JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 713–717 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.713–717.2004

Identification and H_2O_2 Production of Vaginal Lactobacilli from Pregnant Women at High Risk of Preterm Birth and Relation with Outcome

Mark Wilks,[1] Rebecca Wiggins,[2] Angela Whiley,[1] Enid Hennessy,[3] Simon Warwick,[1] Helen Porter,[4] Anthony Corfield,[2] and Michael Millar[1]*

Department of Microbiology, Barts and The London NHS Trust,[1] and Department of Environmental and Preventive Medicine, Wolfson Institute of Preventive Medicine, Barts and The London, Queen Mary School of Medicine and Dentistry,[3] London, and Division of Medicine[2] and Department of Pathology and Microbiology,[4] University of Bristol, Bristol, United Kingdom

Received 31 March 2003/Returned for modification 28 May 2003/Accepted 22 October 2003

Lactobacilli, principally the strains that are hydrogen peroxide (H_2O_2) producing, may have a protective effect against vaginal colonization by pathogenic species such as those that cause bacterial vaginosis. Previous reports have also suggested that H_2O_2-producing lactobacilli in the vagina may protect pregnant women against ascending infection of the chorioamniotic membranes and uterine cavity. We report the identification and H_2O_2 production of lactobacilli isolated from vaginal swabs collected at 20 weeks' gestation from a population of pregnant women at high risk of preterm birth. We also report the correlation between identification and H_2O_2 production in relation to the outcomes of chorioamnionitis and preterm birth. Lactobacilli were identified by partial 16S rRNA gene sequencing. H_2O_2 production by isolates was determined by a semiquantitative method. The most commonly isolated species were *L. crispatus*, *L. gasseri*, *L. vaginalis* and *L. jensenii*. Amounts of H_2O_2 produced by lactobacilli varied widely. The presence of lactobacilli producing high levels of H_2O_2 in the vagina of this population of pregnant women was associated with a reduced risk of bacterial vaginosis at 20 weeks' gestation and subsequent chorioamnionitis. *L. jensenii* and *L. vaginalis* produced the highest levels of H_2O_2. We postulate that H_2O_2-producing lactobacilli are able to reduce the incidence of ascending infections of the uterus and the subsequent production of proinflammatory molecules which are important in the pathogenesis of chorioamnionitis and preterm birth.

The adult human vagina is a complex ecosystem containing an abundance of microorganisms. In women of childbearing age this system is dominated by *Lactobacillus* spp., a genus of gram-positive, nonmotile rod-like bacteria, a defining characteristic of which is the ability to grow in acid media and tolerate acid conditions (pH < 4.5); lactobacilli also ferment carbohydrates to produce lactic acid. In bacterial vaginosis (BV) the balance of flora is changed with reduced numbers of lactobacilli and an increase in numbers of other facultative and anaerobic species such as anaerobic cocci *Prevotella* spp., *Gardnerella vaginalis*, and *Mobiluncus* spp. BV is associated with a number of poor health outcomes, including preterm birth (31). Lactobacilli, principally the strains that are H_2O_2 producing, may have a protective effect against vaginal colonization by pathogenic species such as those that cause BV (16) and possibly human immunodeficiency virus and gonorrhoea (26). Previous reports have also suggested that H_2O_2-producing lactobacilli in the vagina may protect pregnant women against ascending infection of the chorioamniotic membranes and uterine cavity (12, 18). There have been few studies in which lactobacilli have been identified to species level and in which H_2O_2 production in pregnant women has been determined. We report the identification and H_2O_2 production of lactobacilli isolated from vaginal swabs collected at 20 weeks' gestation from a population of pregnant women at high risk of preterm birth. We also report the correlation between identification and H_2O_2 production in relation to the outcomes of chorioamnionitis and preterm birth.

* Corresponding author. Mailing address: Department of Medical Microbiology, Royal London Hospital, 37 Ashfield St., London E1 1BB, United Kingdom. Phone: 44(0)20 7377 7080. Fax: 44(0)20 7377 7330. E-mail: m.r.millar@qmul.ac.uk.

