length of stay (days)	87 (8-431)	41 (7-168)	<0.001

agnosis of respiratory distress syndrome (RDS) was given in 813 infants (70%), 613 infants (52%) needed surfactant therapy. Thirty-three infants (2.8%) had diagnosis of pneumothorax, 30 had wet lung (2.6%), 16 atelectasis (1.4%), 14 lung hypoplasia (1.2%), and 10 (0.9%) emphysema. Twenty-one had diagnosis of asphyxia (1.8%), and 16 (1.4%) had diagnosis of ileus. Four hundred and four (35%) infants were fed human milk, 225 (19%) were fed formula and 540 (46%) were fed both. Antibiotics were given in 986 infants (84%) for mean 9.6 days (range 1 – 83).

Neonatal intensive care complications were evident in 173 infants (14.8%), with at least one per infant. One hundred and thirty-six infants (79%) had one, 32 (18%) had two, and five (3%) had three complications (total number of complications being 215). Infants with and without complications are depicted in table 2 (regarding gestational age distribution) and table 3 (regarding differences of perinatal parameters).

LOS was diagnosed in 141 infants (12.1%) with at least one episode. Their mean gestational age (GA) was 27 weeks; birth weight (BW) was 934 grams (range 355 – 2200). Male gender was predominant (54%) and 40 infants (29%) ware SGA. Pathogens included Staphylococcus epidermidis (24), Staphylococcus haemolyticus (7), Enterococci (5), Enterobacter cloacae (2) and Staphylococcus aureus (2). None was positive for LCR. Nine infants with LOS died, 3 of them directly following LOS.

VAP was diagnosed in 31 infants (2.7%). Mean GA was 26 weeks (range 23.7 – 28.7); BW was 773 grams (range 355 – 1400). Male gender was predominant (61%) and 9 infants (19%) were SGA. Mean duration of mechanical ventilation was 67 days (range 20 – 128). None of these infants died.

A MODS was diagnosed in 32 infants (2.7%); mean GA was 28.6 weeks (23.3 – 32.7), and BW was 1072 grams (370 – 1970). Female gender was predominant (61%), 15 infants (34%) were SGA. Nine infants (20.5%) died, eight following MODS (7/8 due to palliative care)

Eleven infants (0.9%) had diagnosis of NEC IIa or more (additional 4 infants had diagnosis of NEC I). Male gender was predominant (64%) and two infants (18%) were SGA. Mean GA was 27.8 weeks (25.8 – 31), BW 1065 grams (650 – 1496). Two infants (18%) died.

Comparison of infants with LOS, VAP, MODS, or NEC is depictured in table 4.

In total 29 infants (2.48%) of the study population died; 26 (90%) experienced palliative care due to poor prognosis.

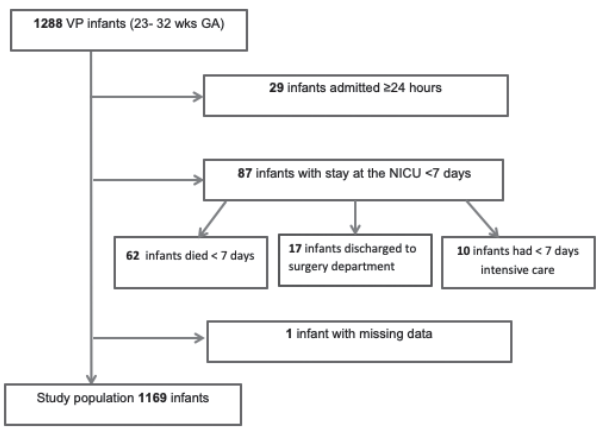
Days on ventilation	43 (1- 264)	5 (0- 134)	<0.001
Surfactant	143 (83)	470 (47)	<0.001
Antibiotics (days)	15 (0 -83)	4 (0-59)	<0.001
Infants died	12 (6.9)	17 (1.7)	<0.001

