Received Date : 21-Dec-2016

Revised Date : 22-Feb-2017

Accepted Date : 07-Mar-2017

Article type : Regular Article

Probiotics and antimicrobial protein and peptide levels in preterm infants

Running title: Probiotics and APP in preterm infants

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Trial Registration: Australia New Zealand Clinical Trials Registry ACTRN 12609000374268

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ABSTRACT

Aim: To characterise the secreted and inducible antimicrobial protein and peptide (APP) levels in a prospective cohort of preterm infants (<30 weeks gestational age) with or without *Bifidobacterium breve* M16V supplementation during the first month of life.

Methods: We analysed serial biosamples of infants who did (n=13) or did not receive (n=62) *B. breve* (3×10^9 cfu/day). Peripheral blood was obtained on days 1, 14 and 28, and infant stool prior to commencement of probiotic supplementation and on day 21. Levels of APP (bactericidal/permeability inducing protein (BPI), beta defensins 1 and 2, lactoferrin, human cathelicidin, secretory phospholipase A2) in plasma and stool were determined. Further, we characterised induced APP levels in whole blood cultured with live *S. epidermidis* or with agonists of Toll-like receptors 2/6 and 4.

Results: Stool, plasma and stimulated blood APP levels changed significantly during the first month of life. Supplementation with *B. breve* did not affect basal or stimulated APP levels except for a transient increase in inducible BPI.

Conclusion: Supplementation with *B. breve* does not appear to act via modulation of systemic or enteric APP expression in preterm infants although small effects cannot be excluded. Further work with other probiotic preparations are warranted.

Key words: Probiotics, B. breve, preterm infant, antimicrobial proteins and peptides

Abbreviations:

Antimicrobial proteins and peptides (APP)

Birth weight (BW)

Gestational age (GA)

Human beta defensin (HbD)

Late-onset sepsis (LOS)

Lipopolysaccharide (LPS)

Necrotizing enterocolitis (NEC)

Key notes:

- The biological mechanisms underlying benefits of probiotics in preterm infants are incompletely defined.
- We therefore investigated if supplementation with the probiotic *Bifidobacterium breve* M16V modulates innate immune function.
- This study shows that systemic levels of key antimicrobial protein and peptides in very preterm infants are not altered by supplementation with *B. breve*.

INTRODUCTION

Preterm infants are particularly susceptible to late-onset sepsis (LOS) and necrotising enterocolitis (NEC), affecting ~30% and 3-10% of those born <28 weeks gestational age (GA), respectively (1). Relative immaturity of the neonatal immune system, especially regulation of inflammatory responses, is central to both LOS and NEC and the resulting systemic inflammation results in worse short- and long-term outcomes (2).

Probiotics are defined as dietary supplemenents containing live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Supplementation with probiotics is one of the most extensively studied interventions in neonatal medicine. Data from high quality clinical trials and large-scale routine use provide clear evidence of numerous benefits (3). Probiotic supplemententation results in reduced risk of NEC and all-cause mortality, as well as shorter duration of parenteral nutrition and hospital admission (4). Two recent meta-analyses demonstrate that probiotics reduce the risk of acquiring LOS in preterm infants (5, 6).

Colonisation with microorganisms begins during delivery and the assembly of the neonatal microbiome is easily perturbed by common early-life interventions, such as delivery by cesearean section or administration of peri- and/or postpartum antibiotics. Establishment of a healthy, site-specific microbiome is increasingly recognised as critical for health outcomes, including reducing susceptibility to infections, autoimmune and metabolic conditions (7).

A number of mechanisms have been suggested for the beneficial effects of probiotic supplementation, but the effects on the preterm infant immune system are poorly characterised. Antimicrobial proteins and peptides (APP) are key components of the innate immune system, which provides critical first line defense in newborn infants. These cationic host defence peptides are have broad-spectrum antimicrobial activity, including activity against neonatal pathogens, and act as important immunodulators (8). APP are expressed in all bodily fluids and are found in biologically active concentrations in amniotic fluid, breast milk, blood, vernix caseosa and other epithelial fluids, including the respiratory and gastrointestinal tracts. In the adult gastrointestinal tract, APP are constitutively expressed and upregulated in reponse to microbial colonisation, bacterial components and their metabolic products, including lipopolysaccharide and butyrate (9, 10). There are limited neonatal data suggesting that colonisation with commensal bacteria may alter intestinal APP expression (11). In addition to their direct role in infection control, APP likely are involved in regulating the local microbiome and immune homeostasis, a putative mechanism essential to the substantially lower risks of NEC and LOS afforded by probiotics (12, 13). It is unknown if probiotic supplementation directly alters local or systemic APP expression in preterm infants, contributes to reduced enteric colonisation with pathogens, improves mucosal integrity and reduced gut-associated sepsis (14-17).

