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Original article

Probiotics and the immunological response to infant vaccinations; a double-blind randomized controlled trial

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ABSTRACT

Objectives: To examine the effect of a combination of probiotics on the antibody response to pneumococcal and pertussis vaccination in healthy Danish children, aged 8–14 months, at the time of starting day care. Moreover, the cytokine response to lipopolysaccharide of whole blood was assessed.
Methods: A total of 290 children were randomly allocated to receive a combination of *Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus rhamnosus* GG daily for a 6-month intervention period, and blood samples were drawn at the start and end of the study. Specific antibody response towards *Streptococcus pneumoniae* serotypes and *Bordetella pertussis* toxin, as well as endotoxin-induced interleukin-6 (IL-6) and interferon-γ (IFN-γ) production in blood were analysed by Luminex and ELISA.
Results: There was no significant difference between the average individual changes from baseline to end of study in antibody concentrations for *S. pneumoniae* for both the probiotics (340.4% ± 11.2%) and the placebo group (382.9% ± 10.4%) (p 0.525), nor for *B. pertussis* toxin in the two groups (probiotics 190.1% ± 12.6% versus placebo 238.8% ± 1.1%, p 0.340). The average individual change in IL-6 concentration was significantly lower in the probiotics versus the placebo group (2.9% ± 10.3% versus 33.7% ± 9.0%, p 0.024), whereas there was no difference in IFN-γ concentration (0.0% ± 0.2% versus −0.2% ± 0.1%, p 0.279).
Conclusions: The probiotic intervention did not affect the antibody response against *S. pneumoniae* and *B. pertussis* toxin in healthy Danish children. **C. Adler Sørensen, Clin Microbiol Infect 2019;25:511.e1–511.e7**

Introduction

Around 90% of all Danish children aged 1–2 years attend day care [1]. These children have an increased risk of respiratory and gastrointestinal infections compared with children in home-care [2,3]. Especially the first 6 months of enrolment into day care have been associated with a high incidence of hospitalizations for acute respiratory infections [2]. Therefore, strategies to reduce and prevent infections at this stage are of great importance.

The 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in the Danish Childhood Immunization Programme in 2010 [4]. The PCV13 has led to a 71% reduction in invasive pneumococcal disease in Danish children <2 years old (considered as the vaccine effectiveness) [4]. Vaccination against *Bordetella pertussis* is an acellular vaccine of pertussis toxoid [5] and has a reported efficacy of >70% [6]. It was suggested that the intestinal microbiota may improve vaccine responses as dysbiosis resulted in systemic inflammation and lower vaccine responses [7]. Likewise, probiotics may facilitate stimulation of the host immune system. Studies and meta-analyses have addressed the effects of probiotics on vaccine

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responses in both adults and children, and the results indicate a variation in efficacy of stimulating the immune system depending on the use of probiotic strains, population and vaccination type [8–10]. In the studies reported so far about half report a beneficial effect. As the studies, apart from the vaccine regimen, vary with respect to type and number of strains, dose, intervention period, age and group size, no clear conclusions of the effect of probiotics on antibody response to vaccination is available. Similarly, there are no indications of which children may benefit immunologically from a probiotic intervention.

The ProbiComp study investigated the effect of a daily administration of a probiotic mixture, for 6 months to children from the enrolment in day care at the age of 8–14 months, on absence due to infections [11]. As a secondary outcome, we here report the effects of the probiotics on immunological responses to vaccine exposure to PCV13 and to pertussis toxoid. In addition, as an alternative measure of the probiotic effect on the humoral response, we assessed the capability of cytokine production after *ex vivo* lipopolysaccharide (LPS) -stimulated whole blood.

Methods

Design and study population

This study was part of the ProbiComp study, a double-blind, placebo-controlled parallel study described in detail by Laursen et al. [11]. In short, 290 Danish children (8–14 months) were randomly assigned to a combination of probiotics *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium animalis* ssp. *lactis* (BB-12) at a dose of 2×10^9 CFUs or placebo (maltodextrin) each for a 6-month period. LGG and BB-12 are registered trademarks of Chr. Hansen A/S. Recruitment was carried out for two seasons, from mid-August to mid-December in 2014 and 2015. The children were recruited from the National Danish Civil Registry by the principal investigators of the ProbiComp study [11]. The probiotic and placebo powders were provided in identical sachets and did not differ in smell, taste, or colour. Study personnel and parents were unaware of the nature of the product. The children were assigned to receive either probiotics or a placebo by using block randomization with a block size of eight and unblinding was performed only after completion of data collection and statistical analyses. Children underwent clinical examinations and blood samples were taken at two visits, before the start of day care and entering intervention, and after 6 months, at the end of the intervention. The parents were interviewed upon inclusion about household characteristics and infant history of illness since birth.

