Human Reproduction Vol.16, No.9 pp. 1809–1813, 2001

# Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source

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**BACKGROUND: The origin of the lactic acid that acidifies the vagina is not well established. It is widely accepted that during times of high oestrogen (during the neonatal period and again during a woman's reproductive years) large amounts of glycogen are deposited in the vaginal epithelium and that the glycogen is anaerobically metabolized to lactic acid. What is not established is whether lactic acid is primarily produced by vaginal bacteria or by vaginal epithelial cells. Human cells can make only L-lactate, while bacteria can produce both D- and L-, thus the D- to L-lactate ratio can indicate the relative contribution of bacterially derived lactic acid. METHODS: In this study, we used chiral HPLC to examine the percentages of D- and L-lactate in vaginal secretions, in primary cultures of bacteria from these vaginal secretions, and in cultures of lactobacillus isolates of vaginal origin. RESULTS: We found that in most vaginal secretion samples, >50% of the lactic acid was the D-isoform (mean 55%, range 6–75%, $n = 14$). CONCLUSIONS: Our results thus support the hypothesis that vaginal bacteria, not epithelial cells, are the primary source of lactic acid in the vagina.**

*Key words:* female reproductive tract/lactic acid/lactobacilli/pH/vagina

## Introduction

Vaginal acidity is important for the maintenance of vaginal health. The healthy vagina is generally found to have a pH of $4 \pm 0.5$ (Cohen, 1969; Paavonen, 1983; Andersch *et al*., 1986; Tevi-Benissan *et al*., 1997), and this acidity has been shown to be microbicidal for many sexually transmitted disease (STD) pathogens (Stamey and Timothy, 1975; Stamey and Kaufman, 1975; Hanna *et al*., 1985), including HIV (Tevi-Benissan *et al*., 1997). Sperm are also rapidly inactivated at pH 4 (Shedlovsky *et al*., 1942; Olmsted *et al*., 2000), but the presence of semen abolishes vaginal acidity for several hours, which allows an opportunity for fertilization (Masters and Johnson, 1966; Tevi-Benissan *et al*., 1997).

One of the hallmarks of bacterial vaginosis (BV) is a reduction in vaginal acidity. BV is a common syndrome in which there is an overgrowth of a variety of mostly anaerobic bacteria and a reduction in lactobacilli, as well as a marked increase in vaginal pH to >4.5. BV has been associated with premature birth (Minkoff *et al*., 1987; Eschenbach 1993; Sagawa *et al*., 1995), increased risk of HIV infection (Taha *et al*., 1998) and pelvic inflammatory disease (Hillier *et al*., 1996).

Vaginal acidity is thought to be produced by the anaerobic metabolism of glycogen to lactic acid (Moller and Kaspersen, 1992). Glycogen, which is deposited in large amounts in the vaginal epithelium during times of high oestrogen availability (Gregoire *et al*., 1971; Paavonen, 1983), could be metabolized to lactic acid either by vaginal bacteria and/or by the epithelium itself, since the vaginal lumen is anaerobic and the cells on the surface of the vaginal epithelium are relatively distant from the oxygen supply of the capillaries, especially when oestrogen concentrations are elevated and the thickness of the vaginal epithelium is correspondingly increased. Although bacteria are widely assumed to be the primary source of lactic acid, critical evidence is lacking and the primary source of lactic acid in the vagina is still under debate (Moller and Kaspersen, 1992; Pybus and Onderdonk, 1999).

Recently we reported that vaginal lactobacilli are capable of producing acid *in vitro* at a rate sufficient to account for the rate at which the vagina acidifies after being neutralized by the ejaculate (Boskey *et al*., 1999). This is consistent with vaginal acidity being primarily of bacterial origin, but does not exclude the possibility that anaerobic metabolism by vaginal epithelial cells plays a major role in acidifying the vagina.