There is a paucity of studies on APP expression in infants and the majority of studies have focussed on faecal levels of a limited number of APP (18-21). We have previously reported that APP cord blood levels correlate with gestational age (GA) and the relative deficiency at earlier GA may contribute to the susceptibility to invasive infections in extremely preterm infants and there are no data on potential effects of probiotic supplementation on immune responses in preterm infants (22, 23).

We aimed to characterise the APP levels of peripheral whole blood and stool during the first month of life in very preterm infant in releation to early-life supplementation with the probiotic strain *B. breve*. In addition to basal APP plasma levels, we also assessed the capacity of whole blood from preterms infant to release APP in response to the most common neonatal pathogen, live *S. epidermidis* and to prototypical Toll-like receptor agonists.

METHODS

Recruitment and sample collection

Eligible preterm infants were enrolled in two independent, but partly overlapping prospective studies and had common repeated biosamples collected at time points indicated below. The first study was a randomised, placebo-controlled clinical trial of probiotic supplementation with *B. breve* M16V (n=159; Trial Registration: Australia New Zealand Clinical Trials Registry ACTRN 12609000374268) (24). The second study was a prospective cohort study of innate immune system ontogeny in very preterm infants (n=129) (25). Both studies were approved by the institutional ethics committee at King Edward Memorial Hospital, Perth, Australia and written informed consent was obtained prior to study participation. Infants were recruited between July 2009 and May 2012. We excluded infants who developed LOS or NEC, as these are common confounders of innate immune responses and the intestinal microbiome (26). Therefore, 13 infants in study 1 and 62 infants (matched by gestational age, birth weight and gender) in study 2 had corresponding biosamples available and were included in the analysis. Table 1 describes the relevant clinical characteristics of the study cohort.

Probiotic supplementation and stool samples: Infants in this clinical trial received $3x10^9$ CFU/day of *B. breve* once any enteral feeds were commenced. Probiotic supplementation was continued until hospital discharge or 37 weeks GA, whichever came first, as described (24). Two stool samples were collected from each infant; before and 21 days after commencing probiotic supplementation. Samples were frozen after collection and stored at –80°C until analysis.

Peripheral blood was collected by venepuncture on days 1, 14, and 28 into lithium-heparin tubes (BD Biosciences). To obtain plasma, blood was centrifuged at 6,000*g* for 2 min, and aliquoted and stored at -80°C until analysis.

Whole blood stimulation assay

Live S. epidermidis preparation

A *S. epidermidis* isolate (wild-type strain 1457, kindly provided by Dr Michael Otto NIAID, Bethesday, MD, USA) was originally obtained from a patient with an infected central venous catheter. Stocks were grown to mid-log phase (OD_{600}: 0.7–0.8) in Heart Infusion Broth (Oxoid, Adelaide, Australia) and stored frozen in Tryptone Soya Broth (Pathwest Laboratories, Western Australia, Australia) containing 15% glycerol. Bacteria were streaked from the frozen stock onto blood agar plates and incubated overnight at 37°C. The following day three separate colonies were exctracted from the blood agar plate and resuspended in PBS. The bacteria in the suspension were counted using a Helber Counting Chamber and the concentration was adjusted to $2.5x10^9$ CFU/ml with PBS (Gibco, Life Technologies, VIC, Australia) and stored on ice until ready for use. Viability of working live bacterial stock were assessed by streaking a known number of bacteria on a blood agar plate, incubating overnight at 37°C and quantifying the growth the following day.

Whole blood stimulation model

Whole blood (25µL) was cultured in RPMI 1640 GlutaMAX (75µL), supplemented with 0.01M HEPES, 1mM sodium pyruvate, 5.5µM beta-mercaptoethanol (all from Gibco, Life Technologies, VIC, Australia), for 24h at 37°C and 5% CO_2 with either supplemented RPMI media, $1x10^6$ CFU/mL live *S. epidermidis*, 10 ng/mL lipopolysaccharide (LPS) from *E. coli* serotype R515 (Alexis Biochemicals, Lausen, Switzerland) or 100ng/mL of the lipopeptide FSL-1 (InvivoGen, CA, USA). Following the overnight incubation 100µL of RPMI 1640 supplemented, as above along with the addtion of 5% foetal bovine serum (SAFC Biosciences, KS, USA), was added and supernatants harvested and stored at -80°C.