Vaccination data

In Denmark, pneumococcal conjugated vaccine (PCV13) and vaccination against *B. pertussis* toxin (PT), are both administered at 3 months, 5 months and 12 months of age. At baseline, children were at the ages 8–13 months and by end of the study their ages were 14–19 months. Vaccination data were also obtained for each participating child to assess the time between last given vaccination and blood sampling time. Vaccination data were obtained from the Danish Vaccination Register. Vaccination coverage of participating children are presented in Table 1.

Table 1
Characteristics of study participants with blood samples from visits 1 and 2 ($n = 213$)

Parameter	Probiotics group ($n = 104$)	Placebo group ($n = 109$)	p value
Female, *n* (%)	47 (45.2)	53 (48.6)	0.616[a]
Siblings, *n* (%)	56 (51.4)	53 (48.6)	0.446[a]
Breastfeeding prevalence:			
Visit 1, *n* (%)	51 (49)	48 (44)	0.464[a]
Visit 2, *n* (%)	11 (10.6)	8 (7.3)	0.407[a]
Indoor pets, *n* (%)	23 (22.1)	23 (21.1)	0.857[a]
Daily smoking indoors, *n* (%)	1 (0.2)	0 (0)	0.305[a]
Health status since birth:			
Doctor-diagnosed asthma or allergic rhinitis, *n* (%)	0 (0)	0 (0)	1.000
Doctor-diagnosed atopic dermatitis, *n* (%)	10/20 (50)	8/21 (38.1)	0.443[a]
Age:			
Visit 1 (median months, IQR)	10 (9.4–10.4)	10.1 (9.6–10.5)	0.347[b]
Start day care (median months, IQR)	10.3 (9.9–11.2)	10.4 (10–11.2)	0.428[b]
Visit 2 (median months, IQR)	16.1 (15.6–16.5)	16.0 (15.6–16.6)	0.527[b]
Vaccination:			
PCV13 coverage ≤1 at visit 1, *n* (%)	6 (5.8)	9 (8.3)	0.490[a]
PCV13 coverage ≥2 at visit 1, *n* (%)	97 (93.3)	100 (91.7)	0.490[a]
PCV13 coverage ≤2 at visit 2, *n* (%)	16 (15.4)	12 (11)	0.345[a]
PCV13 coverage ≥3 at visit 2, *n* (%)	88 (87.6)	97 (89)	0.345[a]
No PCV13 vaccinations	1 (1)	0 (0)	0.305[a]
PT coverage ≤1 at visit 1, *n* (%)	6 (5.8)	7 (6.4)	0.842[a]
PT coverage ≥2 at visit 1, *n* (%)	98 (94.2)	102 (93.6)	0.088[a]
PT coverage ≤2 at visit 2, *n* (%)	15 (14.4)	12 (11)	0.954[a]
PT coverage ≥3 at visit 2, *n* (%)	88 (84.6)	97 (89)	0.438[a]
No PT vaccinations	1 (1)	0 (0)	0.303[a]
No info visit 1 or visit 2	2 (1.9)	0(0)	0.146[a]
PCV13 vaccine before visit 1 (median days, IQR)	141 (123.2–159.2)	141 (126.8–160)	0.858[b]
PCV13 vaccine before visit 2 (median days, IQR)	105 (89–137.2)	104 (80–126)	0.093[b]
PT vaccine before visit 1 (median days, IQR)	141 (125–160)	141.9 (126.8–229)	0.509[b]
PT vaccine before visit 2 (median days, IQR)	105 (89–137)	108.5 (80–126)	0.073[b]
Compliance:			
Second visit (median % of consumed probiotics, IQR)	97 (93–99)	97 (94–99)	0.987[b]

IQR, interquartile range; PCV, pneumococcal conjugated vaccine; PT, pertussis toxin; Visit 1, baseline at inclusion; Visit 2, end-point at end of study.
[a] Pearson's chi-squared test.
[b] Welch two sample *t*-test.

Blood sampling and sample preparation

Peripheral whole blood was drawn at baseline and end of study, and aliquots (approx. 0.5 mL) were subsequently sampled according to the type of analysis. Serum was collected and stored at −80°C until examination by Luminex and ELISA. For LPS stimulation of whole blood, freshly drawn blood was used within 30 min.

