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Growth and Acidification by Vaginal Lactobacilli in Anaerobic Liquid Medium Over the pH Range 5.5 – 8.0

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Abstract

We studied the growth of three vaginal lactobacilli in a defined liquid medium under anaerobic conditions across the pH range 5.5 – 8.0. Growth and acidification profiles of the lactobacilli varied significantly. This model could aid the selection of strains – or combinations of strains – with probiotic potential.

The flora of the healthy vagina is simple and usually dominated by one or a few species of lactobacilli (LB) [7,10]. LB maintains the acidity of the vagina and are critical for vaginal health. Bacterial vaginosis (BV) is the most prevalent vaginal syndrome with major public health implications worldwide [6] and is characterized by a depletion of vaginal LB. Recurrence of BV is seen in approximately 30% of affected females and LB-containing probiotic preparations have been evaluated for treatment and prevention. A means of quantifying the growth and acidification potential of LB over a range of pH values would be useful for intra- and inter-species comparisons of LB considered as potential probiotic candidates. To address this, we studied three vaginal LB under anaerobic conditions in liquid medium over a range of pHs.

A defined liquid medium rendered anaerobic with 'Oxyrase for Broth' (Oxyrase Inc, Ohio, USA) was prepared and used as described previously [11] at initial pHs 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. In brief, media with and without buffer - and for each of these, with and without 1.25g/L human hemoglobin (Hb) - were adjusted to the required initial pH using 1M HCl before filter sterilizing through a 0.22μm filter. The buffers used were 30mM MES for pH 5.5 - 6.5 and 30mM MOPS for pH 7.0 - 8.0. Media were pre-reduced in 7mL screw-top plastic bijoux (Bibby Sterilin) by incubation at 37°C for at least 2h before inoculation [11]. Suspensions of *Lactobacillus crispatus* NCTC 4505 (LC), *Lactobacillus gasseri* ATCC 9857 (LG), *Lactobacillus jensenii* ATCC 25258 (LJ) and *Fusobacterium nucleatum* ATCC 25586 (FN) in phosphate-buffered saline (PBS) were prepared such that 100 - 200µL added to 7mL of medium gave $\approx 10^4$ cfu/mL. Controls for each medium at each initial pH included: uninoculated media to measure the pH effect of Oxyrase; rezasurin sodium at 0.002g/L as a reduction indicator; and FN to demonstrate ability to support anaerobic growth. Also, each strain was inoculated in to MES- and MOPS-containing media adjusted to pH 6.6 - which is at the overlap of the buffers' ranges - to identify any potential inhibition by the buffers. To provide growth curves for each strain in unbuffered medium, each was inoculated in to medium without buffer adjusted to pH 6.6. Bijoux were incubated statically at 37°C and counts done after vortexing for 5s on an undiluted aliquot and serial 10^{-1} dilutions in PBS on blood agar (Oxoid, UK) using a spiral plater (Don Whitley, Shipley, UK) at inoculation and at 25h, at which time pH was measured. For growth curves, additional samples were taken at 15h and 20h. Plates were incubated in 5% CO_2 for 24 - 48h for LB and in anaerobic jars for 48h for FN. All chemicals were from Sigma, Poole, UK and all experiments done at least in triplicate. The $\log_{10}$cfu/mL change in colony counts between time 0 and 25h were calculated for all medium-bacterium combinations and the pH change over 25h calculated using the uninoculated Oxyrase-containing control as baseline. For each strain the Wilcoxon Rank-sum test was used to compare the $\log_{10}$cfu/mL changes in MES at pH 6.6 with those in MOPS at pH 6.6.

At each initial pH, resazurin controls indicated reduction at inoculation and up to 25h. In medium without Hb at inital pH 5.5 Oxyrase alone increased pH by median 0.30 pH points with and 0.83 without, buffer. This alkalinization at low initial pH reduced steadily to negligible for media at initial pH 7.5; for the pH 8.0 media there was minimal acidification (median decrease of 0.13 pH points with and 0.31 without, buffer); almost identical results were seen in the presence of Hb. Each strain grew in unbuffered medium (Figure 1). For each bacterium, growth from initial pH 6.6 in MES did not differ significantly from that in MOPS (P = 0.13 to P = 1.00). FN grew in all media with median increase in $\log_{10}$cfu/mL of 4.48 at pH 6.0 reducing to 2.93 at pH 8.0. However, at pH 5.5 the $\log_{10}$cfu/mL increase with buffer (with Hb: 1.3, without Hb: 2.3) was substantially less than without buffer (4.04 and 4.72, respectively) (Figure 2).