Antimicrobial protein and peptide determination

Stool samples were thawed and fats/lipids removed by a combination of mechanical disruption and centrifugation immediately prior to APP assay. Stool supernatants were stored at 4°C until analysis by multiplex or ELISA.

Human beta defensin 1 (HbD1) and 2 (HbD2), Lactoferrin, LL-37, BPI and sPLA2 circulating levels in plasma and levels produced following stimulation with purified LPS, FSL-1 and live *S. epidermidis* were quantified using either an in-house multiplex bead-based assay or commerical kits.

HbD1, HbD2, LL37 and Lactoferrin levels were quantified using in-house multiplex bead-based assays. Primary antibodies and biotinylated secondary antibodies against, HbD1, HbD2 and Lactoferrin were purchased from Abcam (Melbourne, Vic, Australia) and LL-37 from Innovagen (Lund, Sweden). Data were acquired electronically in real-time and analysed using BioPlex Manager 5.0 software. Data in pg/mL were generated from a seven-point, four- and five-parameter logistic standard curves. All values below the lowest standard were assigned an arbitrary cut-off value of half the lowest standard for analysis.

BPI and sPLA2 levels were quantified using commerical ELISA kits according to the manufacturers' instructions (Hycult Biotech, Plymouth Meeting, PA, USA, and Cayman Chemical, Ann Arbor, MI, USA, respectively). Data in pg/mL were generated from a seven-point four-parameter logistic standard curve.

Statistical analysis

Categorical data were summarised using frequency distributions. Basic contonuous clinical characteristics were summarised using means and standard deviations and medians and ranges. Antimicrobial proteins and peptides were summarised using non-parametric data summaries, medians and interquartile ranges (25^{th} -75^{th} percentiles), as appropriate for the non-normally distributed data. Univariate comparisons between the 'probiotics' and 'no probiotics' groups were conduted using Mann-Whitney tests for continuous outcomes and Chi-square tests for the categorical outcomes. All statistical tests were two-sided and p-values<0.05 were considered statistically significant. Data analysis was conducted using IMB SPSS statistical software (version 20, Armonk, NY, IBM Corp).

RESULTS

Concentrations of antimicrobial proteins and peptides in infant feces

Faecal APP levels were assessed in stool samples collected prior to starting probiotic supplementation and again on d21. We observed comparable changes in APP concentrations between infants receiving probiotic supplementation and those who did not (Figure 1). sPLA2 levels remained stable whereas HDB1 and lactoferrin levels increased and HBD2 concentrations declined between d1 to d21. LL-37 was detected at low low levels in all but five infants (3 with probiotic supplemention), whereas BPI was not detectable at all in stool samples. Further, we found no correlation between faecal load of probiotic bacteria and the levels of any of the measured APP (data not shown).

Circulating levels of antimicrobial proteins and peptides in infant peripheral blood

Circulating APP levels were assessed in plasma samples collected prior to starting probiotic supplementation (d1) and then again on d14 and d21. The changes in plasma APP levels were comparable between supplemented and non-supplemented infants (Figure 2). HBD1, sPLA2 and BPI levels remained constant over the 28 days, whereas lactoferrin and LL-37 levels declined from d1. HBD2 levels were at least a log-fold higher than all other APP at d1 and increased again by d14.

Capacity of infant peripheral blood to release antimicrobial proteins and peptides upon stimulation

Fresh peripheral preterm infant whole blood was cultured with stimulants resembling key neonatal pathogens and APP levels determined in culture supernatants. We found significantly increased levels of BPI (days 14 and 28, $p<0.05$ comparing stimulated to unstimulated samples), LL37 and Lactoferrin (days 1, 14 and 28, $p<0.05$) in response to stimulation with *S. epidermidis*, LPS and FSL (Figure 3a). In contrast, HBD1 and sPLA2 levels were not inducible by any of the stimuli (Figure 3b). HBD2 responses were highly variable among infants and between stimuli. HBD2 was not induced in response to FSL in the majority infants. but it was induced in some infants in response to LPS and LSE with peak responses. Importantly, we did not find any major significant differences in inducible APP levels between the probiotic and not probiotic supplemented infants. There was a trend for increased BPI in probiotic-supplemented infants but this was only apparent at d14 and only with *S. epidermidis* stimulation ($p=0.049$).