MATERIALS AND METHODS

Women attending an antenatal clinic in Bristol, United Kingdom, and considered at high risk of preterm birth according to Creasy criteria (6) were asked to give informed consent to join the study. Ninety-nine women, 96 of whom had had a previous preterm birth, gave informed consent to join the study. Samples were taken at 20 weeks' gestational age when pregnant women in the United Kingdom at high risk of preterm birth are invited to the hospital for a 20-week anomaly ultrasound scan of the fetus and cervical length measurement. Seventeen women were excluded because their samples were not immediately frozen; in a further eight cases, no microbiological sample was taken, and in one case the sample was lost. We report the results from 73 samples. Placentas were examined for histological evidence of chorioamnionitis from 37 of these 73 women. Two high vaginal swabs were taken. One swab was rolled onto a glass slide for Gram staining and microscopy. The second swab was placed in 2 ml of 0.1 M Tris-HCl buffer, pH 7.4, and vortexed for 30 s to disperse adherent bacteria into the buffer solution. Two hundred microliters of the buffer solution was inoculated into an anaerobic cryogenic preservative (prereduced brain heart infusion broth, 10% glycerol, 0.002% resazurin) and stored at −70°C for subsequent microbiological analysis. Whenever possible, placentae from women in the study were sent for histological analysis for evidence of chorioamnionitis and/or inflammation of the umbilical cord (funisitis). Histologic chorioamnionitis was defined as inflammation of the placental surface, with polymorphonuclear leukocyte infiltration into the subchorionic space, intravillous space, or amniotic cavity. Gestational age at delivery was determined from early ultrasound scans or reliable last menstrual period dates when these were within 10 days of the scan.

Microbiology methods. The microbiological studies were carried out by staff with no access to clinical information with respect to the study population. Lactobacilli were recovered from frozen samples by inoculation of serial dilutions onto MRS agar (Unipath, Basingstoke, United Kingdom), which was incubated for 48 h at 35°C in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2. Serial dilutions were used to allow identification of distinct colonial types. Colony types were Gram stained, and single colonies were subcultured onto MRS agar and incubated overnight anaerobically. These cultures were used for DNA extraction (see below) and for determination of H_2O_2 production. H_2O_2 production was initially measured by the method of Song et al. (32). This method was replaced by a semiquantitative assay (Merckoquant Peroxide Test; Merck, Poole, United Kingdom) when it became clear that there was a considerable variation in the amount of H_2O_2 produced by individual strains. Results from this test are expressed in bands of H_2O_2 production: negative, 1 to 3, 3 to 10, 10 to 30, and 30 to 100 mg/liter of H_2O_2.

Identification of lactobacilli. Lactobacilli were identified to species level by 16S rDNA sequencing. Briefly, either genomic DNA was extracted from pure cultures using a QIAmp DNA minikit 4 (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions or a single colony from an isolation plate was suspended in 20 μl of water and added directly to the PCR mixture. A 1,350-bp fragment of 16S rRNA gene was amplified using oligonucleotide primers 5′-GAA CGC TGG CGG CGT GCC (Z1-forward) and 5′-TCC GCG ATT ACT AGC GAT TCC (Z2-reverse), targeted at highly conserved regions of bacterial 16S rRNA genes. The PCR was carried out in a total volume of 25 μl, with a 0.2 μM concentration of each primer, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega, Southampton, United Kingdom), 1× PCR buffer B (Promega), and 1 μl of genomic DNA template or cell suspension. The reaction mixture was incubated at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 58°C for 60 s, and 72°C for 60 s. Initially the PCR product was sequenced using the forward and reverse primers, along with the internal primers B4 5′-TAT TAC CGC GGC TGC TGG CA and 5′-TGC CAG CAG CCG CGG TAA TA on an ABI prism 3700 DNA analyzer (PE Biosystems) (23). These sequences were aligned using the Clustal W algorithm to produce a consensus sequence for each isolate. This was analyzed using BLAST at the NCBI site (1). Subsequently the isolates were identified by sequencing the first 530 bases of the PCR product using the internal primer B4 alone. The results were compared with sequences deposited at GenBank. All isolates were identified based on similarity scores between 97 and 100% (majority > 99%).

Antibiotic susceptibility testing. The antibiotic susceptibility of a representative group of strains was determined using a the British Society of Antimicrobial Chemotherapy disk diffusion method guidelines (see http://www.bsac.org.uk). These included *L. gasseri* (seven isolates); *L. crispatus* (six isolates); *L. vaginalis* (seven isolates); *L. jensenii* (nine isolates); and single isolates of *L. delbreukii*, *L. rhamnosus*, and *L. casei*. Lactobacilli were grown on Iso-Sensitest agar (Unipath, Basingstoke, United Kingdom) containing 5% horse blood at 37°C for 48 h under anaerobic conditions. Selected strains were also tested with E-test strips (AB Biodisk, Solna, Sweden) for penicillin using the same guidelines.