Antibody measurements of S. pneumoniae serotypes and B. pertussis toxin

Antibody measurements against specific anti-pneumococcal capsular IgG (IgG-PN) were performed for 12 *S. pneumoniae* serotypes: 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F, using a Luminex-based assay previously described by Kantsø et al. [12]. The geometric mean of the antibody measurements against the 12 serotypes was used for analysis and all 'out of range' high values were set to 50 mg/L for all calculations.

IgG antibodies to *B. pertussis* toxin (IgG-PT) were measured by an ELISA previously described by Dalby et al. [13,14]. Results were expressed as IU/mL according to the WHO International Standard Pertussis Antiserum National Institute for Biological Standards and Control code 06/140.

Interleukin-6 and interferon-γ production in LPS-stimulated whole blood

Freshly drawn whole blood was stimulated essentially as previously described [15]. In brief, duplicates of 50 μL EDTA-treated whole-blood were diluted in 450 μL RPMI-1640 medium, stimulated with 1 mg/L LPS from *Escherichia coli* O26:B6 (Sigma Aldrich, St Louis, MO, USA), and incubated at 37°C and 5% CO_2 for 24 h. Interleukin-6 (IL-6) and interferon-γ (IFN-γ) concentration in the supernatants were measured by sandwich-ELISA using antibody pairs (R&D Systems, Minneapolis, MN, USA). The detection limits for the IL-6 and IFN-γ assays were 147 ng/L and 15 ng/L, respectively, and intra- and inter-assay coefficient of variation were 14% and 8% for IL-6 and 18% and 4% for IFN-γ, respectively.

Ethical considerations

The protocol for the study was approved by the Committees on Biomedical Research Ethics for the Capital Region of Denmark (H-4-2014-032). Furthermore, the study was registered at clinicaltrials.gov (identifier NCT02180581 posted 2 July 2014). This study required informed consent from parents and legal guardians of the children. Participation in the study was voluntary and parents could withdraw their consent at any time [11]. Vaccination data were obtained at the Danish Vaccination Register (record number 2015-57-0102).

Statistics

Baseline characteristics are shown as the mean (SD) or median (interquartile range (IQR)) for continuous variables and *n* (%) for categorical variables, and difference between the probiotics and placebo groups was evaluated by Pearson's chi-squared test or Welch two-sample *t*-test. The effect of probiotics on the change from baseline to end of study was evaluated by using mixed linear models with simultaneous tests for general linear hypothesis. Changes from baseline to end of study and differences between treatment groups at baseline or end of study were tested by selected pairwise comparisons. Due to the logarithmic nature of antibody titre data, the % change from baseline to end of study and % difference between treatment groups were calculated from the estimate given by R using the formula (exp(Estimate) − 1)*100, and the SD was similarly calculated from the standard error given by R using the formula (exp(standard error) − 1)*100. Both % change and % difference and their corresponding SD values for cytokine responses were directly provided by R. Only children with blood samples available for both visits were included in the analyses. All statistical analyses were performed in the software R (version 3.4.3; R Core Team, 2017. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria), using the LME4 package.

Results

Study population

In total, 260 of the 290 children completed the study (Fig. 1, see ref. [11] for more details). It was possible to collect blood from 252 children at intervention start and 231 children at end of study, and 213 children delivered blood samples at both visits. The aliquot volume of collected blood was, however, not always sufficient, limiting the analyses to be made. The numbers of analyses are shown in Fig. 1.

The probiotic and placebo groups had similar distributions regarding sociodemographic factors, duration of breastfeeding, health status since birth, time between sampling and vaccinations, and study compliance (Table 1).

Antibody response towards S. pneumoniae and B. pertussis vaccination

We assessed the geometric mean of the antibody concentrations against 12 *S. pneumoniae* serotypes for a total of 160 children and measured human IgG antibodies against PT in serum samples from 152 children.

No significant difference was observed in the *S. pneumoniae* antibody response measured between the group of children given probiotics and placebo, neither at baseline nor end of study (Table 2). The antibody concentrations measured at end of study (probiotics 5.18 mg/L, IQR 2.15–9.26 and placebo 5.62 mg/L, IQR 2.35–11.91) were significantly higher than baseline concentrations (probiotics 1.11 mg/L, IQR 0.58–1.68 and placebo 1.02 mg/L, IQR 0.72–1.81, $p \leq 0.001$) (Table 2). However, there was no difference in the change from baseline to end of study in antibody response between the treatment groups (p 0.525) (Table 2, see Supplementary material, Fig. S1). Also, the time (measured in days) since last vaccination at both baseline and end of study significantly affected the concentrations (at baseline a decrease of 0.95%/day; at end of study a decrease of 0.97%/day, $p \leq 0.001$) (see Supplementary material, Fig. S2).