DISCUSSION

This is the first study to investigate whether early-life supplementation with a specific probiotic (*B. breve*) modifies the expression of key APP in human preterm infant stool, peripheral blood plasma, and the capacity of peripheral whole blood immune cells to release APP in response to the neonatal pathogen, *S. epidermidis,* and prototypical TLR agonists

(14, 15, 27). We interrogated unique serial biosamples from very preterm infants to characterise the potential effects of probiotic supplementation on the expression of APP during the first month of life. Despite the widespread routine adminstration of probiotics in preterm infants, this key facet of the innate immune response has not been previously investigated in this population.

Probiotic supplementation resulted in high intestinal colonisation rates, comparable to other probiotic trials (24). There are limited data on faecal levels of APP; human beta-defensin 2 was detected in feces of both term and preterm infants during the first weeks of life, however, the potential relationship with specific intestinal bacteria was not investigated (19, 21). Our study showed that increasing colonisation with *B. breve* in very preterm infants during the first month of life does not modulate basal APP levels in peripheral blood or stool. Furthermore, there was no correlation between the faecal load of *B. breve* and any APP levels (data not shown). Effects of probiotics may be strain-specific; supplementation of human adults with the probiotic strain *Escherichia coli* Nissle 1917 resulted in elevated faecal human beta-defensin 2 levels and was suggested as an important mechanism of probiotic supplementation, whereas administration of *Bifidobacterium lactis* did not alter faecal beta-defensin 2 content (15, 28).

Limited data from our and other groups demonstrate reduced levels of APP in cord blood of preterm infants (22, 23, 29). However, this has not been studied in peripheral blood after birth nor in relation to bacterial colonisation. In addition to basal plasma levels, we also interrogated the capacity of peripheral blood immune cells to secrete APP following stimulation with pathogens or TLR agonists. Our data demonstrate similar levels of the inducible APP Lactoferrin and LL37 in infants with and without probiotic supplementation. While we observed only temporarily higher inducible BPI levels in the probiotic group, it occurred at the time of highest incidence of LOS and in light of the recent publications demonstrating reduced risk of LOS afforded by probiotic supplementation, this finding may be of clinical relevance. Further, there was some evidence of maturation of APP responses

induced by S. epidermidis. The magnitude and rate of this might vary among individual infants which could affect their risk of sepsis, but this needs a detailed study with more time points, especially in the first 2 weeks when risk is highest.

Our study has number of strenghts, including the serial assessments of APP levels in biologically relevant body fluids during the first month of life. This is the most comprehensive characterisation to date of preterm infant systemic and intestinal expression of key human APP with potential relevance for mucosal immunity, intestinal health and protection against invasive infection. Our study also has some unavoidable limitations. Similar to published probiotic trials, there was significant horizontal transmission of the probiotic bacteria within the NICU (24). After three weeks of probiotic supplementation, the rate of colonisation with *B. breve* was 91% in the treatment group versus 38% in the placebo group; this may obscure small differences in APP expression afforded by *B. breve* colonisation. However, we did include additional GA-matched, contemporaneous infants who did not receive probiotic supplementation to increase statistical power. Further, we interrogated a whole blood sepsis model to reveal any inducible differences in the capacity to release APP upon physiological challenges that may not be apparent when assessing basal APP plasma levels alone. It is conceivable that the intestinal expression of APP at mucosal level be different from APP concentrations excreted in stool, but invasive procedures to obtain such samples in healthy preterm infants are not justified. The dynamic exchange of commensal bacteria between a breastfeeding mother and her infant are well described (30), and vertical transmission of may probiotic bacteria occur in both directions, however, we did not assess breast milk for *B. breve* colonisation.

It is unknown whether they are specific to *B. breve* and if supplementation with different probiotic strain may yield different results. A number of clinical trials of probiotic supplementation are currently being conducted to define the optimal probiotic strain(s), dose and length of adminstration, providing the opportunity to characterise potentially relevant biological correlates of superior strategies and maximise the benefits of this intervention. The

impact of intestinal microbiome on immune responses warrants further systematic characterisation. Ongoing clinical studies of different probiotic products, especially single versus multi-strain combinations, may be informative for future prophylactic interventions.

