Research article

Dietary supplementation with probiotics during late pregnancy: outcome on vaginal microbiota and cytokine secretion

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Abstract

Background

The vaginal microbiota of healthy women consists of a wide variety of anaerobic and aerobic bacterial genera and species dominated by the genus *Lactobacillus*. The activity of lactobacilli helps to maintain the natural healthy balance of the vaginal microbiota. This role is particularly important during pregnancy because vaginal dismicrobism is one of the most important mechanisms for

preterm birth and perinatal complications. In the present study, we characterized the impact of a dietary supplementation with the probiotic VSL#3, a mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains, on the vaginal microbiota and immunological profiles of healthy women during late pregnancy.

Results

An association between the oral intake of the probiotic VSL#3 and changes in the composition of the vaginal microbiota of pregnant women was revealed by PCR-DGGE population profiling. Despite no significant changes were found in the amounts of the principal vaginal bacterial populations in women administered with VSL#3, qPCR results suggested a potential role of the probiotic product in counteracting the decrease of *Bifidobacterium* and the increase of *Atopobium*, that occurred in control women during late pregnancy. The modulation of the vaginal microbiota was associated with significant changes in some vaginal cytokines. In particular, the decrease of the anti-inflammatory cytokines IL-4 and IL-10 was observed only in control women but not in women supplemented with VSL#3. In addition, the probiotic consumption induced the decrease of the pro-inflammatory chemokine Eotaxin, suggesting a potential anti-inflammatory effect on the vaginal immunity.

Conclusion

Dietary supplementation with the probiotic VSL#3 during the last trimester of pregnancy was associated to a modulation of the vaginal microbiota and cytokine secretion, with potential implications in preventing preterm birth.

Trial registration

ClinicalTrials.gov NCT01367470

Background

The vaginal microbiota of healthy women consists of a wide variety of anaerobic and aerobic bacterial genera and species dominated by the facultative, microaerophilic anaerobic genus *Lactobacillus*[1]. The activity of lactobacilli helps to maintain the natural healthy balance of the vaginal microbiota. This role is particularly important during pregnancy because abnormalities in vaginal communities, such as bacterial vaginosis (BV) and aerobic vaginitis (AV), have been claimed as important mechanisms responsible for preterm birth and perinatal complications [2].

The association of lower genital tract infection with an increased risk of preterm delivery and

preterm rupture of the fetal membranes has recently attracted great interest in the pathogenesis of such infection-related mechanisms [3,4]. Earlier studies showed an increased rate of prematurity in women with BV, an alteration of the endogenous vaginal microbiota associated with decreased levels of hydrogen peroxide-producing *Lactobacillus* species [4-6]. The mechanisms linking BV with preterm delivery have not been fully identified, but local immune response is hypothesized to be crucial. Despite the notion that BV is a non-inflammatory condition, evidence exists that demonstrates altered levels of certain pro-inflammatory cytokines in women with BV [7,8].

Parturition is characterized by cervical ripening and myometrial maturation with subsequent uterine contractions leading to cervical dilatation and birth [9]. The process of labor displays many of the hallmarks of inflammation. Acute inflammatory features, such as increased influx of leucocytes and elevated expression of pro-inflammatory cytokines, have been observed in cervical tissues and fetal membranes during both term and preterm labor [10-12].

A potentially novel way to protect against infection-mediated preterm birth is to use probiotic bacteria, especially lactobacilli. Probiotics, defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [13], are being studied for their ability to replenish vaginal lactobacilli and modulate immunity [14-16]. In addition, administration of probiotics to the mother during pregnancy and breast-feeding has been described by some studies as a safe and effective mode of enhancing the immunoprotective potential of the breast milk and preventing atopic eczema in the infant [17,18].

In recent years, culture-independent techniques based on the analysis of rRNA gene sequences have been developed, providing powerful tools to reveal the phylogenetic diversity of the microorganisms found within vaginal microbiota and to understand community dynamics [19-24]. In particular, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been successfully used to identify the bacterial composition of different ecological niches, including the vaginal microbiota [22,25,26]. Real-time PCR is a powerful technique for the quantitative analysis of specific microbial populations belonging to complex ecosystems [22,27,28]. Specific primers can be used to focus the quantitative analysis on a particular genus, species or strain of interest.

Several bacterial species are known to colonize both the gastrointestinal and the reproductive tract, and the rectum has been suggested to play an important role as a source or reservoir for organisms that colonize the vagina [15,29]. On this basis, the aim of the present study was to evaluate the impact of a dietary supplementation with the probiotic product VSL#3, a mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains, on the vaginal microbiota and immunological profiles of

asymptomatic healthy women during late pregnancy. The dynamics of the vaginal bacterial communities prior and after probiotic ingestion were assessed by PCR-DGGE and real-time PCR, while the modulation of the cytokine secretion in vaginal fluids was measured by Luminex® Immunoassay. Although previous studies demonstrated the therapeutic efficacy of VSL#3 in the management of gastrointestinal disorders, especially inflammatory bowel disease [30], as well as the ability of the VSL#3 strains to colonize the gut environment [31] and to modulate the immune response of the colonic mucosa [32], this is the first study that investigates the indirect effects of this probiotic formula on the vaginal microbiota.

Results

Bacterial population profiling with PCR-DGGE

PCR-DGGE analysis with universal primers for bacteria (HDA1-GC/HDA2) was used to investigate: (i) the stability of the predominant vaginal bacterial communities over a period of 4 weeks in the last trimester of pregnancy, from the 33rd (W33) to the 37th (W37) week of gestation, and (ii) the influence of the oral consumption of the probiotic VSL#3 from W33 to W37 on the predominant vaginal microbiota (Figure 1).

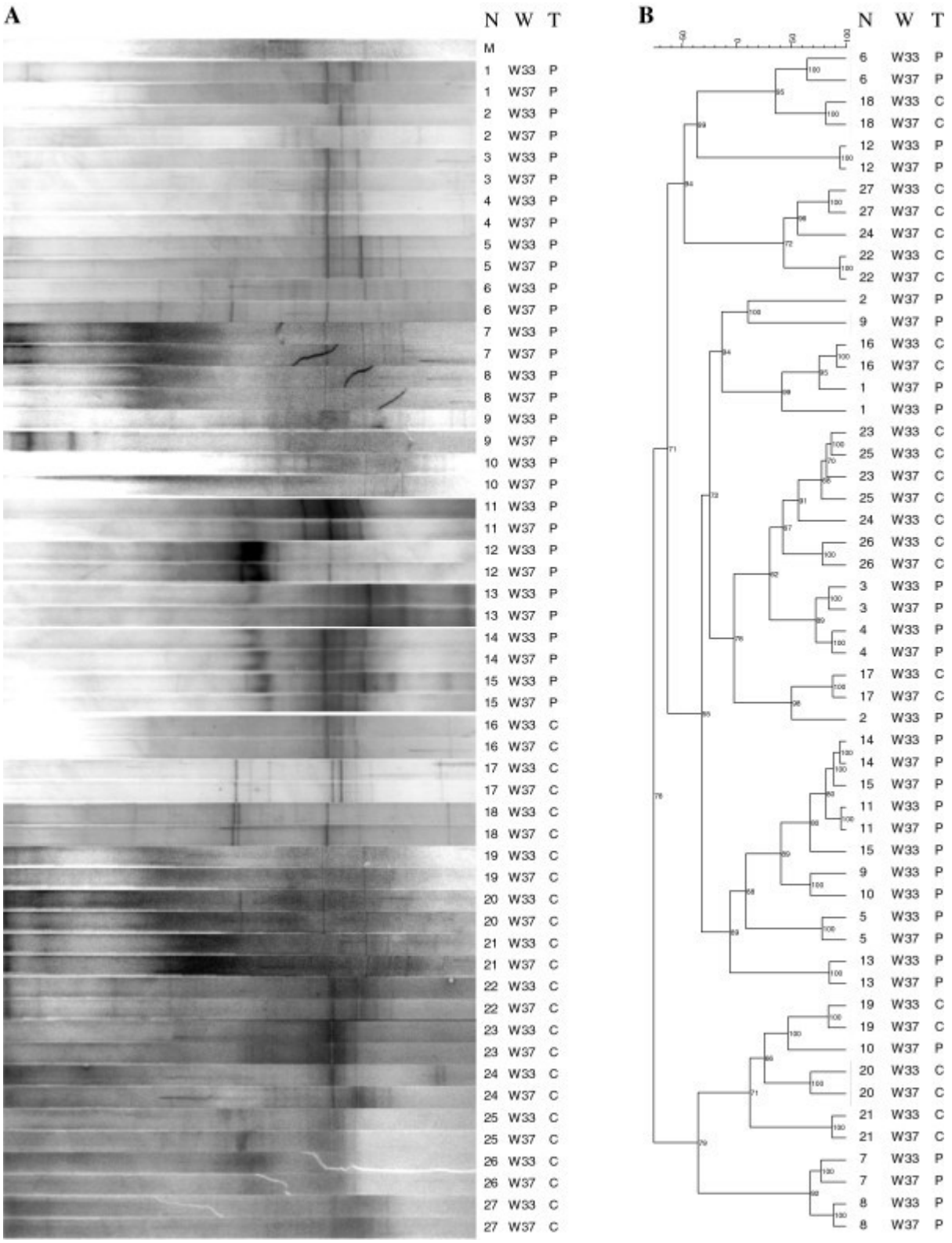

Figure 1. PCR-DGGE analysis with universal primers for bacteria. Analysis was conducted on the vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from 15 women supplemented with the probiotic VSL#3 [(P) N. 1–15] and 12 control women [(C) N. 16–27]. N: woman number; W: week of gestation; T: type of supplementation. (**A**) PCR-DGGE fingerprints. M, external reference marker. (**B**) Dendrogram of the DGGE profiles shown in panel **A**. Pearson correlation was used to calculate the similarity in DGGE profiles.

DGGE band profiles displayed a relatively low complexity for both probiotic (P) and control (C)

groups, as assessed by the richness index. Mean values of the richness index were 6.6 at both W33 and W37 for C group and shifted from 8.4 (W33) to 7.4 (W37) for P group without significant variations between W33 and W37.