Likewise, there was no significant difference in the PT antibody response measured between the group of children given probiotics and placebo, neither at baseline nor end of study (Table 2). The concentrations of PT antibodies were significantly different from baseline to end of study. In the probiotic group, the antibody concentrations measured at end of study (57.29 IU/mL, IQR 35.30–101.67) were significantly higher than baseline concentrations (19.46 IU/mL, IQR 11.09–33.53, $p \leq 0.001$) (Table 2). In the placebo group, the antibody concentrations measured at end of study (57.87 IU/mL, IQR 32.27–101.99) were significantly higher than baseline concentrations (17.64 IU/mL, IQR 9.55–30.64, $p \leq 0.001$) (Table 2). There was no difference in the change from baseline to end of study in antibody response between the treatment groups (p 0.340) (Table 2, see Supplementary material,

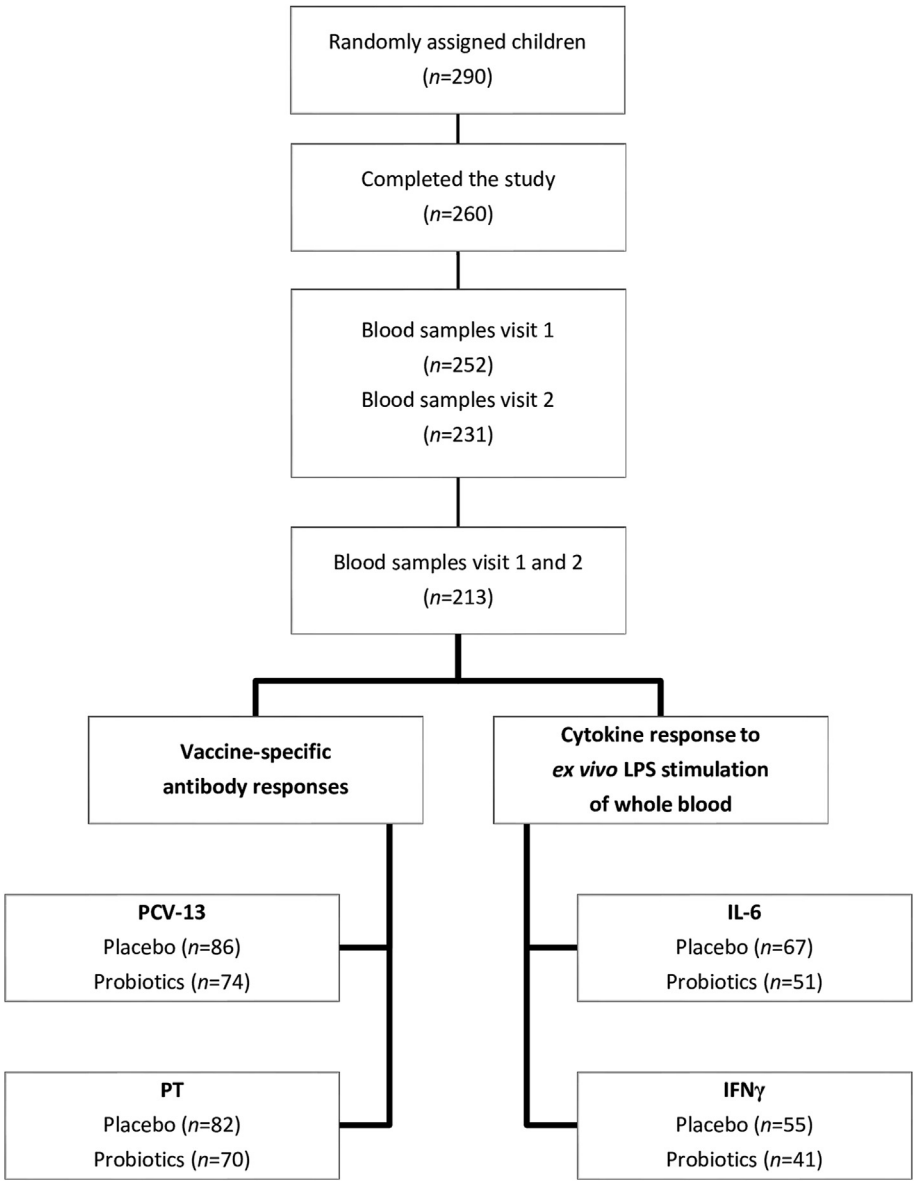

Fig. 1. Flowchart of participants and number of blood samples analysed.