Statistical methods. Univariate analyses were undertaken. The dependent factors were the risk of the following adverse outcomes: BV, preterm birth, chorioamnionitis, and a combined preterm birth or chorioamnionitis. The predictors that were considered were presence or absence of vaginal lactobacilli, the maximum H_2O_2 production level of any of the lactobacillus isolates from an individual woman, and the presence or absence of each of the most common lactobacillus isolates. Multivariate analysis was not undertaken because the numbers were small. Chi-square tests or Fisher's exact tests were used as appropriate for data that could be put into tables with mutually exclusive and exhaustive cells. A chi-square test for trend was also used to see if there was likely to be a trend in the relationship between maximum H_2O_2 production and adverse outcomes. STATA software (release 7.0; StataCorp., College Station, Tex.) was used for all the tests.

RESULTS

Identification of lactobacilli. A total of 92 isolates of lactobacilli were obtained. Twelve samples had no lactobacilli, 33 had one distinct isolate, 26 had two isolates, and 1 each had three or four isolates. Five isolates could not be identified by sequencing as their DNA could not be amplified by PCR. There were significant differences between the amount of H_2O_2 produced by the four most commonly isolated species of lactobacilli (chi-square = 11.3, df = 3, P = 0.01) (Table 1).

TABLE 1. H_2O_2 production by different *Lactobacillus* species[a]

Lactobacillus species (no. of isolates tested)	No. of species that produced the following amount of H_2O_2 (mg/liter):				
	Negative	1–3	3–10	10–30	30–100
L. gasseri (27)	0	6	13	6	2
L. crispatus (21)	0	0	15	4	2
L. vaginalis (16)	0	1	6	7	2
L. jensenii (15)	0	0	3	3	9
L. oris (2)	0	0	1	1	0
L. casei (1)	1	0	0	0	0
L. rhamnosus (1)	0	0	1	0	0
L. fermentum (1)	1	0	0	0	0
L. delbrueckii (1)	0	0	0	0	1
L. reuteri (1)	0	0	1	0	0
L. acidophilus (1)	0	0	1	0	0
Unidentified (5)	0	0	1	2	2

[a] In the group including all women (n = 73), various maximum levels of H_2O_2 production from isolates were observed, as follows: negative, 12 women; 1 to 3 mg/liter, 2 women; 3 to 10 mg/liter, 22 women; 10 to 30 mg/liter, 19 women; 30 to 100 mg/liter, 18 women. In the group including women with chorioamnionitis data (n = 37), various maximum levels of H_2O_2 production from isolates were observed, as follows: negative, 8 women; 1 to 3 mg/liter, 2 women; 3 to 10 mg/liter, 8 women; 10 to 30 mg/liter, 9 women; 30 to 100 mg/liter, 10 women.

Seventy-seven percent of the *L. jensenii* isolates produced the equivalent of >10 mg of H_2O_2/liter, a significantly higher percentage than the two most commonly isolated common species *L. crispatus* and *L. gasseri* (31 and 29%, respectively) (P = 0.015 and 0.016, respectively). Sixty percent of *L. vaginalis* isolates also produced high levels of H_2O_2.

Lactobacillus identification, H_2O_2 production, and pregnancy outcome. Table 2 shows the relationship between BV at the time of sampling (20 weeks' gestational age), preterm birth, and chorioamnionitis and the H_2O_2 status of lactobacillus isolates. The higher the level of H_2O_2 production by lactobacillus isolates at 20 weeks' gestational age was, the lower the probability of BV was at that time of sampling and the lower the probability was of chorioamnionitis at the time of delivery. Table 3 shows the relationship between the commoner species of lactobacilli, BV, and pregnancy outcomes. The presence of either of the two species (*L. jensenii* and *L. vaginalis*) with the highest levels of H_2O_2 production was associated with rates of preterm birth and/or chorioamnionitis that were half those found in their absence. There was very little difference in the rates of preterm birth and/or chorioamnionitis in those with or without *L. crispatus* or *L. gasseri*. Differences were not statistically significant.