Data given as median (range) or n (%); GA = gestational age; LOS = late onset sepsis; MODS multi organ dysfunction syndrome; VAP = ventilator associated pneumonia; NEC = necrotizing enterocolitis

Table 4: Gestational age, birth weight, SGA, and gender of 173 out of 1169 very preterm infants with one or more complications (total 215) born between 2005 and 2015

	LOS (n=141)	**VAP (31)**	**MODS (32)**	**NEC (n=11)**
Gestational age (weeks)	27.1 (23.3 – 32.5)	25.4 (23.4 – 28.7)	28.7 (23.3 – 32.7)	27.4 (25.8 – 31)
Birth weight (grams)	890 (355-2200)	750 (355-1400)	1032 (370-1970)	1065 (650 – 1496)
Small for GA*	40 (28)	6 (19)	15 (34)	2 (18)
Male gender	76 (54)	19 (61)	17 (39)	7 (64)
Death	9 (6.4)	0 (0)	9 (20.5)	2 (18.2)

Data are given as n (%) or median (range); SGA small for gestational age (< 10th percentile); LOS = late onset sepsis; MODS multi organ dysfunction syndrome; VAP = ventilator associated pneumonia; NEC = necrotizing enterocolitis



GA = gestational age; NICU = neonatal intensive care unit; VP = very preterm; wks = weeks

Figure 1: Flow chart of study population recruitment (preterm infants 23 to 32 weeks of gestational age) from 2005 to 2015 at the NICU Graz, Austria

Mean cumulative dosage per infant of LCR was 40.5 x 109 CFU (range 5.3 – 324 x 109 CFU), of gentamycin 907 mg (44 – 7008 mg), and nystatin 60 ml (3 – 467 ml). Total amount of LCR given during the study period was 31.629 packages, 1,060.522 mg gentamycin, and 70.702 ml nystatin. This treatment was safe and no adverse events were observed. Neither an invasive LCR nor a fungal infection was detected during the study time period. There was neither an increase in detection rates of ESBL producing enterobacteria in stool surveillance cultures (done twice a week) nor an increase in gentamycin resistive bacteria as shown in the yearly published local hygiene report (data not shown).

DISCUSSION

Main findings of our study address the safety aspect of probiotic therapy with LCR in the vulnerable population of very low birth weight infants. Over the 11 years lasting study period no side effects were observed. In particular, no systemic LCR infection happened over the study period. Oral aminoglycosides have successfully been investigated in the prevention of NEC in some small past studies (20,21). Aminoglycosides have recently been reported to be effective in preterm animal models of NEC prevention given in contrast to parenteral route (22). Our use of enteral gentamycin is often a matter of discussion. But there was neither an increase in detection rates of ESBL producing enterobacteria in stool surveillance cultures (done twice a week), nor an increase in gentamycin resistive bacteria as shown in the yearly published local hygiene report (data not shown). The third part of our triple anti-infective regimen is nystatin for prophylaxis of fungal infection. During study period, we observed no proven invasive fungal infection. A further aspect of our anti-infective regimen is based on the preferred feeding of human milk. Our data showed higher rates of human milk feeding in the group without complications

Limitations of our study, which have to be mentioned, include the lower level of evidence of a retrospective cohort analysis and the missing control group. We decided against a control group (in order not to harm) due to the fact that a historical control might not be adequate and due to the belief in our concept. We aimed to publish the results of this large cohort of very low birth weight infants having received large amounts of LCR, gentamycin and nystatin. Strengths of our study include the low drop-out rate and the careful evaluation regarding predefined complications.

Regarding the efficacy of this treatment regimen, in general we found low complication rates regarding NEC, LOS, VAP, and MODS, and no AAD. In our cohort analysis the NEC rate of 0.9 % was extremely low compared to studies included in the most recent Cochrane review (8) of median 2.3 % with probiotics and 5.4 % without (9,23-42); the LOS rate of 12.1% (culture proven and clinical sepsis) was comparable to the median 14.8 % with probiotics and lower than the median 19.3 % without probiotics (23-28,32-35).