Table 2
Antibody response towards vaccination

Streptococcus pneumoniae (IgG-PN_{GM} mg/L) ($n = 160$)					*Bordetella pertussis* toxin (IgG-PT IU/mL) ($n = 152$)				
	Baseline median (IQR)	End of study median (IQR)	Change %	p value		Baseline median (IQR)	End of study median (IQR)	Change %	p value
Probiotic group ($n = 74$)	1.11 (0.58–1.68)	5.18 (2.15–9.26)	340.4	≤0.001[a]	Probiotic group ($n = 70$)	19.46 (11.09–33.53)	57.29 (35.30–101.67)	190.1	≤0.001[a]
Placebo group ($n = 86$)	1.02 (0.72–1.81)	5.62 (2.35–11.91)	382.9	≤0.001[a]	Placebo group ($n = 82$)	17.64 (9.55–30.64)	57.87 (32.27–101.99)	238.8	≤0.001[a]
Difference %	4.6	−14.7			Difference %	13.4	−1.2		
p value	0.988[a]	0.749[a]		0.525[b]	p value	0.727[a]	1.000[a]		0.340[b]

IQR, interquartile range.
[a] Mixed linear model with selected pairwise comparisons.
[b] Mixed linear model with simultaneous tests for general linear hypothesis.

Fig. S1). However, the PT antibody concentrations measured at both baseline and end of study depended on the time (measured in days) since last vaccination (at baseline decrease of 0.75%/day and at end of study 0.63%/day, $p \leq 0.001$ (see Supplementary material, Fig. S2).

Whole blood LPS-stimulation-induced IL-6 and IFN-γ responses

Concentrations of IL-6 changed from baseline (6139 ng/mL, IQR 2840–9625) to end of study (9798 ng/mL, IQR 5547–12 322) in

Table 3
Lipopolysaccharide-induced cytokine responses in whole blood

Interleukin-6 (ng/mL) ($n = 118$)					Interferon-γ (ng/mL) ($n = 96$)				
	Baseline median (IQR)	End of study median (IQR)	Change %	p value		Baseline median (IQR)	End of study median (IQR)	Change %	p value
Probiotic group ($n = 51$)	8234 (3078–12055)	8278 (3276–9625)	2.9	0.993[a]	Probiotic group ($n = 41$)	41.88 (6.00–95.00)	56.00 (15.67–87.33)	0.0	1.000[a]
Placebo group ($n = 67$)	6139 (2840–9625)	9798 (5547–12322)	33.7	≤0.001[a]	Placebo group ($n = 55$)	42.00 (6.05–106.17)	55.33 (19.33–80.67)	−0.2	0.383[a]
Difference %	−12.2	18.7			Difference %	−0.2	0.1		
p value	0.616[a]	0.235[a]		0.0238[b]	p value	0.728[a]	0.979[a]		0.279[b]

IQR, interquartile range.
[a] Mixed linear model with selected pairwise comparisons.
[b] Mixed linear model with simultaneous tests for general linear hypothesis.

LPS-stimulated whole blood in the placebo group (33.7% ± 9.0%, p ≤ 0.001) (Table 3). No change from baseline (8234 ng/mL, IQR 3078–12 055) to end of study (8278 ng/mL, IQR 3276–9625) was observed in the probiotics group (2.85% ± 10.27%, p 0.993) (Table 3). This resulted in an overall significant difference in the change in LPS-stimulated IL-6 concentration from baseline to end of study between treatment groups (p 0.0238) In contrast, there was no difference in the change of LPS-stimulated IFN-γ production from baseline to end of study between the two groups (p 0.279) (Table 3, see Supplementary material, Fig. S3). Specifically, concentrations of IFN-γ changed from baseline (42.00 ng/mL, IQR 6.05–106.17) to end of study (55.33 ng/mL, IQR 19.33–80.67) in LPS-stimulated whole blood in the placebo group (−0.23% ± 0.14%, p 0.383) (Table 3). No change from baseline (41.88 ng/mL, IQR 6.00–95.00) to end of study (56.00 ng/mL, IQR 15.67–87.33) was observed in the probiotics group (0.01% ± 0.17%, p 1.000) (Table 3).