Antibiotic susceptibility. Thirty-three strains of lactobacilli were tested using the British Society of Antimicrobial Chemotherapy disk diffusion method. All of the 33 isolates were ciprofloxacin resistant, and all were erythromycin sensitive. Six of seven isolates of *L. vaginalis* were vancomycin resistant, compared with none of nine strains of *L. jensenii*. Single isolates of *L. crispatus* and *L. gasseri* were vancomycin resistant. Six of the seven isolates of *L. vaginalis* were moderately resistant to penicillin (MIC > 0.1 mg/liter), compared with none of nine isolates of *L. jensenii*. The MICs of penicillin for isolates of *L. jensenii* were <0.032 mg/liter. Isolates of *L. crispatus* and *L. gasseri* showed variable results for penicillin susceptibility.

TABLE 2. Association of adverse pregnancy outcomes with isolation of lactobacillus from the vagina and with maximum H_2O_2 isolate production

Adverse outcome (no. of women affected/total)	% (no.) of women with adverse outcome						P (χ^2 for trend)
	Vaginal lactobacilli were:		Maximum H_2O_2 isolate production (mg/liter) was:				
	Absent	Present	0–3[a]	3–10	10–30	30–100	
Bacterial vaginosis (11/72)	42 (5)[c]	10 (6)	36 (5)	27 (6)	0 (0)	0 (0)	0.0007
Preterm birth (18/73)	42 (5)	21 (13)	36 (5)	32 (7)	11 (2)	22 (4)	0.19
Chorioamnionitis (12/37)	56 (5)	25 (7)	50 (5)	50 (4)	22 (2)	10 (1)	0.031
Chorioamnionitis and/or preterm birth (26/73)	67 (8)[b]	30 (18)	57 (8)	45 (10)	16 (3)	28 (5)	0.028

[a] Includes women from whom no lactobacillus isolates were isolated.
[b] $P = 0.014$.
[c] $P = 0.005$.

DISCUSSION

There are few reports describing the species of lactobacillus found in the vagina of pregnant women and even fewer which have considered the relationship between those species and pregnancy outcome. One of the major problems has been the difficulty in accurately ascertaining the species by phenotypic methods (2, 22). Vaginal lactobacilli were described as *L. acidophilus* mainly on the basis of the site of isolation rather than any specific biochemical or genetic property. In this study we isolated *L. acidophilus* sensu stricto only once. The work of Johnson and coworkers using DNA homology studies led to the recognition of six groups within the *L. acidophilus* group, which were later accorded species rank (8, 20). Since then several other species have been described and their 16S RNA genes have been sequenced (7, 10). Although the genus *Lactobacillus* is currently defined primarily on the basis of DNA homology results, the more recent introduction of 16S RNA-based methods marks a further significant advance, removing the inevitable uncertainties and ambiguities over preparation of probes and choice of hybridization conditions, which can affect the interpretation of DNA hybridization studies. The widespread availability and reliability of DNA sequencing methods make 16S RNA the current method of choice for identification of lactobacilli. Several different *Lactobacillus* species may be present in the human gut (36) and in the oral cavity (25) of an individual, but this may not be the case in the human vagina during childbearing years. In this study only one lactobacillus species was present in most subjects. A recent study of 202 isolates from 23 Swedish women also reported isolation of single species in the majority of subjects (37). The range of species isolated from the human vagina is small and different from those isolated from the mouth and intestine. The most common vaginal species in this and other studies are *L. gasseri*, *L. crispatus*, *L. vaginalis*, *L. jensenii*, *L. iners*, and *Lactobacillus* sp. 1086V. The relative proportion of these species varies between studies. Whether differences in the prevalence of species in studies reflect geographical variations in species carriage is not clear. Two recently described vaginal species, *L. iners* and *L. fornicalis*, were not isolated in this study (7, 10). *L. iners* does not grow on MRS agar, which was used in this study and most other recent studies, so the failure to grow this strain is not surprising. *L. fornicalis* does grow on MRS agar and is related to, but distinct from *L. johnsonii* and *L. gasseri*. It can be differentiated by various methods including 16S rRNA sequencing.