Pearson correlation was used to calculate the similarity index (SI) between DGGE patterns related to the time points W33 and W37 for each pregnant woman (Table 1). The SI median values of P group and C group were 73% and 79%, respectively. In particular, 3 women belonging to P group (N. 2, 9 and 10) and only one woman belonging to C group (N. 24) showed SI values lower that 50%. For each woman, significant differences between DGGE profiles related to W33 and W37 were searched by Wilcoxon Signed Rank Test. No significant variations were detected between W33 and W37 in control women. Significant differences ($P < 0.05$) were found for 5/15 (33%) women belonging to P group (N. 4, 5, 9, 10, 11). Interestingly, women N. 9 and 10 were the same presenting SIs < 50%. These data suggested a potential role of the probiotic formula in modulating the vaginal bacterial communities. The peak heights of the DGGE densitometric curves were analyzed using the Wilcoxon Signed Rank Test in order to search for significant differences in single species abundances between W33 and W37. No significant changes in species abundance were found for both P and C groups, even in women N. 4, 5, 9, 10, 11.

Table 1

Similarity index (SI) of DGGE profiles related to W33 and W37 obtained with universal (HDA1/HDA2) and Lactobacillus-specific (Lac1/Lac2) primers

Woman N	HDA1-GC/HDA2 SI (%)	Lac1/Lac2-GC SI (%)
Probiotic (P)		
1	55.2	21.6
2	28.4	62.0
3	84.0	84.0
4	87.7	84.1
5	78.0	87.8
6	64.5	68.1
7	77.2	85.6
8	88.5	95.5
9	37.5	86.2
10	41.3	91.9
11	95.3	96.6
12	94.5	93.3
13	84.7	96.9
14	94.3	94.3
15	81.1	44.5
Control (C)		
16	91.2	90.9
17	87.8	93.7
18	81.6	76.9
19	83.7	91.5

Table 1

Similarity index (SI) of DGGE profiles related to W33 and W37 obtained with universal (HDA1/HDA2) and Lactobacillus-specific (Lac1/Lac2) primers

Woman N	HDA1-GC/HDA2 SI (%)	Lac1/Lac2-GC SI (%)
20	67.7	81.3
21	87.1	94.3
22	94.6	74.4
23	85.3	74.1
24	25.4	46.0
25	84.7	84.2
26	78.3	68.1
27	84.5	86.3

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Cluster analysis showed that the DGGE profiles related to the time points W33 and W37 clustered together for all the control women, except for the woman N. 24 (Figure 1). Four supplemented women (N. 2, 9, 10 and 15) showed W33 and W37 DGGE profiles not closely related. However, the DGGE patterns of the majority of the women administered with VSL#3 grouped according to the subject and not to the time point, revealing that the inter-individual variability was higher than the variability induced by the probiotic supplementation.

Because of the importance of lactobacilli in the establishment of a healthy vaginal environment [2], DGGE analysis with *Lactobacillus*-specific primer set (Lac1/Lac2-GC) was also carried out. This analysis allowed us to investigate the variations in lactobacilli population occurring physiologically from W33 and W37 and potentially associated with the VSL#3 intake (Figure 2).

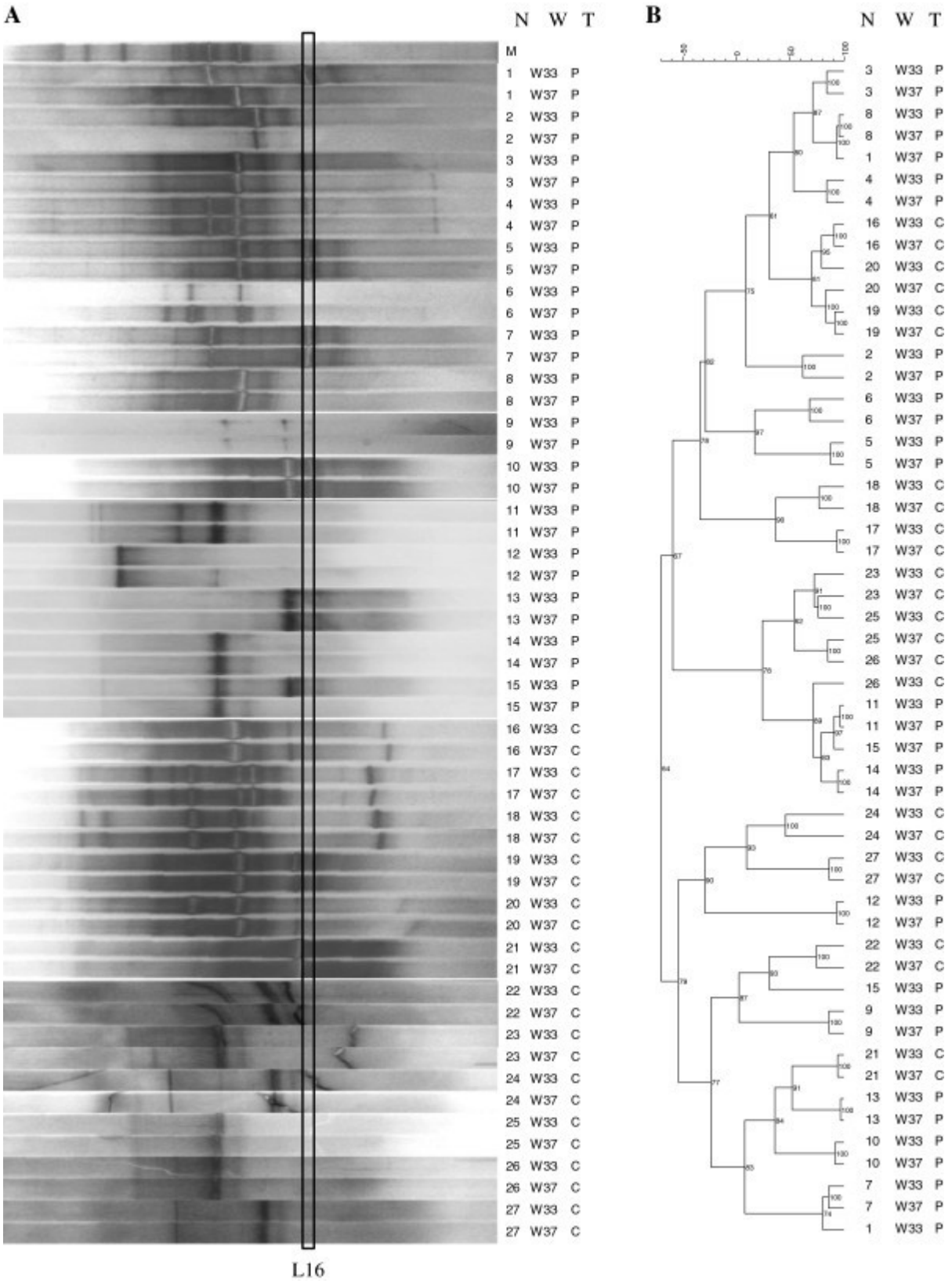

Figure 2. PCR-DGGE analysis with *Lactobacillus*-specific primers. Analysis was conducted on the vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from 15 women supplemented with the probiotic VSL#3 [(P) N. 1–15] and 12 control women [(C) N. 16–27]. N: woman number; W: week of gestation; T: type of supplementation. (**A**) PCR-DGGE fingerprints. M, external reference marker. Band L16 corresponds to *L. helveticus* (GenBank accession number: AB571603) (**B**) Dendrogram of the DGGE profiles shown in panel **A**. Pearson correlation was used to calculate the similarity in DGGE profiles.

Richness indexes ranged from 5.7 (W33) to 5.4 (W37) for P group and from 6.3 (W33) to 6.8 (W37) for C group. Mean values of SI were 79% and 80% for P and C groups, respectively (Table 1). Only 2 women included in P group showed SIs < 50% (N. 1 and 15). Wilcoxon Signed Rank Test highlighted significant differences between DGGE profiles related to W33 and W37 for women N. 7 and 10, accounting for 13% of women included in P group. Comparing this percentage with the 33% obtained by DGGE analysis with HDA1-GC/HDA2 primer set, the probiotic intake seemed to have a more extended impact on total bacteria than lactobacilli. Notably, only for woman N. 10, significant differences were found between W33- and W37-related DGGE patterns for both HDA1-GC/HDA2 and Lac1/Lac2-GC primer sets.

The peak height analysis by Wilcoxon Signed Rank Test allowed us to identify a band, denominated L16 (Figure 2), which significantly changed after probiotic supplementation. Sequencing of the DNA extracted from this band revealed 100% homology with *L. helveticus* strains. The nucleotide sequence of this DGGE fragment was deposited in DDBJ Nucleotide Sequence Database under the accession number AB571603. *L. helveticus* was found to be a representative species within lactobacilli population since it was detected in 9 women supplemented with VSL#3 and 2 control women, corresponding to a frequency of occurrence of 40.7%. Notably, a general decrease in the intensity of *L. helveticus* band was observed in P group while no variations were appreciable in C group.

Cluster analysis showed that *Lactobacillus*-specific DGGE profiles related to the time points W33 and W37 were closely related for all control women and for the majority of women administered with VSL#3, except for the subjects N. 1 and 15 (Figure 2).