Chiral molecules (optical isomers) are molecules with identical chemical composition, but which differ in structure by the asymmetric arrangement of functional groups around one or more of their carbons atoms (chiral centres). They are described by their ability to rotate light to the left (L-) or right (D-). Human metabolism can only produce L-lactate (Brin, 1965), while bacteria can produce both D- and L-lactate (Smith *et al*., 1989; Bongaerts *et al*., 1997; McCabe *et al*., 1998). Thus, D-lactate in serum has been found to be a marker for the

presence of a bacterial infection (Smith *et al*., 1986; Smith, 1991), and the presence of D-lactate can be similarly used as a marker for bacterial metabolism in other bodily fluids. This assay is related to the use of the succinate/lactate ratio for the diagnosis of vaginitis (Hillier, 1993). We used chiral HPLC, a method of separating molecules by the orientation of their chiral centres, to determine the relative amounts of D- and L-isoforms of lactate in vaginal secretions, in primary cultures of bacteria from these secretions, and in cultures of lactobacillus isolates of vaginal origin. Chiral HPLC was used to determine the D/L ratio of lactate isoforms since, unlike more conventional enzymatic methods, HPLC identifies and quantifies both isomers concurrently and is not sensitive to pH.

## Materials and methods

### *Ethics*

Vaginal secretions were collected under a protocol approved by the Human Subjects Review Board of Johns Hopkins University. Signed consent forms were obtained from each participant in the study. In addition, serum samples were provided by two women who gave additional consent.

### *Recruitment*

Eleven women of reproductive age (aged 19–32) were recruited by word of mouth from within the investigator's department, and women were requested not to donate if they were menstruating or were within 24 h after an episode of unprotected intercourse. Due to the small sample size, time of menstrual cycle and oral contraceptive use were not used as study variables; however, all samples were Gram stained to check for the presence of a lactobacillus-dominated vaginal flora (no samples needed to be discarded). Due to multiple donations from some women, over the course of the study there were 14 samples in all.

### *pH measurements*

In all cases, sample pH was measured using a pH meter (Beckman Φ11; Wilmington, DE, USA) with a calibrated microelectrode (M1-414-6 cm; Microelectrodes Inc., Bedford, NH, USA). All vaginal samples had pH between 4 and 4.5.

### *Collection of vaginal secretions*

Women were instructed to insert a commercial menstrual collection device (Instead® cup; Ultrafem, Missoula, MT, USA), wait 5 s, and then remove it and place it in a 50 ml conical tube (labelled with the woman's age and day of her menstrual cycle). They were provided with printed instructions for the use of the device, and a female investigator experienced in its use (E.R.B.) reviewed the instructions with them and was available to answer questions. The tube was spun at ~600 *g* for 15 min to pool the secretions at the bottom of the tube. The cup was then removed from the tube and discarded. Small aliquots of each sample were removed to measure pH and to inoculate media for primary cultures (see below). The samples were then diluted ~1:4–1:10 in phosphate buffered saline (PBS) as necessary to allow for filtration through a 0.2 μm syringe filter to prepare for HPLC. Dilution varied due to the range of viscosity of the vaginal secretion samples and the need to filter them prior to analysis. Three serum samples were collected from two women, with one of these being collected immediately after an extended period of exercise in an attempt to increase serum lactic acid concentration. These samples were similarly filtered (no dilution was necessary) for HPLC.

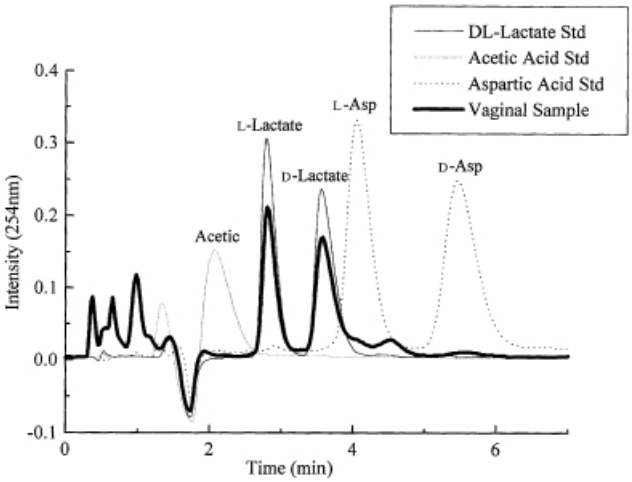


**Figure 1.** HPLC trace of a vaginal secretion sample (diluted ~1:7) compared with lactic acid (1.1 mmol/l), acetic acid (1.6 mol/l) and aspartic acid (3.8 mmol/l) standards.