There is a need for further work looking at the natural history of lactobacillus colonization in pregnant women. Although there is general agreement that at any one time there is only one species of lactobacillus colonizing the vagina, the lack of longitudinal studies in both pregnant and nonpregnant women means that the factors affecting the stability of lactobacillus populations have not been well characterized. In a recent study of 101 nonpregnant women, Vallor et al. found that 78 (77%) women were initially colonized by lactobacilli and 60 (77%) of these 78 maintained this state when samples were taken at 4 and 8 months. Of the 23 (23%) women who were negative for lactobacilli at baseline, 18 (78%) had acquired some lactobacilli at 4 or 8 months (35). However, only 8 of the 23 women became colonized with H_2O_2-producing strains of *L. crispatus* or *L. jensenii*, the predominant species in their study. There was some evidence that H_2O_2-producing lactobacilli were better able to persist in the vagina than non-H_2O_2-producing strains and that this persistence was associated with protection against BV (35). Exposure to antibiotics

TABLE 3. Association of adverse outcomes in pregnancy with common lactobacillus isolates

Adverse outcome (no. of women affected/total)	% (no.) of women with adverse outcome							
	L. jensenii		*L. vaginalis*		*L. crispatus*		*L. gasseri*	
	Absent	Present	Absent	Present	Absent	Present	Absent	Present
Bacterial vaginosis (11/72)	19 (11)	0 (0)	17 (10)	7 (1)	19 (10)	5 (1)	14 (7)	17 (4)
Preterm birth (18/73)	28 (16)	13 (2)	29 (17)	7 (1)	25 (13)	24 (5)	24 (12)	26 (6)
Chorioamnionitis (12/37)	39 (11)	11 (1)	31 (10)	40 (2)	29 (7)	39 (5)	39 (10)	18 (2)
Chorioamnionitis and/or preterm birth (26/73)	40 (23)	20 (3)	39 (23)	21 (3)	35 (18)	38 (8)	36 (18)	35 (8)

was a risk factor for loss of lactobacillus strains. In this study testing of a small number of isolates suggests that species vary in their in vitro antibiotic susceptibility. The extent to which in vitro results predict the response of the vaginal flora to patient exposure to antibiotics has not been determined. It may be that some antibiotics differentially damage the different species of lactobacilli present in the vagina. The strains of *L. jensenii* that we tested were particularly susceptible to antibiotics.

Most isolates of vaginal lactobacilli produced some detectable H_2O_2. The mechanism by which lactobacilli resist the antimicrobial activity of H_2O_2 is unknown. In this study and in most other recent studies (see Table 1), H_2O_2 production was tested after growth on solid medium for 48 h and incubation under anaerobic conditions. The optimum conditions for H_2O_2 production have not been established. It is not clear whether production is constitutive or inducible or the extent of dependence on phase of growth, medium constituents, and redox potential. Shaken aerobic cultures may result in higher levels of H_2O_2 production than anaerobic cultures (11, 33). Vallor et al. found isolates of *L. crispatus* and *L. jensenii* that did not produce H_2O_2, whereas all of our isolates of these species did produce H_2O_2. In our study, the production of hydrogen peroxide by lactobacilli was initially tested by looking for a color change produced in media containing tetramethylbenzidine incubated anaerobically as used in many other studies. However, we found the results difficult to interpret and only qualitative, and several alternatives were considered. H_2O_2 is used in a wide variety of industrial applications, such as decontamination and water purification, and the Merckoquant peroxidase strip test is used for testing levels of hydrogen peroxide and other peroxides in the field, where an easy to read semiquanititative or quantitative test is required. In our hands, this test proved easier to read and provided a semiquantitative assessment of how much hydrogen peroxide was produced. There were striking differences between the amounts of H_2O_2 produced by different isolates and by different species. Nearly all isolates had at least some detectable H_2O_2 activity with only two isolates, one isolate of *L. casei* and one isolate of *L. fermentum*, not producing any detectable H_2O_2. Nearly half (44 of 91) of all the isolates of lactobacilli produced from 3 to 10 mg of H_2O_2/liter. Two-thirds of the *L. jensenii* produced a very high level of H_2O_2 (30 to 100 mg/liter). It has been demonstrated in some studies that H_2O_2-producing vaginal lactobacilli have an inhibitory effect on colonization by the bacteria that predominate in BV (21). However, the extent of the contribution to the inhibition of vaginal colonization by potential pathogens due to H_2O_2 production by comparison with production of bacteriocins or organic acids, and competition for adhesion sites is not known (3).