Quantitative variations of vaginal bacterial populations

Quantitative real-time PCR (qPCR) was performed to analyze changes in concentration of *Lactobacillus*, *Bifidobacterium* and *Streptococcus thermophilus*, that were included in the probiotic VSL#3, and *Gardnerella vaginalis*, *Atopobium*, *Prevotella* and *Veillonella*, that are important BV-related genera and species [22,28]. qPCR efficiency for all assays was between 90% and 110% and correlation coefficients for genomic DNA standards were > 0.99. The sensitivity of qPCR assays was 9.1×10^{-3}, 1.5×10^{-4}, 3.7×10^{-4}, 1.7×10^{-1}, 1.4×10^{-2}, 4.9×10^{-4}, 3.3×10^{-1} ng of target DNA for *Lactobacillus*, *Bifidobacterium*, *S. thermophilus*, *G. vaginalis*, *Atopobium*, *Prevotella* and *Veillonella*, respectively. All subjects naturally harbored strains belonging to *Lactobacillus*, *Bifidobacterium*, *Atopobium* and *Prevotella*, as demonstrated by the presence of these genera in the

vaginal samples collected at W33. Woman N. 9 (P group) was the only exception lacking lactobacilli at both the baseline and after one-month intake of VSL#3 (Table 2). *G. vaginalis* was found in two women belonging to C group (N. 18 and 20) at both time points at the concentration of $5.5 \times 10^1 \pm 3.8$ (N. 18: W33), $7.5 \times 10^1 \pm 4.6$ (N. 18: W37), $2.2 \times 10^2 \pm 1.8 \times 10^1$ (N. 20: W33) and $1.9 \times 10^2 \pm 3.2 \times 10^1$ (N. 20: W37). *S. thermophilus* and *Veillonella* were not detected in any pregnant woman enrolled in this study. Statistical elaboration of qPCR data related to *Lactobacillus*, *Bifidobacterium*, *Atopobium* and *Prevotella* was performed to search for significant variations of these genera associated with the going on of pregnancy or the probiotic supplementation (Figure 3). No significant changes in the amounts of these bacteria were found between W33 and W37 in both P and C groups. However, in spite of the lack of statistical relevance, a weak modulation was observed for *Bifidobacterium* and *Atopobium*. Regarding bifidobacteria (Figure 3B), a physiological tendency to decrease was observed in vaginal samples of control women at the end of the study period (mean value, W33: $4.3 \pm 2.2 \times 10^{-1}$; W37: $2.0 \pm 1.7 \times 10^{-1}$). This trend seemed to be counterbalanced in women consuming VSL#3 since amount of bifidobacteria slightly increased during the supplementation period (mean value, W33: $9.9 \times 10^{-1} \pm 1.6 \times 10^{-1}$; W37: $1.4 \pm 1.2 \times 10^{-1}$). An opposite trend was observed for *Atopobium* (Figure 3C). This genus increased at W37 (mean value, 9.2 ± 3.2) compared to W33 (mean value, 7.0 ± 2.8) in C group, while it remained constant after VSL#3 supplementation (mean value, W33: $1.4 \times 10^1 \pm 3.8$; W37: $1.3 \times 10^1 \pm 5.2$).

Table 2

qPCR data of Lactobacillus, Bifidobacterium, Atopobium and Prevotella

		ng of target DNA/μg vaginal genomic DNA (mean ± SD)			
Woman N.	**Time point**	*Lactobacillus*	*Bifidobacterium*	*Atopobium*	*Prevotella*
Probiotic (P)					
1	W33	$2.4 \times 10^1 \pm 1.1$	$1.9 \times 10^{-2} \pm 7.4 \times 10^{-3}$	3.6 ± 1.5	$2.1 \times 10^{-2} \pm 1.0 \times 10^{-2}$
	W37	$3.0 \times 10^1 \pm 3.1$	$3.1 \times 10^{-2} \pm 2.7 \times 10^{-4}$	$1.3 \times 10^1 \pm 6.8$	$9.1 \times 10^{-2} \pm 1.6 \times 10^{-2}$
2	W33	$9.6 \pm 8.7 \times 10^{-1}$	$3.1 \times 10^{-2} \pm 8.8 \times 10^{-3}$	$5.4 \times 10^1 \pm 7.4$	$1.4 \times 10^{-1} \pm 4.8 \times 10^{-2}$
	W37	$5.9 \times 10^{-1} \pm 4.9 \times 10^{-2}$	$2.4 \times 10^{-2} \pm 1.2 \times 10^{-2}$	$2.4 \times 10^1 \pm 1.9 \times 10^1$	$1.1 \times 10^{-1} \pm 1.1 \times 10^{-2}$
3	W33	$2.4 \times 10^1 \pm 2.9$	$2.4 \times 10^{-2} \pm 4.2 \times 10^{-3}$	$1.1 \times 10^1 \pm 6.0$	$1.1 \times 10^{-1} \pm 7.7 \times 10^{-3}$
	W37	$2.2 \times 10^1 \pm 2.4$	$3.0 \times 10^{-2} \pm 2.4 \times$	4.0 ± 2.3	$5.2 \times 10^{-2} \pm 8.2 \times$

Table 2

qPCR data of Lactobacillus, Bifidobacterium, Atopobium and Prevotella

		ng of target DNA/μg vaginal genomic DNA (mean ± SD)			
			10^{-3}		10^{-3}
4	W33	$2.2 \times 10^{1} \pm 2.0$	$6.8 \times 10^{-2} \pm 8.3 \times 10^{-3}$	4.7 ± 1.9	$7.3 \times 10^{-2} \pm 2.9 \times 10^{-2}$
	W37	$1.5 \times 10^{1} \pm 1.4$	$2.1 \times 10^{-2} \pm 7.2 \times 10^{-3}$	5.2 ± 2.0	$4.6 \times 10^{-2} \pm 9.5 \times 10^{-3}$
5	W33	$2.5 \times 10^{1} \pm 4.5$	$2.1 \times 10^{-2} \pm 3.4 \times 10^{-3}$	$1.2 \times 10^{1} \pm 3.0$	$9.3 \times 10^{-2} \pm 8.3 \times 10^{-3}$
	W37	$2.2 \text{ x } 10^{1} \pm 4.5$	$1.4 \text{ x } 10^{-2} \pm 3.2 \text{ x } 10^{-3}$	$1.5 \text{ x } 10^{1} \pm 1.9$	$3.0 \text{ x } 10^{-2} \pm 1.1 \text{ x } 10^{-2}$
6	W33	$1.1 \times 10^{-1} \pm 3.4 \times 10^{-3}$	$7.1 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$1.0 \times 10^{1} \pm 4.1$	$1.2 \times 10^{-1} \pm 1.3 \times 10^{-2}$
	W37	$2.2 \pm 6.0 \times 10^{-1}$	$2.1 \pm 1.7 \times 10^{-1}$	$2.4 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.5 \times 10^{-1} \pm 1.2 \times 10^{-2}$
7	W33	$4.1 \times 10^{1} \pm 8.5$	$3.7 \times 10^{-2} \pm 5.4 \times 10^{-3}$	$2.9 \times 10^{1} \pm 9.2$	$1.2 \times 10^{-1} \pm 2.1 \times 10^{-2}$
	W37	$2.0 \times 10^{1} \pm 2.6$	$1.7 \times 10^{-2} \pm 4.4 \times 10^{-3}$	$2.6 \times 10^{1} \pm 7.7$	$1.1 \times 10^{-1} \pm 1.1 \times 10^{-3}$
8	W33	$1.0 \times 10^{1} \pm 1.7 \times 10^{-1}$	$1.3 \times 10^{-2} \pm 1.9 \times 10^{-3}$	5.5 ± 1.2	$4.2 \times 10^{-2} \pm 1.9 \times 10^{-2}$
	W37	$2.1 \times 10^{1} \pm 2.0$	$1.5 \times 10^{-2} \pm 2.6 \times 10^{-3}$	$1.6 \times 10^{1} \pm 6.6$	$5.1 \times 10^{-2} \pm 3.3 \times 10^{-3}$
9	W33	0.0 ± 0.0	$7.1 \times 10^{-3} \pm 2.8 \times 10^{-5}$	$1.8 \times 10^{1} \pm 7.1$	$6.7 \times 10^{-2} \pm 1.5 \times 10^{-2}$
	W37	0.0 ± 0.0	$1.1 \times 10^{1} \pm 1.0$	$1.5 \times 10^{1} \pm 6.8$	$2.3 \times 10^{-1} \pm 8.0 \times 10^{-2}$
10	W33	$6.7 \pm 6.1 \times 10^{-1}$	$2.0 \times 10^{-2} \pm 4.8 \times 10^{-3}$	$1.4 \times 10^{1} \pm 4.3$	$8.6 \times 10^{-2} \pm 2.0 \times 10^{-2}$
	W37	$1.1 \times 10^{1} \pm 1.4$	$2.3 \times 10^{-2} \pm 1.5 \times 10^{-2}$	$1.7 \times 10^{1} \pm 9.7$	$8.0 \times 10^{-2} \pm 2.9 \times 10^{-2}$
11	W33	$2.7 \times 10^{1} \pm 1.7$	$2.9 \text{ x } 10^{-3} \pm 1.7 \times 10^{-3}$	2.3 ± 1.8	$3.2 \times 10^{-2} \pm 3.3 \times 10^{-3}$
	W37	$3.0 \times 10^{1} \pm 5.6$	$1.3 \text{ x } 10^{-2} \pm 8.5 \times 10^{-3}$	$1.3 \pm 7.5 \times 10^{-1}$	$3.6 \times 10^{-2} \pm 1.3 \times 10^{-2}$
12	W33	$2.2 \pm 5.6 \times 10^{-1}$	$1.5 \times 10^{1} \pm 2.3$	$1.4 \times 10^{1} \pm 2.9$	$2.2 \times 10^{-1} \pm 2.1 \times 10^{-2}$
	W37	$2.0 \pm 3.1 \times 10^{-1}$	$8.7 \pm 5.6 \times 10^{-1}$	$1.2 \times 10^{1} \pm 2.3$	$1.0 \times 10^{-1} \pm 1.8 \times 10^{-2}$
13	W33	$3.7 \times 10^{1} \pm 5.4$	$3.0 \times 10^{-2} \pm 4.5 \times 10^{-3}$	$7.0 \pm 2.6 \times 10^{-1}$	$2.7 \times 10^{-2} \pm 5.0 \times 10^{-4}$
	W37	$6.6 \times 10^{1} \pm 5.9$	$1.1 \times 10^{-2} \pm 2.2 \times 10^{-3}$	$6.8 \pm 6.6 \times 10^{-1}$	$5.7 \times 10^{-2} \pm 2.0 \times 10^{-3}$