### *Primary culture*

To examine lactate production from primary cultures of vaginal bacteria, a disposable plastic loop was used to inoculate a small amount of vaginal secretions into 1.5 ml MRS broth (BBL, Sparks, MD, USA) prepared at pH 5 to favour the growth of lactobacilli. After vortexing, the inoculated broth was split into two tubes. The pH was measured in the first tube (control), which was then filtered immediately through a 0.2 μm syringe filter to remove the bacteria. The second tube (primary culture) was incubated for 24 h at 37°C in 5%$CO_2$/95% air, then the pH of this primary culture was measured, and the culture was also filtered to prepare it for HPLC.

### *Stock culture*

To examine lactate production by stock cultures of vaginal isolates of lactobacilli, we tested three $H_2O_2$ positive vaginal isolates from each of the two species *L. jensenii* and *L. crispatus* (gift of Dr Sharon Hillier, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA), $H_2O_2$ negative vaginal isolates of *L. vaginalis* (ATCC no. 49540) and *L. gasseri* (ATCC no. 9857) and human isolates of *G. vaginalis* (ATCC no. 14018) [American type culture collection (ATCC), Manassas, VA, USA]. Freshly thawed aliquots of lactobacillus stock were inoculated in MRS broth (pH 6), and grown overnight at 37°C in 5%$CO_2$/95% air. As an additional control, freshly thawed aliquots of *G. vaginalis* stock were grown overnight in basal broth as described previously (McLean and McGroarty, 1996). Final sample pH was measured, and samples were filtered through a 0.2 μm syringe filter to prepare them for HPLC.

### *Chiral HPLC*

A D-penicillamine column (Phenomenex, Torrance, CA, USA) was used for all separations with 2 mmol/l $CuSO_4$: methanol (85:15) as the mobile phase. D-penicillamine binds more strongly to the D- form of the chiral centre of lactic acid allowing for easy separation of the L- and D-isomers. Dilute solutions of acetic acid, DL-aspartic acid (which has the same chiral centre as lactic acid), D-lactic acid, L-lactic acid, and a 50/50 racaemic DL-lactic acid were used as standards/controls. For all samples, a 5 μl injection volume was used (Waters U6K Injector; Waters Co., Milford, MA, USA). The flow rate was set at 1.0 ml/min (Waters 510 HPLC Pump; Waters Co.), and absorbance was detected at 254 nm (Waters 486 tunable absorbance detector; Waters Co.). Each sample was run a minimum of three times. Standards were run at the beginning and end of each

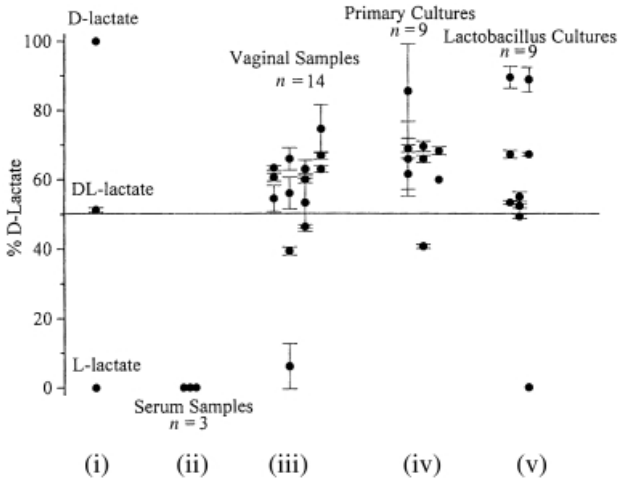


**Figure 2.** Percentage of lactic acid which is of the D- isoform in: (i) control solutions of D-lactate, L-lactate and racaemic (~50/50) DL-lactate; (ii) serum samples; (iii) vaginal secretion samples; (iv) primary cultures derived from a subset of the vaginal secretion samples; (v) lactobacillus cultures from stock vaginal isolates.