Conflicts of interest: The authors have no conflicts of interest relevant to this article to disclose.

Acknowledgments: This project was supported by the National Health and Medical Research Council of Australia (#572548), the Department of Health, Western Australia and Telethon Channel 7 Trust, Western Australia. David Burgner is supported by National Health and Medical Research Council of Australia Fellowship (#1064629) and is an Honorary Future Leader Fellow of the National Heart Foundation of Australia.

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Table 1. Basic clinical characteristics of study cohort.

	B. breve n=13	Control n=62	P value
Gestational age, weeks			
Mean ± SD**	27.8 ± 1.7	27.6 ± 1.6	ns
Birthweight, grams**			
Mean ± SD	1004 ± 239	1043 ± 280	ns
Chorioamnionitis*			
Chorioamnionitis, n (%)	4 (30.8)	26 (41.9)	
No chorioamnionitis, n (%)	5 (38.5)	27 (43.5)	
Chorioamnionitis not reveiwed, n (%)	4 (30.8)	9 (14.5)	ns
Male*, n (%)	8 (61.5)	36 (58.1)	ns
Cesearean section*, n (%)	12 (92.3)	35 (56.5)	0.015
Deaths prior to 28 days*, n (%)	0 (0)	2 (3.2)	ns
Antenatal steroids*, n (%)	12 (92.3)	61 (98.4)	ns
Gestational age and birth weight*			
Small, n (%)	2 (15.4)	4 (6.5)	ns
Appropriate, n (%)	9 (69.2)	53 (85.5)	ns
Large, n (%)	2 (15.4)	5 (8.1)	ns
Prolonged Rupture of Membranes >24 hours, n (%)	3 (23.1)	25 (40.3)	ns