The beneficial use of probiotics has also been systematically evaluated in preterm infants in low- and medium-income countries (43). The review of randomized controlled trials revealed a significant reduction of NEC (number needed to treat – NNT = 25), LOS (NNT = 25) and all-cause mortality (NNT = 50); additionally, no significant adverse effects were reported (43). Another network meta-analysis investigated the efficacy and safety of probiotics,

probiotics plus fructo-oligosaccharides, pentoxifylline, arginine, and lactoferrin in NEC prevention (44). Probiotics and Arginine performed better compared with placebo, but only probiotics achieved a considerable decrease of mortality rates.

The main problem discussed in meta-analyses and systematic review is related to heterogeneity of organisms and dosing regimens studied (45); hence it is difficult to find out the most effective and safe single strain or the best combination of probiotics. Lactobacilli alone or in combination with Bifidobacteriae were found to be effective in most of the reviews (46) All included trials reported no systemic infection with supplemental probiotics (47) Thus, our study adds to the favorable profile of LCR reporting on 11 years of safe and well-tolerated use in very low birth weight infants. The question of safety has also been addressed by the European Society for Pediatric Gastroenterology (ESPGAN) and the ESPGAN considered the use of probiotics in general safe (47).

Our data open the discussion of whether feeding all preterm infants with probiotics might be justified. It seems to be better justified to use one probiotic strain compared to multiples due to a better control for adverse events and efficacy, as shown by our data.

We found a further low rate of VAP (2.7%). VAP has recently been reported to occur in 13.2% of a cohort of 605 low birth weight infants with a mean gestational age of 27 weeks (48); and an even two-fold increased rate of 28.3% has been observed in a cohort of 67 extremely preterm infants <28 weeks of gestational age (2). Risk factor for VAP was a preceding bloodstream infection and VAP was per se an independent predictor of mortality (2). As far as VAP is a factor associated with prolonged NICU length of stay, the reduction of VAP by probiotics as shown by our data seems to be highly cost-effective at a first glance. Multi-organ dysfunction is often described as a common terminal pathway in the pathophysiology of sepsis and may include respiratory, cardiovascular, central nervous system, adrenal, clotting, immune (leukopenia or neutropenia), and renal dysfunction (49). However, the timing of onset and frequency of these phenomena among hospitalized preterm infants with fatal disease are mainly unknown. The majority of fatal episodes have been reported to occur in extremely low birth weight infants during the first four weeks of life. Hence, our rate of MODS is hardly comparable due to the lack of reported rates.

Overall, our mortality rate was low with 2.48 % compared to the literature with median 4.4 % with, and 8.0 % without probiotics (9,23-34,37-39,50).

In conclusion, the use of LCR over 11 years in very low birth weight infants proved to be safe and complication rates including NEC, LOS, VAP and MODS were low compared to the literature

"WHAT IS ALREADY KNOWN"

Probiotics are known to lower the risk for necrotizing enterocolitis (NEC) and sepsis (LOS) and all-cause mortality by meta-analysis.

Lactobacilli and Bifidobacteriae might be most effective alone or in combination.

"WHAT THIS STUDY ADDS"

Lactobacillus casei rhamnosus (LCR) as part of a triple anti-infective regimen is effective in prevention of NEC and LOS and ventilator associated pneumonia.

LCR proved to be safe without any case of invasive infection as demonstrated by its use over 11 years in more than 1.100 VLBW infants.

Conflict of Interest

B. Resch received honoraria for oral lectures from Abbvie, Milupa and Germania.
C. Hofer has nothing to declare
B. Urlesberger received Honoraria from Chiesi, Milupa, Nestle and Dräger

Authorship and responsibilities

B.R. planned the study, wrote the manuscript and was responsible for editing the paper, C.H. did data collection and provided the tables and figures, B.U. was responsible for the discussion of the study findings and editing of the final version of the manuscript.

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