Table 2

qPCR data of Lactobacillus, Bifidobacterium, Atopobium and Prevotella

		ng of target DNA/μg vaginal genomic DNA (mean ± SD)			
14	W33	$2.2 \times 10^{1} \pm 8.5$	$1.7 \times 10^{-2} \pm 4.9 \times 10^{-3}$	$9.0 \pm 4.4 \times 10^{-1}$	$6.7 \times 10^{-2} \pm 6.6 \times 10^{-3}$
	W37	$1.6 \times 10^{1} \pm 4.9$	$2.8 \times 10^{-2} \pm 4.7 \times 10^{-3}$	$1.1 \times 10^{1} \pm 1.1$	$1.1 \times 10^{-1} \pm 1.8 \times 10^{-3}$
15	W33	$2.2 \times 10^{1} \pm 7.1$	$1.4 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$1.8 \times 10^{1} \pm 5.6$	$1.1 \times 10^{-1} \pm 1.4 \times 10^{-2}$
	W37	$2.8 \times 10^{1} \pm 3.4$	$4.7 \times 10^{-3} \pm 2.3 \times 10^{-3}$	$1.1 \times 10^{1} \pm 2.4 \times 10^{-1}$	$7.4 \times 10^{-2} \pm 2.4 \times 10^{-3}$
Control (C)					
16	W33	$5.4 \times 10^{1} \pm 4.0$	$2.1 \times 10^{-2} \pm 5.6 \times 10^{-3}$	$1.1 \times 10^{1} \pm 4.6$	$6.8 \times 10^{-2} \pm 1.1 \times 10^{-2}$
	W37	$2.0 \times 10^{1} \pm 1.7$	$2.0 \times 10^{-2} \pm 7.4 \times 10^{-3}$	$1.4 \times 10^{1} \pm 5.0$	$5.6 \times 10^{-2} \pm 5.4 \times 10^{-3}$
17	W33	$5.5 \pm 5.3 \times 10^{-1}$	$6.0 \pm 1.6 \times 10^{-1}$	$1.2 \times 10^{1} \pm 4.3$	$5.9 \times 10^{-2} \pm 2.3 \times 10^{-2}$
	W37	$1.5 \times 10^{1} \pm 2.9$	$9.3 \pm 5.3 \times 10^{-1}$	$1.9 \times 10^{1} \pm 8.7$	$5.4 \times 10^{-2} \pm 1.0 \times 10^{-2}$
18	W33	$2.6 \pm 1.6 \times 10^{-1}$	$1.8 \pm 3.5 \times 10^{-2}$	$1.3 \times 10^{1} \pm 5.5$	$8.8 \times 10^{-2} \pm 1.7 \times 10^{-2}$
	W37	$1.2 \times 10^{1} \pm 2.0$	$2.9 \pm 7.5 \times 10^{-2}$	$3.3 \times 10^{1} \pm 4.4$	$4.5 \times 10^{-2} \pm 2.8 \times 10^{-3}$
19	W33	$7.6 \times 10^{1} \pm 3.3 \times 10^{-1}$	$1.2 \pm 7.9 \times 10^{-3}$	$1.3 \times 10^{1} \pm 3.6$	$1.9 \times 10^{-1} \pm 3.2 \times 10^{-3}$
	W37	$2.7 \times 10^{1} \pm 3.8$	$2.7 \times 10^{-2} \pm 4.7 \times 10^{-3}$	8.2 ± 4.6	$1.1 \times 10^{-1} \pm 2.6 \times 10^{-2}$
20	W33	$1.6 \times 10^{1} \pm 1.4$	$1.1 \times 10^{1} \pm 1.2$	$1.2 \times 10^{1} \pm 5.5$	$8.6 \times 10^{-2} \pm 1.5 \times 10^{-2}$
	W37	$1.0 \times 10^{1} \pm 6.4 \times 10^{-2}$	$1.1 \times 10^{1} \pm 1.4$	$1.2 \times 10^{1} \pm 4.7$	$1.1 \times 10^{-1} \pm 3.1 \times 10^{-2}$
21	W33	$5.6 \times 10^{1} \pm 8.3$	$1.7 \times 10^{-2} \pm 1.7 \times 10^{-3}$	$2.1 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.3 \times 10^{-1} \pm 2.0 \times 10^{-2}$
	W37	$6.4 \times 10^{1} \pm 1.5$	$3.3 \times 10^{-2} \pm 8.7 \times 10^{-3}$	$2.2 \times 10^{1} \pm 1.0 \times 10^{1}$	$1.2 \times 10^{-1} \pm 2.4 \times 10^{-2}$
22	W33	$4.3 \times 10^{1} \pm 2.0$	$1.2 \times 10^{-1} \pm 2.8 \times 10^{-2}$	$2.3 \times 10^{-1} \pm 1.5 \times 10^{-2}$	0.0 ± 0.0
	W37	$6.8 \times 10^{1} \pm 5.1$	$2.7 \times 10^{-2} \pm 6.6 \times 10^{-3}$	$1.9 \times 10^{-1} \pm 2.0 \times 10^{-2}$	0.0 ± 0.0
23	W33	$2.6 \times 10^{1} \pm 5.6$	$2.3 \times 10^{-1} \pm 3.6 \times 10^{-2}$	0.0 ± 0.0	0.0 ± 0.0
	W37	$6.3 \times 10^{1} \pm 2.0$	$8.2 \times 10^{-3} \pm 1.9 \times$	$1.6 \times 10^{-1} \pm 2.9 \times$	$5.3 \times 10^{-1} \pm 1.8 \times$

Table 2

qPCR data of Lactobacillus, Bifidobacterium, Atopobium and Prevotella

		ng of target DNA/μg vaginal genomic DNA (mean ± SD)			
			10^{-3}	10^{-2}	10^{-1}
24	W33	$1.2 \times 10^{1} \pm 1.0$	$2.7 \times 10^{1} \pm 2.1 \times 10^{-1}$	$1.8 \pm 1.5 \times 10^{-1}$	$6.8 \times 10^{-1} \pm 3.4 \times 10^{-2}$
	W37	$7.5 \times 10^{1} \pm 3.8$	$9.7 \times 10^{-3} \pm 3.7 \times 10^{-3}$	$3.7 \times 10^{-1} \pm 3.4 \times 10^{-2}$	0.0 ± 0.0
25	W33	$6.5 \times 10^{1} \pm 1.0 \times 10^{1}$	$3.0 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$7.5 \times 10^{-2} \pm 7.5 \times 10^{-3}$	0.0 ± 0.0
	W37	$6.6 \times 10^{1} \pm 7.1$	$9.1 \times 10^{-3} \pm 5.1 \times 10^{-4}$	$2.5 \times 10^{-1} \pm 2.7 \times 10^{-2}$	0.0 ± 0.0
26	W33	$8.5 \times 10^{1} \pm 6.3$	$4.4 \pm 9.3 \times 10^{-1}$	$3.2 \times 10^{-1} \pm 3.9 \times 10^{-2}$	0.0 ± 0.0
	W37	$5.4 \times 10^{1} \pm 4.5$	$2.0 \times 10^{-2} \pm 6.1 \times 10^{-4}$	$3.6 \times 10^{-1} \pm 4.2 \times 10^{-2}$	0.0 ± 0.0
27	W33	$7.0 \times 10^{1} \pm 1.5 \times 10^{1}$	$3.3 \times 10^{-2} \pm 4.7 \times 10^{-3}$	$2.8 \times 10^{-1} \pm 2.6 \times 10^{-2}$	0.0 ± 0.0
	W37	$6.6 \times 10^{1} \pm 3.6 \times 10^{-1}$	$2.1 \times 10^{-2} \pm 1.6 \times 10^{-2}$	$4.0 \times 10^{-1} \pm 3.8 \times 10^{-2}$	0.0 ± 0.0

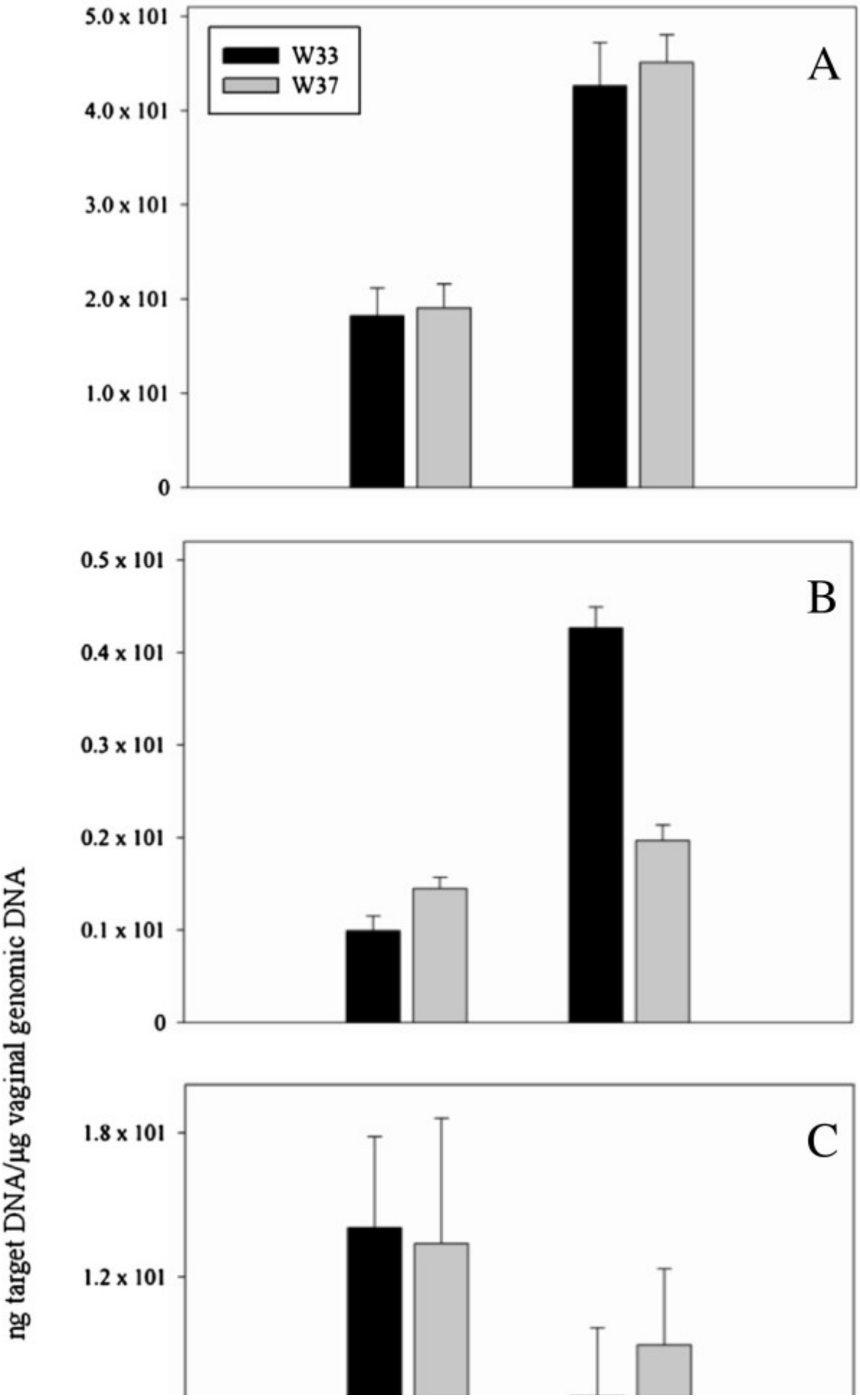
W33
W37
A
B
C
5.0 x 101
4.0 x 101
3.0 x 101
2.0 x 101
1.0 x 101
0
0.5 x 101
0.4 x 101
0.3 x 101
0.2 x 101
0.1 x 101
0
1.8 x 101
1.2 x 101
ng target DNA/µg vaginal genomic DNA