day, and also after every 5–6 samples. Using Origin 4.0 software (Microcal Software Inc., Northhampton, MA, USA), absorbance curves were fit to a baseline, and peaks were identified and measured. D- and L-lactate peaks were identified by their location relative to the DL standard. The percentage of D-lactate present was calculated by dividing the area under the D-lactate peak by the sum of the areas under both (D and L) peaks. Percentage of D-lactate = 100× (D-lactate)/[(D-lactate) + (L-lactate)].

### *Calculation of the relative amount of bacterial derived lactate*

Assuming that the D/L ratio of bacterially produced lactate is the same *in vitro* as *in vivo*, the ratio of bacterially-derived to epithelial cell (VEC)-derived lactic acid was calculated as follows.

Let C = ratio of D/L lactate in culture = $D_{bacteria}/L_{bacteria}$

Let V = ratio of D/L lactate in the vagina = $D_{bacteria}/(L_{bacteria} + L_{cells})$ Then

F = fraction of lactate produced by VEC = $L_{cells}/(D_{bacteria} + L_{bacteria} + L_{cells})$

$$F = \frac{(C/V)-1}{C + (C/V)}$$

## Results

### *Validation of the method*

As can be seen in Figure 1, the lactic acid peaks in a vaginal secretion sample were easily identifiable, and D- and L-lactate were easily distinguished using chiral HPLC. Due to the increased binding affinity of the chiral centre, D- peaks tended to be shorter and wider than L- peaks, but with 50/50 racaemic mixtures of D- and L-acids the areas under each peak were identical (see control results in Figure 2). D- and L-lactate were the predominant low molecular weight constituents found by HPLC in all vaginal samples. By comparing the areas under the D- and L-peaks with those obtained with the controls, the total lactate (D plus L) concentration in the vaginal secretions was found to be in the range 10–50 mmol/l. This is comparable with ~20 mmol/l, the concentration previously reported as being present in the vagina (Bauman *et al*., 1982). To assess the stability of the test solutions over time, all samples were measured on at least 2 separate days, and several samples were tested over a period of several weeks. There were no significant changes in the D-/L-lactate ratio in any of these samples.

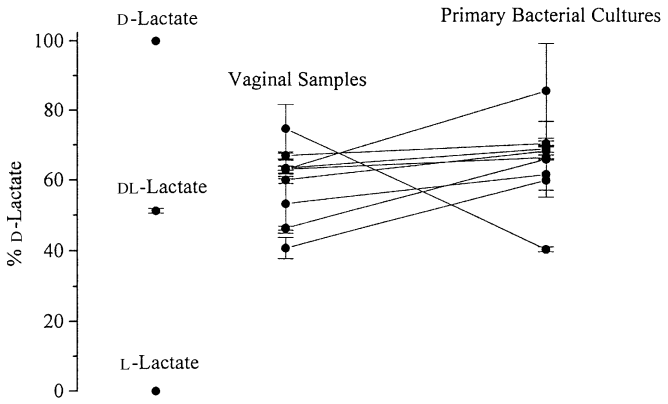


**Figure 3.** Percentage of D-lactate in vaginal samples compared with the percentage of D-lactate in primary cultures grown from those samples. Eight paired samples.

### *Percentage of D-lactate in vaginal secretions*

As shown in Figure 2, in most of the vaginal secretion samples (11 of 14) greater than 50% of the vaginal lactic acid was the D-isoform (mean 55%, range 6–75%, $n = 14$).

### *Percentage of D-lactate in primary cultures*

In primary cultures of vaginal bacteria, the percentage of D-lactate was, also, on average, greater than 50% (mean 66%, range 41–86%, $n = 9$) (Figure 2). The relationship between the percentage of D-lactate in each vaginal sample and its corresponding primary culture is shown in Figure 3.