***Chi-square, ** upaired t-test**

Figure 1

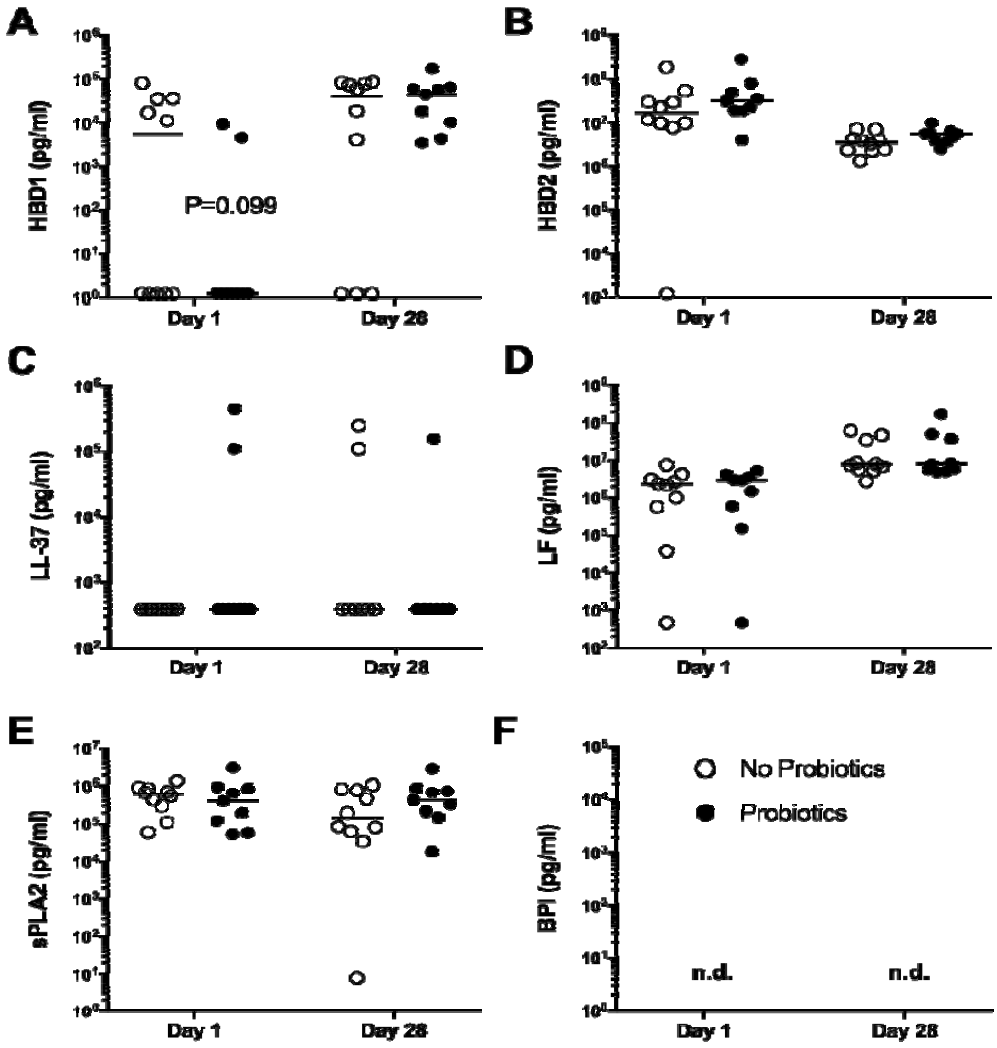

Figure 2

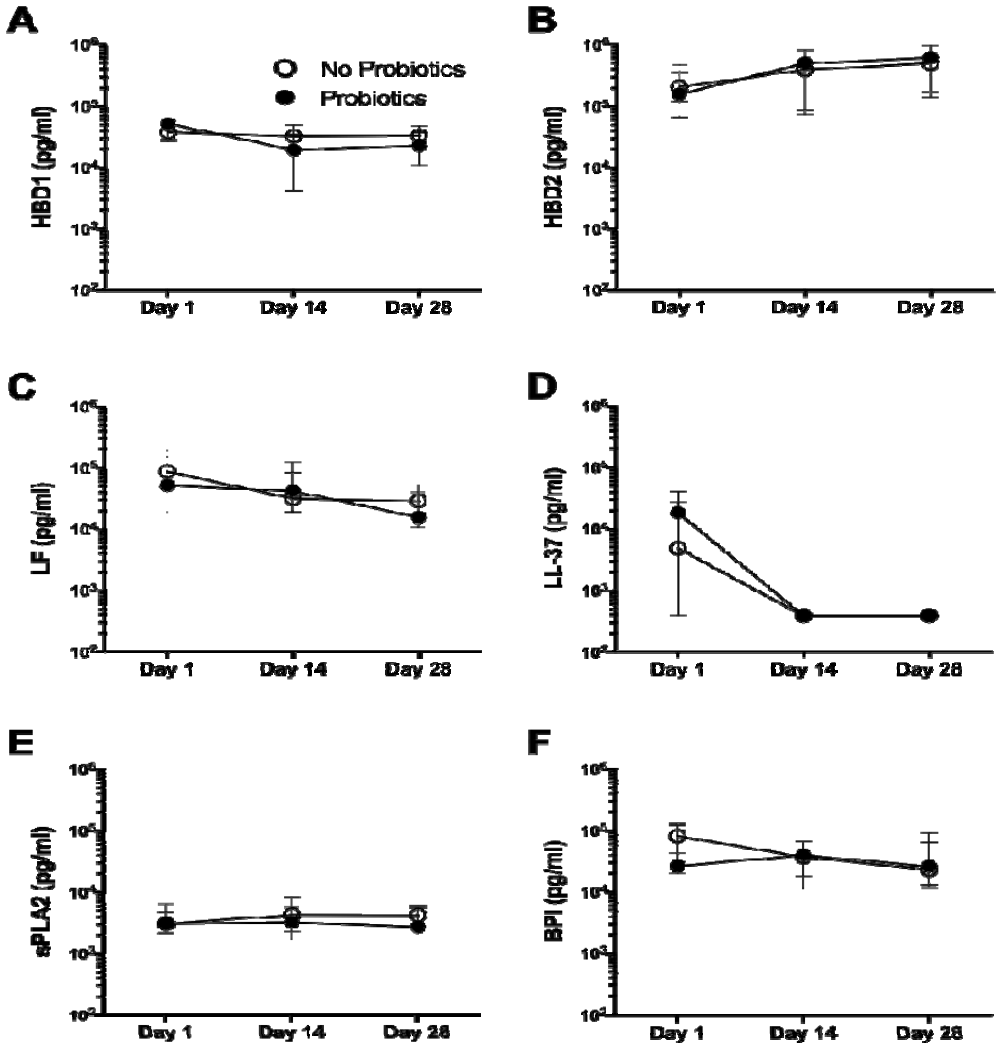

Figure 3

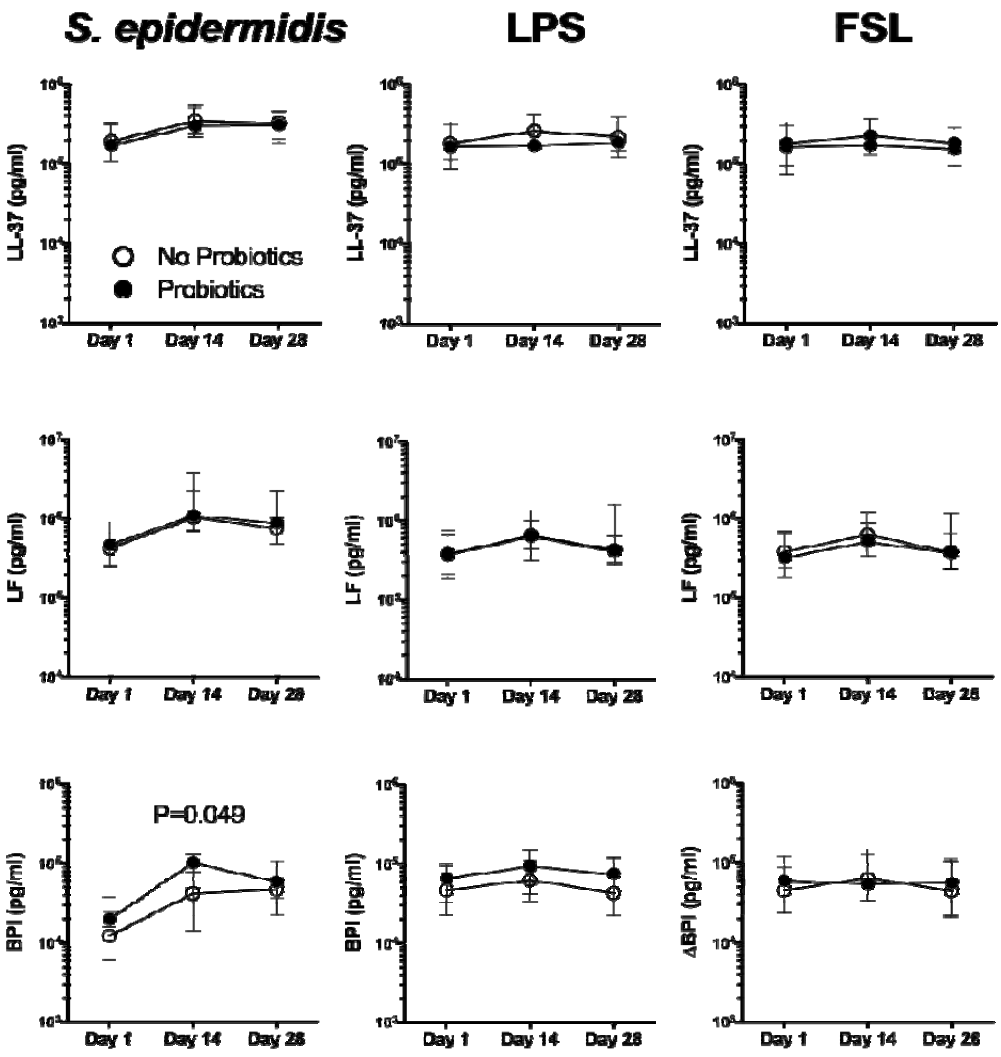

Figure Legends

Figure 1. APP levels (pg/ml) in stool of very preterm infants who did or did not receive probiotics. Data show levels of A) HBD1, B) HBD2, C) LL-37, D) lactoferrin (LF), E) sPLA2, and F) BPI in available stool samples from individual infants with (closed circles, n=9) and without (open circles, n=10) probiotic supplementation. Median is shown as bar.

Figure 2. APP levels (pg/ml) in peripheral blood plasma of very preterm infants with or without probiotic supplementation. Data show median plasma levels (with interquartile range) for infants with (closed circles, n=10-11) and without (open circles, n=31-34) probiotic supplementation.

Figure 3. Inducible levels of antimicrobial proteins and peptides. Culture supernatants of preterm infant peripheral blood stimulated *in vitro* with live *S. epidermidis* (left column), LPS (centrre column) or FSL1 (right column). Data show median plasma levels (with interquartile range; pg/ml) for infants with (closed circles, n=10-11) and without (open circles, n=33-37) probiotic supplementation.