Figure 3. qPCR evaluation of *Lactobacillus*(A),*Bifidobacterium*(B),*Atopobium*(C)and*Prevotella*(D). Analysis was performed on vaginal samples collected at 33rd (W33) and 37th (W37) week of gestation from pregnant women supplemented (P) and not supplemented (C) with VSL#3. Data are expressed as ng of DNA of the target genus per μg of total bacterial DNA extracted from the vaginal sample. The diagrams show the mean values with error bars representing the standard deviations.

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Immunological profiles

The effect of the probiotic intake on the vaginal immune response was evaluated by measuring the levels of 27 cytokines, chemokines and growth factors in the vaginal samples of the pregnant women belonging to P and C groups.

Figure 4 shows the cytokines and chemokines whose concentration significantly changed in P and C groups during the study period ($P < 0.05$). In group C, significant reductions at W37 were found for 5 mediators, 4 cytokines [IL-4 (mean value, W33: $2.8 \times 10^{-2} \pm 1.5 \times 10^{-2}$; W37: $1.3 \times 10^{-2} \pm 6.9 \times 10^{-3}$), IL-7 (mean value, W33: $1.2 \times 10^{-1} \pm 8.6 \times 10^{-2}$; W37: $6.1 \times 10^{-2} \pm 3.5 \times 10^{-2}$), IL-9 (mean value, W33: $1.1 \pm 5.6 \times 10^{-1}$; W37: $3.7 \times 10^{-1} \pm 1.5 \times 10^{-1}$) and IL-10 (mean value, W33: $1.5 \times 10^{-1} \pm 1.1 \times 10^{-1}$; W37: $9.4 \times 10^{-2} \pm 5.4 \times 10^{-2}$)] and 1 chemokine [RANTES (mean value, W33: 4.3 ± 2.9; W37: $1.3 \pm 3.9 \times 10^{-1}$)]. Both IL-4 and IL-10 are produced by Th2 cells and exert a regulatory role in the immune response. IL-7 and IL-9 are hematopoietic growth factors that control proliferation and homeostasis of a variety of hematopoietic cells. RANTES is a pro-inflammatory chemokine which attracts monocytes, lymphocytes, basophils and eosinophils in the inflammatory response. In P group a significant variation was registered only for the chemokine Eotaxin, which decreased after probiotic supplementation (mean value, W33: 5.3 ± 8.8; W37: 2.0 ± 2.1). Eotaxin exerts a pro-inflammatory activity by recruiting eosinophils during allergic responses.

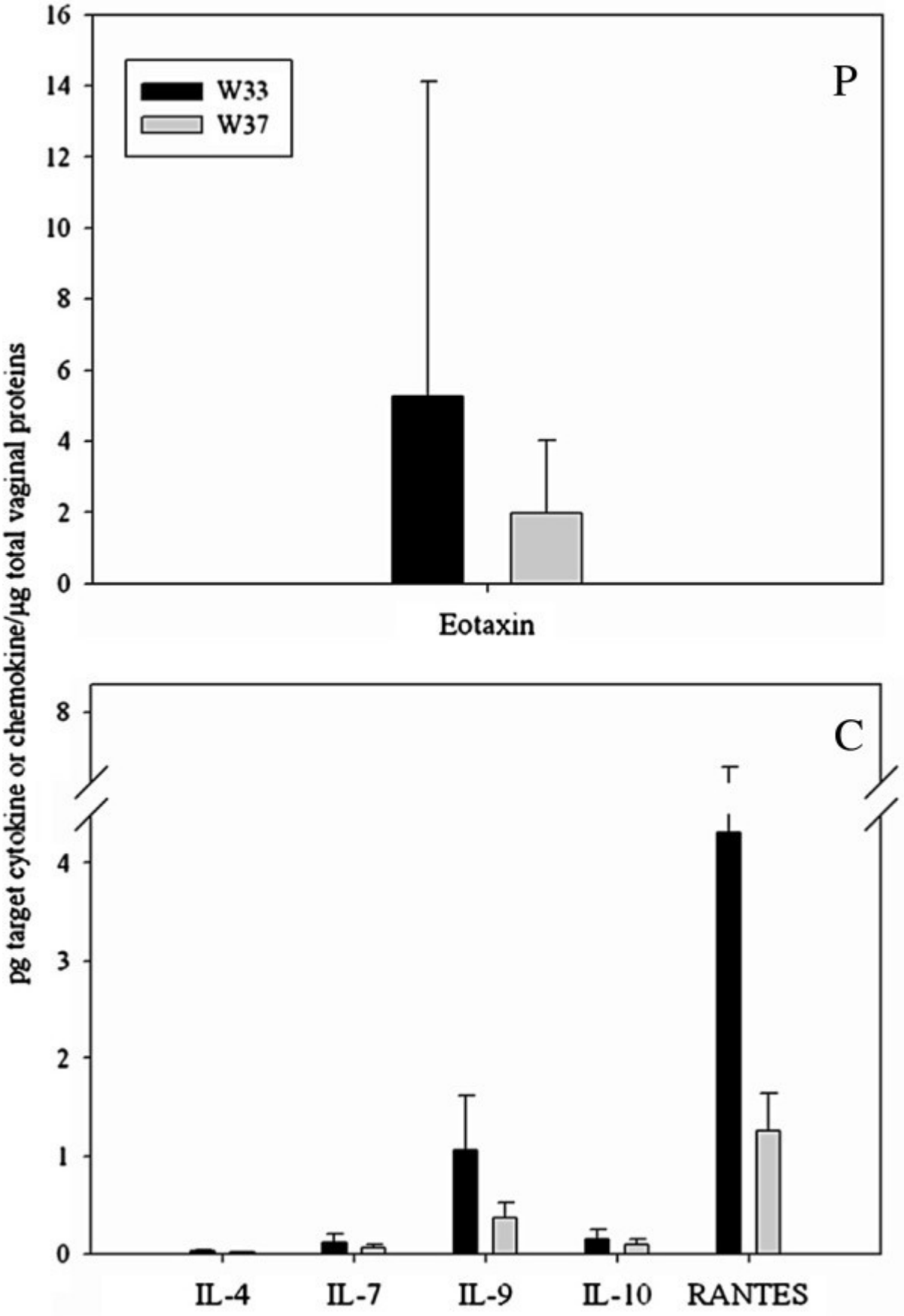

Figure 4. Cytokines and chemokines whose concentration significantly changed during the study period (*P*<0.05). P, probiotic group; C, control group; W33, 33rd gestational week (black colour); W37, 37th gestational week (grey colour). Cytokine or chemokine names are reported in x-

axis. Data are expressed as pg of the target cytokine or chemokine per μg of total proteins present in the vaginal sample (y-axis). The diagrams show means with error bars representing the standard deviations.

Figure 5 shows women, belonging to P and C groups, who registered significant variations in total levels of immune-mediators during the study period ($P < 0.05$). Significant changes were found for women N. 18, 19, 20, 21, 23, 24, 25 and 27 (8/12; 67%) of C group and women N. 1, 2, 3, 10, 11 (5/15; 33%) of P group.