Discussion

We have assessed the effect of a daily intake of a combination of probiotic strains, LGG and BB-12, on the response to vaccinations against *S. pneumoniae* and *B. pertussis* in healthy children starting in day care as a secondary outcome of the ProbiComp study [11]. It was previously shown that the administered probiotic strains could be detected in faeces and proliferated in the gut, but did not change the gut microbiota community structure [16]. In this study, we did not find an effect on the concentration of specific antibody response between the probiotic and placebo group towards the two vaccines. Yet, the vaccine response was high (Table 2). Moreover, to study a systemic effect of the probiotics, we also assessed changes in the capability to produce cytokines upon *ex vivo* LPS stimulation in whole blood and found here significant difference between the IL-6 production in blood from the group receiving probiotics and the placebo group.

Only a limited number of other studies have assessed the effect of probiotics on the outcome of vaccination in children. To our knowledge, seven other studies have addressed the effect of probiotics on the antibody response against toxoid vaccines and carbohydrate vaccines in children/infants. They vary greatly in number of participants, duration, and dose and strains of administered probiotics. Of these, three studies administered probiotics to infants from birth in a period of 4–12 months [17–19]. One study assessed the effect of administering a probiotic mixture of around 1.2×10^{10} CFU daily to newborn infants during the first 6 months of life. Antibody titres against diphtheria and tetanus toxoid in the 6-month-old children showed no significant differences between the probiotic and placebo group, but for *Haemophilus influenzae* type B, concentrations tended to be higher in the probiotic group [17]. The same trend of *Haemophilus influenzae* type B IgG level was observed in another study [18]. A recent study also did not find any differences after probiotic supplementation in diphtheria, tetanus, pertussis, polio and hepatitis B antibody titres [19].

Four studies initiated the probiotic intervention at a later timepoint, between 2 months and 5 years [20–23]. Of these studies, the study by Pérez et al. [21] is most comparable with ours, as they also addressed the IgG response to pneumococcal vaccination and saw no effect of the probiotic intervention. However, in contrast to our study, the enrolled children varied greatly in age (from 9 months to 10 years), and were from families of low socio-economic status and presumed to have been exposed to a high microbial load. Such factors are expected to affect the study outcome, e.g. by higher variation due to time since last vaccination, or by reducing the effect of the probiotic intervention due to the high microbial load. Even though the children in the present study came from families in Denmark where the majority of parents had >15 years of education, so indicating a 'high hygienic lifestyle', no effect of the probiotic intervention was found. Hence, the importance of the microbial stimulation for the strength of the antibody response towards these types of vaccines is questionable. Of note, in our study the probiotic intervention took place during the first months in day care, a time where the infant encounters a high number of microorganisms of high diversity, and so may—with respect to the microbial load—not differ substantially from the Pérez et al. study [21]. Taken together, our study is in line with former studies, which found no or few trends towards an increase in antibody levels towards the injected antigens (notably against *Haemophilus influenzae* type B).

We also performed another analysis of systemic blood parameters; cytokine production (IL-6 and IFN-γ) upon *ex vivo* LPS-stimulated whole blood. The production of IL-6 by whole blood reflects the number and response of innate leucocytes, notably monocytes, while IFN-γ is produced by natural killer cells and T helper type 1 cells [24,25]. Here, we found that the change in IL-6 level differed significantly between the probiotic and placebo groups. This may imply that probiotics may affect the capability to respond to a microbial stimulus or LPS. However, as the difference seems to be greatly driven by a lower IL-6 baseline level in the placebo group, we cannot rule out that the difference is due to regression to the mean in the placebo group.

Number of probiotic bacteria as well as the strain(s) used seems to influence the outcome [10]. In our study, the dose was 2×10^9 CFU/day, which is comparable to most other infant studies. The combination of LGG and BB-12 has not been used in any of the reported studies, hence we cannot exclude that other strains would have given a different outcome.

Most of the studies showing an enhancing effect of the antibody titre enrolled individuals above 65 years [26], indicating that a positive effect of probiotics may depend on the immune status of

the participants, as it is well-known that the immune system deteriorates with age. As the effects of probiotics on vaccination are mostly seen in studies involving elderly individuals, this points towards the fact that vaccines today are so efficient that an effect of probiotics is merely on the general status of the immune system. Hence, we suggest that immunocompromised individuals and elderly will benefit from probiotics by improved protection upon vaccination.

A strength in the current study is that blood samples were drawn at enrolment as well as at the termination of the intervention, allowing us to baseline adjust end-point values. A limitation in the study is that we did not test against viral vaccines (rubella, mumps, measles), which might have given another outcome. However, in other studies children did not show markedly different outcome of probiotic intervention on the viral vaccines compared with the toxoid and carbohydrate vaccines [10,19]. We studied the effects of probiotics in healthy Danish children assumed to have a well-developed immune system. A group of immunocompromised children might have given other results.