The relationship between BV and pregnancy outcome has been studied extensively, whereas the relationship between lactobacilli colonizing the vagina and pregnancy outcome has received less attention. Very recently a mathematical model has been developed which uses the total microbiological findings at 20 weeks' gestational age to predict preterm birth. The model is currently being validated (27). There is evidence to suggest that the protective effect of lactobacilli is not restricted to women who are at high risk of preterm birth as in our study. In a prospective study of the presence of vaginal lactobacilli and preterm birth in 1,958 women with singleton pregnancies, 95% of 1,616 women who delivered at >37 weeks of gestation, 90% of 224 women who delivered between 33 and 36 weeks, and only 80% of 224 women who delivered at 33 weeks were colonized with lactobacilli (34). BV after 26 weeks gestation is associated with a relative risk of preterm delivery of 1.4 to 1.9, whereas before 16 weeks it is associated with relative risk of 5 to 7.5 (17). Attempts to reduce the incidence of preterm birth by treatment of BV in pregnant women have given variable results (14, 15). In the largest randomized control trial there was no effect on the rate of preterm birth when women were treated with metronidazole alone (4), although in that study women were not treated until after 16 weeks gestation. The timing of treatment may be critical in determining efficacy. Another possible explanation for the variable results of trials designed to reduce the frequency of preterm birth by treatment of BV is that treatment may also eliminate H_2O_2-producing lactobacilli. The antimicrobial susceptibility of a variety of *Lactobacillus* spp. have been reported in a number of studies, but there have been no reports pertaining to clinical isolates identified by molecular methods from the vagina of pregnant women. Although assessment of chorioamnionitis was only possible in 39 cases in this study, there was a significant correlation between the occurrence of chorioamnionitis and preterm birth, as has been reported by other groups (38). A current hypothesis for the pathogenesis of preterm birth is that pathogenic bacteria gain access to the lower pole of the uterus, where they set up an inflammatory reaction. This leads to the production of prostaglandins and cytokines, which ripen the cervix and eventually cause the uterus to contract. A number of recent studies suggest a role for the production of inflammatory mediators in pathogenesis of chorioamnionitis (5, 28–30). In previous studies chorioamnionitis has been associated with a number of adverse sequelae in addition to preterm birth (13, 31).

Chorioamnionitis is a leading cause of fetal and neonatal death, although studies of the contribution to outcome are complicated because of the association with preterm birth (9). Chorioamnionitis is associated with a decreased risk of respiratory distress syndrome but an increased risk of chronic lung disease (19, 24). Chorioamnionitis may also contribute to intrauterine growth retardation and thymic involution and to be a risk factor for cerebral palsy (39). In this study we found that the isolation of high level H_2O_2-producing strains of lactobacilli at 20 weeks' gestation was associated with a reduced risk of chorioamnionitis. Similarly, Hitti et al. showed that H_2O_2-producing lactobacilli isolated at the time of preterm labor were negatively associated with amniotic fluid infection, although H_2O_2 production was not measured quantitatively (18). Vallor et al. suggest that H_2O_2-producing strains of *L. crispatus* or *L. jensenii* should be considered for vaginal probiotics, as these strains appear not only to produce H_2O_2 but also to persist in the vagina during pregnancy (35). Our findings partially support this approach, but in addition the results presented here suggest that the amount of H_2O_2 produced by probiotic strains may be a crucial factor in determining efficacy. In this study we show that the amounts of H_2O_2 produced by lactobacilli vary widely and that the presence of strong H_2O_2-producing lactobacilli in the vagina of pregnant women is associated with a reduced risk of BV at 20 weeks' gestational age and subsequent chorioamnionitis. If H_2O_2-producing strains of

lactobacilli do a have a role, it is not surprising that the amount of H_2O_2 produced is important. We postulate that this may be because H_2O_2-producing lactobacilli are able to reduce the incidence of ascending infections of the uterus and the subsequent production of proinflammatory molecules which are important in the pathogenesis of chorioamnionitis and preterm birth.

ACKNOWLEDGMENTS

This study was partially supported by The Tommys Campaign.