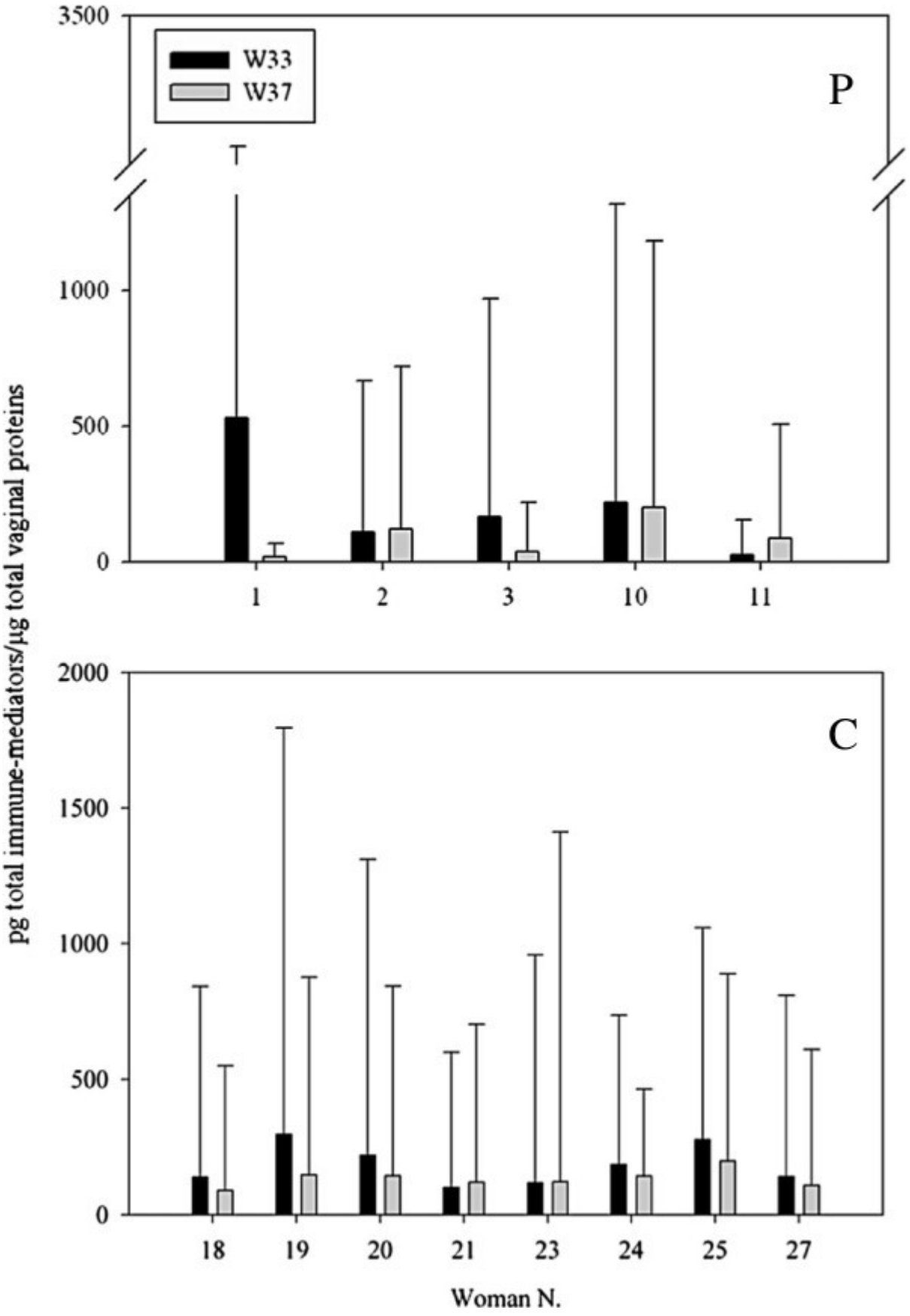

Figure 5. Women registering significant variations in total levels of immune-mediators. P, probiotic group; C, control group; W33, 33rd gestational week (black colour); W37, 37th gestational week (grey colour). Identification numbers of women registering significant variations are reported in x-axis. Data are expressed as pg of total immune-mediators per µg of total vaginal proteins (y-

axis). The diagrams show means with error bars representing the standard deviations.

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Discussion

To our knowledge, this is the first study describing the effect of a probiotic mixture, orally consumed during the last trimester of pregnancy, on the vaginal microbiota and immune response. Although several health-promoting activities of probiotics have been described in relation to the gut homeostasis [16,32], less information is available regarding the interactions between orally administered probiotic bacteria and the vaginal microbial habitat.

The first step in ascertaining the influence of the dietary supplementation with the probiotic VSL#3 on the vaginal microbiota of pregnant women was the characterization of vaginal bacterial communities by using an integrated approach based on PCR-DGGE and qPCR.

DGGE population profiling, conducted with universal primers for bacteria and *Lactobacillus*-specific primers, allowed us to investigate the variations of the predominant vaginal bacterial communities and *Lactobacillus* species occurring both physiologically in the last trimester of pregnancy and potentially associated with VSL#3 intake. The influence of the probiotic intake in modulating the predominant bacterial populations and *Lactobacillus* species could be hypothesized since significant differences between DGGE profiles at W33 and W37 were found only in women belonging to P group. Notably, the lower percentage of women belonging to P group who displayed significant differences in *Lactobacillus*-specific DGGE profiles between W33 and W37, compared to the universal bacterial DGGE patterns, suggested a major stability of lactobacilli population and a more extended impact of the probiotic VSL#3 on total bacteria than lactobacilli. However, no significant changes in single species abundances were found between W33- and W37-related universal DGGE profiles. Differently, the statistical analysis of the peak heights of the *Lactobacillus*-specific DGGE densitometric curves allowed us to identify a band, corresponding to *L. helveticus*, which significantly decreased after probiotic supplementation. Strains belonging to *L. helveticus* are used as starter cultures in the manufacturing of a variety of fermented dairy products, to modulate flavor. The presence of *L. helveticus* in vagina, likely due to the migration from the gut, can be related to a diet rich in yogurt and cheese. This work is not the first describing *L. helveticus* in vaginal samples. Stoyancheva *et al*. [33] identified this species among several *Lactobacillus* isolates from vaginal fluids of healthy Bulgarian women in childbearing age by using three different molecular techniques, amplified ribosomal DNA restriction analysis, ribotyping and PCR with

species-specific primers. The decrease of *L. helveticus* observed in our study could be due to a competition between the *Lactobacillus* strains present in VSL#3 formula and dairy *L. helveticus* strains in colonizing vaginal environment.

Cluster analysis showed that universal and *Lactobacillus*-specific DGGE profiles related to the time points W33 and W37 of the control women were closely related. Also the DGGE patterns of the majority of women administered with VSL#3 grouped according to the subject and not to the time point, revealing that the inter-individual variability was higher than variability induced by the probiotic supplementation.

The hypothesis of a positive action of VSL#3 on the vaginal microbiota of pregnant women was further supported by qPCR results, which suggested a role of the probiotic product in counteracting the decrease of the health-promoting *Bifidobacterium* genus and the increase of the BV-related *Atopobium* genus, that occurred in control women during late pregnancy. Notably, group B *Streptococcus*, which was found in two women (N.1 and 10) before the probiotic intake, was no longer found after the dietary supplementation (data not shown).

The second step of the present research was the investigation of the vaginal immunological profiles of the pregnant women in order to search for correlations between the VSL#3 intake and changes in vaginal immune response. Pregnancy has been referred to as a state of relative immune compromise. This notion has been related to both demonstration of depression of certain aspects of cell-mediated immunity and clinical observations of an increased severity of numerous infectious conditions in pregnant women [7]. On the other hand, preterm cervical ripening can be likened to an inflammatory process with cytokines as important mediators [34].

Bioplex immunoassay was used in the present work to measure levels of 27 cytokines, chemokines and growth factors in the vaginal samples of the pregnant women belonging to P and C groups. In group C a significant reduction at W37 was found for IL-4, IL-7, IL-9, IL-10 and RANTES. IL-4 is a key regulator in humoral and adaptive immunity. It has many biological roles, including the stimulation of activated B-cells and T-cell proliferation, and the differentiation of CD4+ T-cells into Th2 cells. A regulatory role is also exerted by IL-10. In relation to pregnancy, IL-10 decreases the production of pro-inflammatory cytokines, such as IL-8, IL-6, TNFα, IL-1β and prostaglandin E_2 in lipopolysaccharide-stimulated fetal membranes [35,36]. Both IL-4 and IL-10 are produced by Th2 cells. IL-7 and IL-9 are hematopoietic growth factors that act as regulators of cell survival, proliferation and homeostasis of a variety of hematopoietic cells. RANTES is a potent and versatile chemokine, capable of attracting monocytes, lymphocytes, basophils and eosinophils. This cytokine

has been implicated in the regulation of the inflammatory response and recruitment of macrophages to the implantation site in early pregnancy [37]. However, no variations in RANTES levels have been associated with preterm cervical ripening and labor [34]. Immunological profiles related to women belonging to C group indicated that some fluctuations in vaginal immune-modulators occurred physiologically during the last trimester of pregnancy. In particular, it is noteworthy the decrease of IL-10 and IL-4, important regulatory cytokines controlling the inflammatory reaction responsible for uterine contractions and cervical ripening at the labor time [12]. In P group a significant variation was registered only for the chemokine Eotaxin, which decreased after probiotic supplementation. Eotaxin selectively recruits eosinophils, and for this reason is implicated in allergic responses [38]. By comparing the data related to the two study groups, the following hypotheses could be formulated regarding the possible impact of the probiotic intake on cytokine secretion during late pregnancy: (i) probiotics counteracted the decrease of anti-inflammatory cytokine levels occurring in C group; (ii) probiotics induced the decrease of a pro-inflammatory cytokine in P group, showing a global anti-inflammatory effect on the vaginal immunity. In addition, a stabilization effect on the vaginal immunity during late pregnancy could be attributed to the probiotic intake, since the percentage of women with modified amounts of immune-mediators decreased from 67% to 31% in relation to the dietary supplementation.

Conclusion

The impact of the oral intake of the probiotic VSL#3 on the vaginal microbiota and immune response of pregnant women was investigated by molecular fingerprinting techniques (PCR-DGGE and qPCR) and Luminex® immunoassay. The major findings of this study are the following: (i) VSL#3 intake seems to be associated with a modulation of the predominant vaginal bacterial communities; (ii) VSL#3 modulation of *Lactobacillus* population appears to be related to variations of *L. helveticus* species; (iii) a potential role of the probiotic product in counteracting the physiological decrease of *Bifidobacterium* and increase of *Atopobium* could be hypothesized; (iv) the probiotic supplementation can be associated with a global anti-inflammatory effect on the vaginal immunity, with potential implications in preventing preterm birth.

Methods

Study design and sample collection

A pilot, not randomized, controlled and perspective study was conducted. The study protocol was approved by the ethical committee of the University of Bari, Italy. Written informed consent was

obtained from all the participants in the study. A total of 27 healthy pregnant women (21 to 42 years of age; mean, 32) who had no symptoms of vaginal or urinary tract infection were included in the present study (Table 3). None of the subjects had received oral or local antimicrobial therapy within the previous 2 weeks. The recruited subjects were divided into 2 groups: (i) probiotic group [P (n=15)]; (ii) control group [C (n=12)] on the basis of their availability to consume the probiotic product. Women of the P group consumed 1 sachet once/day of VSL#3 (VSL Pharmaceuticals, Inc.,Towson, MD, USA) for 4 weeks from the 33rd (W33) to the 37th (W37) week of gestation. Women of the C group did not receive any dietary supplementation. VSL#3 sachet contains 900 billion viable lyophilized bacteria consisting of 4 strains of *Lactobacillus* (*L. paracasei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*), 3 strains of *Bifidobacterium* (*B. longum*, *B. breve*, *B. infantis*) and 1 strain of *Streptococcus thermophilus*. Mid-vaginal swabs were collected from women of both P and C groups at the time points W33 and W37. Samples were placed in 1 ml of sterile saline and stored immediately at −80°C until use.