### *Percentage of D-lactate in vaginal lactobacillus culture*

The percentage of D-lactate produced by nine isolates of vaginal lactobacilli was again, on average >50% D-lactate (mean 53%, range 0–90%) (Figure 2). Eight of the nine isolates made ~50% or greater D-lactate.

### *Calculation of the relative amount of bacterial derived lactate*

The percentage of vaginal lactic acid produced by bacteria was estimated by assuming that the percentage of D-lactate made by primary cultures of vaginal bacteria *in vitro* was the same as produced by these bacteria *in vivo*. This assumption is of necessity a simplification since it is unlikely that all the vaginal bacterial species metabolized the same way in culture as they did in the vagina. In fact, in one vaginal sample the percentage of D-lactate was higher in the vaginal secretion than in the primary culture from that sample (the markedly discordant pair shown in Figure 3). In the remainder of samples for which we had both primary culture and secretion data, we calculated that ~80% (mean 83%, range 70–96%) of the vaginal lactate was bacterial in origin.

## Discussion

Lactic acid is the primary molecule responsible for the acidification of the vagina (Doderlein, 1892; Zweifel, 1908; Weinstein *et al.*, 1936). Although acetic acid is also reported to be present in most women, we did not detect an acetic acid peak in most samples, and when we did, it was significantly smaller than the lactic acid peaks (see Figure 1). This was due in part to the relatively low sensitivity of our method for measuring acetic acid. However, our results are consistent with the literature which reports that in women with healthy lactobacillus-dominated vaginas the acetic acid concentrations are approximately one tenth those of lactate (Bauman *et al.*, 1982; Owen and Katz, 1999). Acetic acid is not a chiral molecule and is smaller than lactic acid, therefore it appears as a single, easily recognizable, early peak (Figure 1).

That glycogen in shed vaginal epithelial cells is the carbohydrate precursor of vaginal lactic acid was established as early as 1897 (Cruickshank and Sharman, 1934a). Various studies have shown that lactobacilli either can (Zweifel, 1908; Wylie and Henderson, 1969) or cannot (reviewed in Cruickshank and Sharman, 1934b) directly metabolize glycogen to lactic acid without it being pre-reduced to glucose by a mammalian enzyme. However, the two negative reports are open to question since these studies used commercial oyster glycogen that has a different C:H ratio than human vaginal glycogen and that has been shown to be less susceptible to fermentation by lactobacilli (Wylie and Henderson, 1969).

The ionic constitution of vaginal secretions differs significantly from that of plasma, and $Na^+$, $K^+$, and $Cl^-$ appear to play an important role in the regulation of vaginal lubrication and transudation across the vaginal mucosa. In the vagina, $K^+$ is elevated ~600% with respect to plasma, while $Na^+$ and $Cl^-$ are decreased to ~45 and 60% of plasma concentrations (Wagner and Levin, 1978; Moghissi, 1979; Wagner and Levin, 1980; Mende *et al.*, 1990). $Na^+$ and $Cl^-$ continuously enter the vaginal lumen both from the cervical secretions and by transudation from the blood through the vaginal epithelium. The vaginal epithelium actively reabsorbs $Na^+$ back into the interstitial fluid, generating the observed transvaginal potential difference of 25–50 mV negative to the blood (Wagner and Levin, 1978; Moghissi, 1979). $Cl^-$ presumably follows passively. Although it is possible that $K^+$ could be secreted by the vagina (Wagner and Levin, 1978), the $K^+$ concentration measured in the vaginal lumen is compatible with the range of vaginal potential differences observed if the $K^+$ passively distributes between the plasma and the luminal fluid according to the Nernst equilibrium (Wagner and Levin, 1978; Moghissi, 1979). The Nernst equation describes the passive diffusion of ions across a potential difference—in this example the transvaginal potential observed across the vaginal epithelium.