We thank N. Kato, Gifu University, Tokyo, Japan, for the gift of lactobacillus strains and R. Whiley, Barts and the London School of Medicine and Dentistry, for advice on methods of H_2O_2 production.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, W. Willer, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Antonio M. A., S. E. Hawes, and S. L. Hillier.** 1999. The identification of vaginal lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. J. Infect. Dis. **180:**1950–1956.
3. **Aroutcheva, A., D. Gariti, M. Simon, S. Shott, J. Faro, J. A. Simoes, A. Gurguis, and S. Faro.** 2001. Defense factors of vaginal lactobacilli. Am. J. Obstet. Gynecol. **185:**375–379.
4. **Carey, J. C., M. A. Klebanoff, J. Hauth, S. Hillier, E. Thom, J. Ernest, R. P. Heine, R. P. Nugent, M. L. Fischer, K. J. Leveno, R. Wapner, and M. Varner.** 2000. Metronidazole to prevent preterm delivery in pregnant women with asymptomatic bacterial vaginosis. N. Engl. J. Med. **342:**534–540.
5. **Chaiworapongsa, T., R. Romero, J. C. Kim, Y. M. Kim, S. C. Blackwell, B. H. Yoon, and R. Gomez.** 2002. Evidence for fetal involvement in the pathologic process of clinical chorioamnionitis. Am. J. Obstet. Gynecol. **186:**1178–1182.
6. **Creasy, R. K., B. A. Gummer, and G. C. Liggins.** 1980. System for predicting spontaneous preterm birth. Obstet. Gynecol. **55:**692–695.
7. **Dicks, L. T. M., M. Silvester, P. A. Lawson, and M. D. Collins.** 2000. *Lactobacillus fornicalis* sp. nov., isolated from the posterior fornix of the human vagina. Int. J. Syst. Evol. Microbiol. **50:**1253–1258.
8. **Du Plessis, E. M., and L. M. Dicks.** 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*. Curr. Microbiol. **31:**114–118.
9. **Elimian, A., U. Verma, D. Beneck, R. Cipriano, P. Visintainer, and N. Tejani.** 2000. Histologic chorioamnionitis, antenatal steroids, and perinatal outcomes. Obstet. Gynecol. **96:**333–336.
10. **Falson, E., C. Pascual, B. Sjödén, M. Ohlén, and M. D. Collins.** 1999. Phenotypic and phylogenetic characterisation of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. Int. J. Syst. Bacteriol. **49:**217–221.
11. **Fontaine, E. A., and D. Taylor-Robinson.** 1990. Comparison of quantitative and qualitative methods of hydrogen peroxide produced by human vaginal strains of lactobacilli. J. Appl. Bacteriol. **69:**326–331.
12. **Goffeng, A. R., E. Holst, I. Milsom, L. Göran, P.-A. Lundberg, and B. Andersch.** 1997. Fetal fibronectin and microorganisms in vaginal fluid of women with complicated pregnancies. Acta Obstet. Gynecol. Scand. **76:**521–527.
13. **Hagberg, H., W.-B. Wennerholm, and K. Sävman.** 2002. Sequelae of chorioamnionitis. Curr. Opin. Infect. Dis. **15:**301–306.
14. **Hauth, J. C., R. L. Goldenberg, W. W. Andrews, M. B. DuBard, and R. L. Cooper.** 1995. Reduced incidence of preterm delivery with metronidazole and 15 erythromycin in women with bacterial vaginosis. N. Engl. J. Med. **333:**1732–1736.
15. **Hauth, J. C., S. Cliver, P. Hodgkins, W. W. Andrews, J. R. Schwebke, and E. W. Hood.** 2001. Mid-trimester metronidazle and azithromycin did not prevent preterm birth in women at increased risk: a double-blind trial. Am. J. Obstet. Gynecol. **185:**S86.
16. **Hawes, S. E., S. L. Hillier, J. Benedetti, C. E. Stevens, L. A. Koutsky, P. Walner-Hanssen, and K. K. Holmes.** 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J. Infect. Dis. **174:**1058–1063.
17. **Hay, P., R. Lamont, D. Taylor-Robinson, D. Morgan, C. Ison, and J. Pearson.** 1994. Abnormal bacterial colonisation of the genital tract and subsequent pre-term delivery and late miscarriage. Br. Med. J. **308:**295–298.