Table 3

Characterization of the subjects included in the study groups

Woman N	Age	Type of delivery[1]	Gestational age at birth
Probiotic (n = 15)			
1	31	SD	39 week + 6 days
2	32	CD	40 week + 3 days
3	39	SD	40 week + 1 day
4	31	SD	40 week + 2 days
5	33	SD	40 week + 3 days
6	30	SD	39 week
7	33	SD	41 week + 3 days
8	34	CD	39 week
9	36	CD	38 week + 4 days
10	38	SD	38 week + 5 days
11	42	SD	39 week + 4 days
12	30	SD	39 week
13	29	SD	40 week + 2 days
14	33	CD	39 week + 2 days
15	25	SD	40 week + 1 day
Control (n = 12)			
16	28	SD	40 week + 6 days
17	33	SD	39 week + 3 days
18	33	CD	37 week + 4 days
19	32	CD	41 week + 3 days
20	34	SD	40 week
21	21	SD	39 week + 5 days
22	30	SD	38 week + 6 days
23	30	SD	40 week + 2 days

Table 3

Characterization of the subjects included in the study groups

Woman N	Age	Type of delivery[1]	Gestational age at birth
24	34	CD	39 week + 6 days
25	38	CD	41 week + 1 days
26	38	CD	38 week + 5 days
27	30	SD	40 week + 2 days

[1] SD: spontaneous delivery; CD: caesarean delivery.

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The individual characteristics (age, type of delivery and gestational age at birth) of women enrolled in the present study are reported in Table 3. Gestational age was determined by utilizing the last menstrual period and earliest ultrasound.

DNA extraction from vaginal samples

Frozen vaginal swabs were thawed, mixed by vortex shaker for 1 min and then removed from the liquid. The liquid was centrifuged at 10,000 × g for 15 min, and the pellet was washed 3 times in saline at 40°C. The pellet was resuspended in 180 μl of enzymatic lysis buffer (20 mM Tris–HCl, pH 8, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and incubated at 37°C for 30 min. Glass beads (200 mg) were added and the sample was mixed by vortexing for 1 min. Total DNA was extracted by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol “Pretreatment for Gram-positive bacteria”. A slight modification was introduced: a centrifugation step (8000 × g for 5 min) was carried out after incubation with proteinase K to remove glass beads. DNA amounts were quantified by using NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

PCR-DGGE and cluster analysis

Amplification reactions were performed in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). GoTaq Flexi DNA Polymerase (Promega, Madison, WI) was used as thermostable DNA polymerase. The reaction mixture contained 0.5 μM of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$ solution, 1.25 U of GoTaq Flexi DNA Polymerase, 5 μl of Green GoTaq Flexi buffer 5X, and 2 μl of the bacterial DNA template (30–40 ng) in a final volume of 25 μl. The universal primers HDA1-GCclamp and HDA2 for bacteria [39] were used to amplify a conserved region within the 16S rRNA gene. The thermocycle program consisted of the following time and temperature profile: 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 60 s; and

72°C for 8 min. The *Lactobacillus* genus-specific primers Lac1 and Lac2-GCclamp [40] were used to amplify a specific region of the 16S rRNA gene of lactobacilli. The amplification program was 95°C for 5 min; 35 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 60 s; and 72°C for 8 min. A volume of 8 μl of PCR samples was loaded on DGGE gels, containing 30-50% and 25-55% gradients of urea and formamide for universal bacteria and lactobacilli, respectively. DGGE analysis was performed by using the D-Code Universal Mutation System Apparatus (Bio-Rad, Los Angeles, CA), as previously described [22]. Following electrophoresis, gels were silver stained [41] and scanned using a Molecular Imager Gel Doc XR System (Bio-Rad). DGGE gel images were analyzed using the FPQuest software version 4.5 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion to electrophoresis, the DGGE patterns were aligned and normalized using an external reference marker. The marker for the DGGE analysis with the universal primers for bacteria contained PCR amplicons from *Bacteroides*, *Coriobacterium*, *Enterococcus faecalis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Acidaminococcus fermentas* and *Atopobium*. The marker for the DGGE analysis with *Lactobacillus*-specific primers contained PCR amplicons from *L. plantarum*, *L. paracasei*, *L. brevis*, *L. gasseri*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*. After normalization, bands were defined for each sample using the appropriate densitometric curve. The similarity in the profiles was calculated on the basis of the Pearson correlation coefficient with the Ward clustering algorithm. Cluster analysis of the DGGE patterns was performed using the FPQuest software.

Sequencing of DGGE fragment

The DNA fragment of interest was excised from the denaturing gel with a sterile scalpel, washed once in 1X PCR buffer, and incubated in 20 μl of the same buffer overnight at 4°C. Two μl of the buffer solution were used as a template for PCR reaction. Reamplification of the 16S rRNA region was conducted as described above by employing the primers Lac1 and Lac2 (without the GC-clamp). The re-amplified fragment was purified using the Wizard SV Gel and PCR Clean-up system (Promega), and then subjected to automated sequence analysis of both DNA strands with Lac1 and Lac2. BigDye terminators (ABI-PerkinElmer, Foster City, CA) were used with a 377 sequencer (ABI). Sequence identity was determined by comparison with the rRNA gene sequences deposited in GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST webcite).

Quantitative real-time PCR

Quantitative PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) and

SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Each DNA sample was amplified with different genus- or species-specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region: Bact-0011f/Lab-0677r [42] for *Lactobacillus*, Bif164/Bif662 [43] for *Bifidobacterium*, Th1/Th2 [44] for *Streptococcus thermophilus*, F-GV1/R-GV3 [45] for *Gardnerella vaginalis*, c-Atopo-f/c-Atopo-r [46] for *Atopobium*, g-Prevo-f/g-Prevo-r [47] for *Prevotella*, VeilloF/VeilloR [48] for *Veillonella*. Amplifications were carried out in a final volume of 20 µl containing 0.5 µM of each primer, 4 µl of LightCycler-FastStart DNA Master SYBR Green I (Roche) and either 2 µl of template or water (no-template control).

The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min followed by 30 (*Lactobacillus*, *Atopobium*, *G. vaginalis* and *Veillonella*), 35 (*Prevotella*) or 40 (*Bifidobacterium*, *S. thermophilus*) cycles of denaturation at 95°C for 15 s; primer annealing at 63°C (*Lactobacillus*, *S. thermophilus*), 62°C (*Veillonella*), or 60°C (*Bifidobacterium*, *Atopobium*, *Prevotella*, *G. vaginalis*) for 20 s; extension at 72°C for 45 s (*Lactobacillus*, *Atopobium*, *Prevotella*, *G. vaginalis*, *Veillonella*), 30 s (*Bifidobacterium*), or 15 s (*S. thermophilus*) and a fluorescence acquisition step at 85°C (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*, *S. thermophilus*), 87°C (*Prevotella*) or 90°C (*Bifidobacterium*) for 5 s. DNAs extracted from *L. acidophilus* NCFM, *B. longum* NCC2705, *G. vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790, *Atopobium vaginae* ATCC BAA-55 and *S. thermophilus* ATCC 19258 were used as standards for PCR quantification. DNAs extracted from vaginal samples were amplified in triplicate for each primer set and the mean value was used for statistical analysis. Data were expressed as ng of DNA of the targeted genus or species per µg of total DNA extracted from the vaginal sample.

Bioplex immunoassay

Cytokine levels were determined using a multiplexed bead immunoassay. Prior to assay, vaginal samples were concentrated 10 times with Microcon spin devices (YM3, Millipore Corporation, Billerica, MA) and subsequently resuspended in Bio-Plex Assay Buffer. The levels of 27 immune-mediators, 15 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IFN-γ, TNFα), 7 chemokines (MCP-1, MIP-1α, MIP-1β, RANTES, Eotaxin, IL-8, IP-10) and 5 growth factors (PDGF-BB, FGF basic, G-CSF, GM-CSF, VEGF), were measured using the human ultrasensitive cytokine 27-plex antibody bead kit (Bio-Rad). Assays were performed in 96-well filter plates, as previously described [49]. Briefly, the filter plate was prewetted with washing buffer (Bio-Rad) and the solution was aspirated from the wells using a

vacuum manifold (Millipore Corporation). Microsphere beads coated with monoclonal antibodies against the different target analytes were added to the wells. Samples and standards were pipetted into the wells and incubated for 30 min with the beads. Wells were washed using a vacuum manifold (Millipore Corporation) and biotinylated secondary antibodies were added. After incubation for 30 min, beads were washed then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, R-phycoerythrin (streptavidin/R-phycoerythrin). After washing to remove the unbound streptavidin/R-phycoerythrin, the beads (a minimum of 100 per analyte) were analyzed in the Luminex 200 instrument (MiraiBio, Alameda, CA). The Luminex 200 monitors the spectral properties of the beads to distinguish the different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentration of the samples was estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software version 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve. The intra-assay CV including ultrafiltration and immunoassay averaged 19%. Concentrations of cytokines, chemokines and growth factors were then converted in pg of the target molecule per μg of total proteins present in the vaginal sample.

Statistical analysis

Statistical analysis was performed using SigmaStat (Systat Software, Point Richmond, CA). For each subject, variations of the DGGE profiles related to the time points W33 and W37 were analyzed by Pearson correlation. Significant differences in the intensity of each DGGE band among all vaginal samples and in the amounts of the bacterial genera and species determined by qPCR were searched by using Wilcoxon Signed Rank Test. This test was also used to analyze differences in cytokines, chemokines and growth factors. A *P* value below 0.05 was considered statistically significant.