The prevailing theories regarding the mechanism(s) by which probiotics exert their putative effects are still debated. In general, the probiotic bacteria are believed to, like other bacteria, stimulate the innate immune system but may also affect the polarization of the adaptive immune system [27]. We have recently shown that probiotics administered to newborn mice with a depleted microbiota increased granulopoiesis (unpublished data), a mechanism that may explain a positive effect of probiotics on influenza-vaccinated elderly. This mechanism may also explain the significant difference in *ex vivo* IL-6 production in LPS-stimulated whole blood, as a high concentration of neutrophils in blood may affect the concentration of monocytes, the main IL-6 producers in blood. This is, however, purely speculative because we cannot rule out the possibility of regression to the mean in the placebo group. Probiotics may also aid the stimulation of defence mechanisms in the epithelium, notably production of mucins and anti-microbial peptides. This mechanism may explain why more pronounced effects are found in studies of probiotics and mucosal vaccines [28,29].

In conclusion, we did not find an effect of probiotic intervention on the antibody titres to pneumococcal and pertussis toxoid vaccination in 14- to 18-month-old children after their first months in day care. This may reflect how efficient the vaccines are and suggests that only immunocompromised individuals and elderly may benefit from probiotics to strengthen the outcome of vaccination.

Transparency declaration

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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Access to data

Rikke Pilmann Laursen and Kim F. Michaelsen, Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Denmark.

Author contributions

CAS and EF have contributed equally to this article and share first authorship. CAS and EF contributed to analysis and interpretation of data, drafting of the article. CSJ contributed to analysis of antibody concentrations. HF contributed to analysis and interpretation of cytokine data. RPL coordinated and conducted the data collection. AL and CM contributed to design of the ProbiComp study. CR contributed to statistical data analysis. KFM contributed to study design and obtained approvals. KAK and HF contributed to study design, planning analyses and drafting of the article. All authors critically reviewed the manuscript, approved the final draft and take responsibility for the integrity and the accuracy of this research and the interpretation hereof.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2018.07.031.

References

[1] Statistics Denmark. Færre børn i daginstitutionerne efter skolereformen. Available at: http://dst.dk/site/dst/udgivelser/nyt/getpdf.aspx?cid=19245 [Accessed 28 February 2018].
[2] Kamper-Jørgensen M, Wohlfahrt J, Simonsen J, Grønbaek M, Benn CS. Population-based study of the impact of childcare attendance on hospitalizations for acute respiratory infections. Pediatrics 2006;118:1439–46.
[3] Lu N, Samuels ME, Shi L, Baker SL, Glover SH, Sanders JM, et al. Child day care risks of common infectious diseases revisited. Child Care Heal Dev 2004;30:361–8.
[4] Harboe ZB, Dalby T, Weinberger DM, Benfield T, Mølbak K, Slotved HC, et al. Impact of 13-valent pneumococcal conjugate vaccination in invasive pneumococcal disease incidence and mortality. Clin Infect Dis 2014;59:1066–73.
[5] Hviid A. Effectiveness of two pertussis vaccines in preterm Danish children. Vaccine 2009;27:3035–8.
[6] Thierry-Carstensen B, Dalby T, Stevner MA, Robbins JB, Schneerson R, Trollfors B, et al. Experience with monocomponent acellular pertussis combination vaccines for infants, children, adolescents and adults—a review of safety, immunogenicity, efficacy and effectiveness studies and 15 years of field experience. Vaccine 2013;31:5178–91.
[7] Huda MN, Lewis Z, Kalanetra KM, Rashid M, Ahmad SM, Raqib R, et al. Stool microbiota and vaccine responses of infants. Pediatrics 2014;134:e362–72.
[8] Praharaj I, John SM, Bandyopadhyay R, Kang G. Probiotics, antibiotics and the immune responses to vaccines. Phil Trans R Soc B 2015;370:1–12.
[9] Lei W-T, Shih P-C, Liu S-J, Lin C-H, Yeh T-L. Effect of probiotics and prebiotics on immune response to influenza vaccination in adults: a systematic review and meta-analysis of randomized controlled trials. Nutrients 2017;9:1–17.
[10] Zimmermann P, Curtis N. The influence of probiotics on vaccine responses—a systematic review. Vaccine 2018;36:207–13.
[11] Laursen RP, Larnkjær A, Ritz C, Hauger H, Michaelsen KF, Mølgaard C, et al. Probiotics and child care absence due to infections: a randomized controlled trial. Pediatrics 2017;140:1–11.
[12] Kantsø B, Ingdam Halkjær S, Østergaard Thomsen O, Belard E, Gottschalck IB, Jørgensen CS, et al. Immunosuppressive drugs impairs antibody response of the polysaccharide and conjugated pneumococcal vaccines in patients with Crohn's disease. Vaccine 2015;33:5464–9.
[13] Dalby T, Seier-Petersen M, Kristiansen MP, Harboe ZB, Krogfelt KA. Problem solved: a modified enzyme-linked immunosorbent assay for detection of human antibodies to pertussis toxin eliminates false-positive results occurring at analysis of heat-treated sera. Diagn Microbiol Infect Dis 2009;63:354–60.
[14] Dalby T, Petersen JW, Harboe ZB, Krogfelt KA. Antibody responses to pertussis toxin display different kinetics after clinical *Bordetella pertussis* infection than after vaccination with an acellular pertussis vaccine. J Med Microbiol 2010;59:1029–36.