In contrast to the $K^+$ distribution, the $H^+$ distribution across the vaginal epithelium (pH of the acidic vagina compared with that of serum) cannot be explained by passive diffusion of protons across the potential difference. The Nernst equation predicts that for the observed potential difference of 25–50 mV across the vaginal epithelium, a $\Delta$ pH of 0.4–0.8 units from serum would be expected. This would correspond to a vaginal pH of 6.6–7. The observed pH of the vagina, ~4 in reproductive age women, is 3.4 units (on average) lower than that of serum (pH 7.4). For passive distribution of protons to create such a large drop in vaginal pH would require a transvaginal potential difference of almost 200 mV—four to eight times that which was observed. This implies that if the epithelium is the primary source of vaginal acidity it must do so by active transport of protons into the vagina. Even if the glycogen in the epithelial cells was anaerobically metabolized to lactic acid by these cells, in the presence of the observed transvaginal potential most of the protons would passively diffuse to the plasma—not the vaginal lumen. Regardless of whether the epithelium actively secretes acid, or whether bacterial metabolism creates acid, the transvaginal potential difference of 25–50 mV implies that when the vagina is more acidic than ~pH 6.5, protons will passively diffuse out of the lumen of the vagina and into the serum. That is, the vaginal epithelium will continuously passively absorb protons from the lumen whenever the vagina is more acidic than ~pH 6.5.

We have demonstrated in a group of 14 samples from 11 women that the majority of vaginal lactic acid was produced by bacteria. Since only bacteria can make the D- isoform of lactic acid, and since in 11 of 14 vaginal samples >50% of the lactic acid was D-lactate this implies that, at a minimum, 50–60% of their vaginal lactate was bacterial in origin. However, as bacteria also produce L lactate, and since different strains produce different proportions of D- and L-lactate, we measured the percentage of D-lactate primary cultures of vaginal bacteria produced in culture. The relative percentage of D-lactate was comparable, but generally slightly lower, in vaginal secretions then it was in the primary culture, possibly due to a small amount of L-lactate produced by the epithelial cells *in vivo*. Assuming that the bacteria produced the same relative amounts of D- and L-lactate in culture as they did in the vagina, we calculated that vaginal epithelial cells are probably only responsible for ~20% of the lactate in the vagina (mean = 17%, range = 4–30%) and that the majority of the vaginal lactate is bacterial in origin.

At the concentrations reported to be present in the human vagina, (~20 mmol/l) (Bauman *et al.*, 1982), lactic acid is more than capable of acidifying cervical mucus and serum to the approximate pH of the vagina (data not shown). The data presented here show that most of the lactate in the vagina must be bacterial in origin. Recently we reported that lactobacilli of vaginal origin acidify their growth media to a pH comparable with that of the lactobacillus-dominated vagina, and that these bacteria produce lactic acid at a rate sufficient to account for the rate of acid production in the vagina (as indicated by the rate at which the vagina reacidifes after intercourse) (Boskey *et al.*, 1999). Thus the acidity of the healthy, lactobacillus-dominated vagina cannot be produced primarily by the vaginal epithelium. Instead, all results available to date are consistent with the hypothesis that vaginal acidity is primarily produced by bacteria.

The contribution of vaginal acidity to reproductive health is becoming increasingly understood. Our confirmation of the role of lactobacilli in the acidification of the vagina suggests that whenever possible the normal vaginal flora should be

maintained during the course of clinical treatment for STD and other disorders. Furthermore, we have confirmed the need to test vaginal products, such as microbiocides, for harmful effects against lactobacilli before recommending them for regular use. In conclusion, the presence of lactobacilli as a part of the normal vaginal flora is an important component of reproductive health, and further research is needed to understand how such a healthy flora can be maintained.

## Acknowledgements

We thank Dr Sharon Hillier and May Antonio of the University of Pittsburgh School of Medicine for providing the *L. jensenii* and *L. crispatus* isolates used in these studies, and Drs Sharon Achilles and Larry Zeitlin for assistance with sample collection. This work was supported in part by NIH Training Grant T32-GM-0–7231 and by NIH Grant P01-A1-45467.

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*Received on February 9, 2001; accepted on May 8, 2001*