18. **Hitti, J., S. L. Hillier, K. J. Agnew, M. A. Krohn, D. P. Reisner, and D. A. Eschenbach.** 2001. Vaginal indicators of amniotic fluid infection in preterm labour. Obstet. Gynecol. **97:**211–219.
19. **Jobe, A. H., and M. Ikegami.** 2001. Antenatal infection/inflammation and postnatal lung maturation and injury. Respir. Res. **2:**27–32.
20. **Johnson, J. L., C. F. Phelps, C. S. Cummins, J. London, and F. Gasser.** 1980. Taxonomy of the *Lactobacillus acidophilus* group. Int. J. Syst. Bacteriol. **30:**53–68.
21. **Klebanoff, S. J., S. L. Hillier, D. A. Eschenbach, and A. M. Waltersdorph.** 1990. Control of the microbial flora of the vagina by H_2O_2-producing lactobacilli. J. Infect. Dis. **164:**94–100.
22. **Klein, G., A. Pack, C. Bonaparte, and G. Reuter.** 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. **41:**103–125.
23. **Ley, B. E., C. J. Linton, D. M. C. Bennett, H. Jalal, A. B. M. Foot, and M. R. Millar.** 1998. Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **17:**247–253.
24. **Lyon, A.** 2000. Chronic lung disease of prematurity. The role of intra-uterine infection. Eur. J. Pediatr. **159:**798–802.
25. **MacFarlane, T. W., and L. P. Samaranayake.** 1989 Clinical oral microbiology. Wright, London, United Kingdom.
26. **Martin, H. L., B. A. Richardson, P. M. Nyange, L. Lavreys, S. L. Hillier, B. Chohan, K. Mandaliya, J. O. Ndinya-Achola, J. Bwayo, and J. Kreiss.** 1999. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. J. Infect. Dis. **180:**1863–1868.
27. **Onderdonk, A. B., M. L. Lee, E. Lieberman, M. L. Delaney, and R. E. Tuomala.** 2003 Quantitative microbiologic models for preterm delivery. J. Clin. Microbiol. **41:**1073–1079.
28. **Patrick, L. A., and G. N. Smith.** 2002. Proinflammatory cytokines: a link between chorioamnionitis and fetal brain injury. J. Obstet. Gynaecol. Can. **24:**705–709.
29. **Romero, R., J. Espinoza, T. Chaiworapongsa, and K. Kalache.** 2002. Infection and prematurity and the role of preventive strategies. Semin. Neonatal. **7:**259–274.
30. **Shalak, L. F., A. R. Laptook, H. S. Jafri, O. Ramilo, and J. M. Perlman.** 2002. Clinical chorioamnionitis, elevated cytokines, and brain injury in term infants. Pediatrics **110:**673–680.
31. **Sobel, J. D.** 2000. Bacterial vaginosis. Annu. Rev. Med. **51:**349–356.
32. **Song, Y.-L., N. Kato, Y. Matsumiya, C.-X. Liu, H. Kato, and K. Watanabe.** 1999. Identification of and hydrogen peroxide production by fecal and vaginal lactobacilli isolated from Japanese women and newborn infants. J. Clin. Microbiol. **37:**3062–3064.
33. **Tomas, M. S., E. Bru, and M. E. Nader-Macias.** 2003. Comparison of the growth and hydrogen peroxide production by vaginal probiotic lactobacilli under different culture conditions. Am. J. Obstet. Gynecol. **188:**35–44.
34. **Usui, R., A. Ohkuchi, S. Matsubara, A. Izumi, T. Watanabe, M. Suzuki, and H. Minakami.** 2002. Vaginal lactobacilli and preterm birth. J. Perinat. Med. **30:**458–466.
35. **Vallor, A. C., A. D. A. May, S. E. Hawes, and S. L. Hillier.** 2001. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. J. Infect. Dis. **184:**1431–1436.
36. **Vasquez, A., T. Jakobsson, S. Ahrné, U. Forsum, and G. Molin.** 2002. Vaginal *Lactobacillus* flora of healthy Swedish women. J. Clin. Microbiol. **40:**2746–2749.
37. **Walter, J., G. W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. M. Loach, K. Munro, and T. Alatossava.** 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. **66:**297–303.
38. **Wu, Y. W., and J. M. Colford, Jr.** 2000. Chorioamnionitis as a risk factor for cerebral palsy: a meta-analysis. JAMA **284:**1417–1424.
39. **Yoon, B. H., R. Romero, J. B. Moon, S. S. Shimm, M. Kim, G. Kim, and J. K. Jun.** 2001. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. Am. J. Obstet. Gynecol. **185:**1130–1136.