[15] Lauritzen L, Kjær TMR, Fruekilde M-B, Michaelsen KF, Frøkiær H. Fish oil supplementation of lactating mothers affects cytokine production in 2 1/2-year-old children. Lipids 2005;40:669–76.
[16] Laursen MF, Laursen RP, Larnkjær A, Michaelsen KF, Bahl MI, Licht TR. Administration of two probiotic strains during early childhood does not affect the endogenous gut microbiota composition despite probiotic proliferation. BMC Microbiol 2017;17:1–9.
[17] Kukkonen K, Nieminen T, Poussa T, Savilahti E, Kuitunen M. Effect of probiotics on vaccine antibody responses in infancy—a randomized placebo-controlled double-blind trial. Pediatr Allergy Immunol 2006;17:416–21.
[18] Soh SE, Ong DQR, Gerez I, Zhang X, Chollate P, Shek LP, et al. Effect of probiotic supplementation in the first 6 months of life on specific antibody responses to infant hepatitis B vaccination. Vaccine 2010;28:2577–9.
[19] Wu B-B, Yang Y, Xu X, Wang WP. Effects of Bifidobacterium supplementation on intestinal microbiota composition and the immune response in healthy infants. World J Pediatr 2016;12:177–82.
[20] Matsuda F, Chowdhury MI, Saha A, Asahara T, Nomoto K, Tarique AA, et al. Evaluation of a probiotics, *Bifidobacterium breve* BBG-01, for enhancement of immunogenicity of an oral inactivated cholera vaccine and safety: a randomized, double-blind, placebo-controlled trial in Bangladeshi children under 5 years of age. Vaccine 2011;29:1855–8.
[21] Pérez N, Iannicelli JC, Girard-Bosch C, González S, Varea A, Disalvo L, et al. Effect of probiotic supplementation on immunoglobulins, isoagglutinins and antibody response in children of low socio-economic status. Eur J Nutr 2010;49:173–9.
[22] West CE, Gothefors L, Granström M, Käyhty H, Hammarström ML, Hernell O. Effects of feeding probiotics during weaning on infections and antibody responses to diphtheria, tetanus and Hib vaccines. Pediatr Allergy Immunol 2008;19:53–60.
[23] Youngster I, Kozer E, Lazarovitch Z, Broide E, Goldman M. Probiotics and the immunological response to infant vaccinations: a prospective, placebo controlled pilot study. Arch Dis Child 2011;96:345–9.
[24] Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell 2016;167:1125–36.
[25] Mærkedahl RB, Frøkiær H, Stenbæk MG, Nielsen CB, Lind MV, Lundtoft C, et al. In vivo and ex vivo inflammatory markers of common metabolic phenotypes in humans. Metab Syndr Relat Disord 2018;16:29–39.
[26] Yeh T-L, Shih P-C, Liu S-J, Lin CH, Liu JM, Lei WT, et al. The influence of prebiotic or probiotic supplementation on antibody titers after influenza vaccination: a systematic review and meta-analysis of randomized controlled trials. Drug Des Devel Ther 2018;12:217–30.
[27] Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nat Rev Immunol 2017;17:219–32.
[28] Isolauri E, Joensuu J, Suomalainen H, Luomala M, Vesikari T. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 1995;13:310–2.
[29] Paineau D, Carcano D, Leyer G, Darquy S, Alyanakian MA, Simoneau G, et al. Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol 2008;53:107–13.