Competing interests

VSL Pharmaceuticals, Inc. is financing the article-processing charge. The authors declare that they have no other competing interests.

Authors' contributions

BV performed the study design, analysis and interpretation of the data and the writing of the paper. FC, MC and ST performed DGGE and qPCR experiments and statistical analysis of the data. MEB

and TC enrolled the subjects and collected the vaginal samples. ES and MCV carried out the Bioplex immunoassay. PB supervised the study. All authors read and approved the manuscript.

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Science News *... from universities, journals, and other research organizations*

Probiotics Are Secret Weapon for Fighting Symptoms of the Common Cold in College Students, Study Suggests

ScienceDaily (Oct. 22, 2012) — College students are notoriously sleep-deprived, live in close quarters and lead stress-filled lives, making them especially susceptible for contracting colds and upper-respiratory infections. For these reasons, a team lead by researchers at the University of Medicine and Dentistry of New Jersey-School of Health Related Professions (UMDNJ-SHRP) selected this population to study the effects of probiotic supplementation on health-related quality of life (HRQL) during the common cold.

The study, led by Registered Dietitian Tracey J. Smith, an adjunct professor at UMDNJ-SHRP, randomized 198 college students aged 18 to 25 and living on-campus in residence halls at Framingham State University in Massachusetts. Groups received either a placebo (97 students) or a powder blend containing Chr. Hansen's probiotic strains BB-12® and LGG® (101 students) for 12 weeks. Each day, students completed a survey to assess the effect of the probiotic supplementation.

Although there have been previous studies on the effect of probiotics on the duration of colds and severity of symptoms, this is the first study to investigate the effect of probiotic strains on HRQL during upper-respiratory infections, taking into account duration, symptom severity and functional impairment -- all important factors of HRQL. "HRQL is subjectively assessed by the patient and most simply defined as 'the component of overall quality of life that is determined primarily by the person's health and that can be influenced by clinical interventions,'" Smith says.

An article detailing the results of the study was published in the October 2012 issue of the *British Journal of Nutrition*. "We know that certain probiotic strains support immune health and may improve health-related quality of life during upper-respiratory infections," says Smith. "This double-blind study assessed how probiotic supplementation affects the duration and severity of symptoms, and the impact of symptoms on the daily life of infected students."

The study found that while all students caught colds at roughly the same rate, the students who took the probiotic supplementation experienced: • A duration of colds that was two days shorter (four days vs. six days) • Symptoms that were 34% less severe and • A higher quality of life that resulted in fewer missed school days (15 vs. 34 missed by students taking the placebo).

What makes probiotics so effective in treating symptoms of upper-respiratory infections? "Cold symptoms like a stuffy nose and sore throat are the body's inflammatory response toward a virus, not a direct action of the virus itself," explains Smith. "Probiotic microorganisms may soften your immune system's reaction by reducing your body's inflammatory response."

The Take-Away for the Public: "If cost is not an issue, then otherwise healthy persons who are especially stressed, sleep-deprived or living in close quarters [such as a college dormitory] could supplement daily during cold season with both LGG and BB12 to improve their quality of life if/when they do get a cold," says Smith. However she cautions that not all probiotics are created equal. "The study supports the combination of LGG and BB12 -- two very specific strains of probiotics. These two strains also are in a number of supplement-type products that are available over the counter," she says, "but consumers need to read the label to be sure that the product

contains Lactobacillus rhamnosus GG [LGG] and Bifidobacterium animalis lactis BB12 [BB12]. There also are some yogurts that contain LGG and/or BB12 but check the labels, since companies change the probiotics strains often."

"People should also recognize that probiotics are not for everyone," Smith continues. "Those considering probiotic supplementation should consult with their physician first."

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Journal Reference:

Tracey J. Smith, Diane Rigassio-Radler, Robert Denmark, Timothy Haley, Riva Touger-Decker. **Effect of Lactobacillus rhamnosus LGG® and Bifidobacterium animalis ssp. lactis BB-12® on health-related quality of life in college students affected by upper respiratory infections**. *British Journal of Nutrition*, 2012; : 1 DOI: 10.1017/S0007114512004138

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Probiotics Can Reduce Risk of Diarrhea Caused by Antibiotics, Study Finds

ScienceDaily (May 8, 2012) — A new RAND Corporation study finds that taking probiotics can reduce the risk of developing the diarrhea that is a common side effect of taking antibiotics.

Probiotics are microorganisms that are believed to improve health by maintaining a normal balance of microorganisms in the human intestines. They are contained in some food products and also are sold as nutritional supplements.

Pooling evidence from a large number of published research studies examining the effects of probiotics, RAND researchers found consistent evidence that probiotics can benefit people who are taking antibiotics.

The findings are published in the May 9 edition of the *Journal of the American Medical Association.*

"We found a clear beneficial effect of probiotics in preventing or treating antibiotic-associated diarrhea," said study co-author Sydne J. Newberry, a nutritional scientist and a researcher at RAND, a nonprofit research organization. "However, more work is needed to determine which types of probiotics work best, which patients are most likely to benefit from probiotics and whether there are any risks in using them."

As many as 30 percent of patients who take antibiotics suffer from diarrhea, a side effect that is a key reason why some patients do not follow through with a full course of antibiotic treatment. Interest in the possibility that probiotics might help or prevent this type of diarrhea has grown in recent years, resulting in a proliferation of individual studies.

Researchers from the RAND-based Southern California Evidence-Based Practice Center conducted an extensive review of the medical literature to find studies that examined whether probiotic use can prevent and treat antibiotic-associated diarrhea. Researchers pooled results from the studies together to gain a clearer understanding of whether probiotics can prevent or treat antibiotic-associated diarrhea.

Use of probiotics was associated with a 42 percent lower risk of developing diarrhea when taking antibiotics as compared to not using probiotics. The RAND analysis estimates that 13 people would need to use probiotics in order to prevent one case of antibiotic-associated diarrhea.

However, researchers say the evidence was insufficient to show which type of microorganism was better than another because the included strains were poorly documented and often given as blends of several types. Further, there was no indication that the response varied systematically by a person's age, the duration of antibiotic use or other clinical indications.

The RAND team says that future efforts to study the effects of probiotics should try to determine whether some strains or combinations of probiotics work better than others, work best with specific antibiotics and whether use of probiotics for antibiotic-associated diarrhea is associated with any health risks.

The study was supported by RAND's internal funds, and built upon an earlier study supported by the U.S. Agency for Healthcare Research and Quality, the National Institute of Health Office of Dietary Supplements, the NIH National Center for Complementary and Alternative Medicine, and

FDA Center for Food Safety and Applied Nutrition.

Internal funding was provided through RAND's Investment in People and Ideas program, which combines philanthropic contributions from individuals, foundations and private-sector firms with earnings from RAND's endowment and operations to support research on issues that reach beyond the scope of traditional client sponsorship.

Other authors of the study are Susanne Hempel, Dr. Alicia R. Maher, Zhen Wang, Jeremy N.V. Miles, Roberta Shanman and Breanne Johnsen, all from RAND, and Dr. Paul Shekelle from RAND and the West Los Angeles VA Medical Center.

Story Source:

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Prebiotic May Help Patients With Intestinal Failure Grow New and Better Gut

ScienceDaily (Oct. 15, 2012) — Adding the right prebiotic to the diets of pediatric patients with intestinal failure could replace intravenous feeding, says a new University of Illinois study.

"When we fed the carbohydrate fructooligosacharide (FOS) as a prebiotic, the gut grew and increased in function," said Kelly A. Tappenden, a U of I professor of nutrition and gastrointestinal physiology. "The study showed that using the correct pre- and probiotic in combination could enhance these results even more."

When FOS enters the intestines, bacteria convert it into butyrate, a short-chain fatty acid that increases the size of the gut and its ability to digest and absorb nutrients, she said.

But today's IV solutions don't contain butyrate and adding it would entail drug development trials and regulatory red tape. She wanted to see if adding this carbohydrate to the diet while continuing to provide most nutrients intravenously would cause the gut to start producing butyrate on its own. It worked.

According to Tappenden, at least 10,000 U.S. patients are totally reliant on intravenous feeding because their intestines have been surgically shortened.

Many of these patients are premature infants who develop necrotizing enterocolitis, a kind of gangrene of the intestine. In the U.S., one in eight infants is a preemie, and removing necrotized, or dead, intestine is the most common surgical emergency in these babies.

"Surgery saves their lives, but with so much intestine removed, they're unable to digest or absorb nutrients. These babies are also at risk for long-term complications, such as bone demineralization and liver failure. Our goal is to take kids who've had this resection and cause their gut to grow and adapt," she said.

She tested her hypothesis about butyrate using newborn piglets, an excellent model for the human infant in metabolism and physiology. Piglets with intestinal failure were assigned to one of four groups: a control group; a group whose diet contained FOS, a carbohydrate given as a prebiotic to stimulate the production of butyrate by beneficial bacteria; a probiotic, or actual live bacteria; and a combination of pre- and probiotics.

"We believed that bacteria in the gut would use the prebiotic to make butyrate and support intestinal growth. But we thought that might only happen in the group that received both pre- and probiotics because we didn't know if the newborn gut would have enough bacteria to make this important short-chain fatty acid."

Actually, the neonatal piglets did have enough bacteria in their guts, and the prebiotic alone was effective in increasing intestinal function and structure, she said.

"In fact, the probiotic that we used in one of the groups eliminated the beneficial effect of the prebiotic. That shows us that we need to be exceptionally careful in selecting the probiotic we use, matching it to the specific disease," she noted. Many consumers believe all probiotics are equal, but the effect of specific bacterial strains is different, she said.

"At this point, we can only recommend consumption of the FOS prebiotic alone," she added.

The article appears in the September 2012 issue of the *Journal of Parenteral and Enteral Nutrition*. Jennifer L. Barnes of the U of I and Bolette Hartmann and Jens J. Holst of the University of Copenhagen, Copenhagen, Denmark, are co-authors of the study, which was funded by grants from the National Institutes of Health.

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