Growth and acidification profiles of the LB varied significantly across the range of initial pHs (Figure 3). Of the three LB, only LC was capable of growing across the whole range, though growth

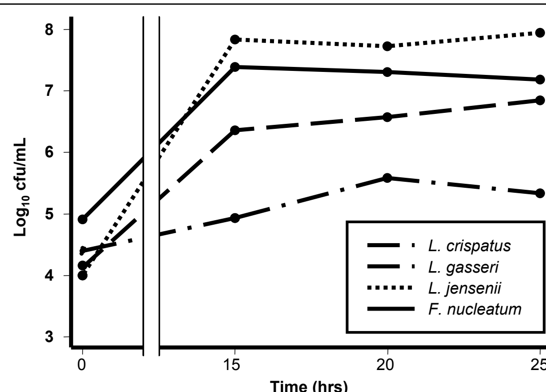

Figure 1: Anaerobic growth curves for *F. nucleatum* and three lactobacilli in medium without buffer at initial pH 6.6.

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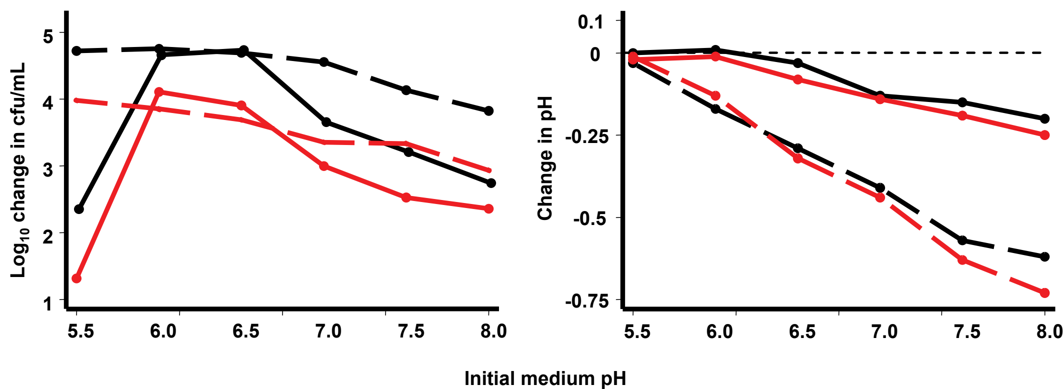

Figure 2: Change in bacterial counts and pH for *F. nucleatum* after 25h of anaerobic incubation with or without haemoglobin (red and black lines, respectively) and with or without MES buffer (intact and dotted lines, respectively).

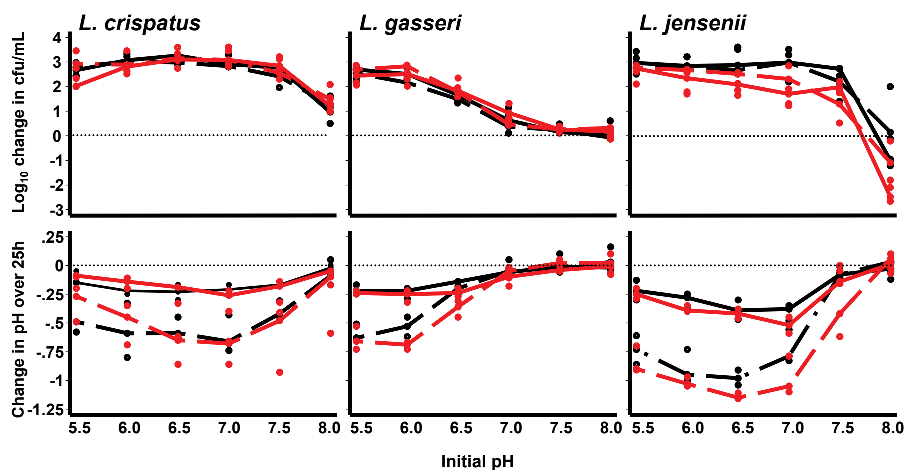

Figure 3: Change in bacterial counts and pH after 25h of anaerobic incubation for three lactobacilli, with or without haemoglobin (red & black lines, respectively), and with or without MES buffer (intact and dotted lines, respectively).

was optimal from pHs 6.0 - 7.5; acidification was maximal from initial pH 6.5 - 7.0, but negligible from initial pH 8.0. LG growth and acidification were maximal when initial pH was 5.5 or 6.0, falling to negligible when ≥7.0. Growth of LJ was consistent when initial pH was 5.5 - 7.5, but reduced by Hb and inhibited markedly at initial pH 8.0; LJ was the most potent acidifier at initial pH <7.0 but this was reduced when initial pH was >7.0.

To reduce any unpredictable buffering effects of standard nutrient broths we used a defined medium with limited inherent buffering capacity and very low concentrations of complex organic substrates. The lower end of the pH range we used is more acidic than the pH range recommended by the manufacturer of Oxyrase (pH 6.8 – 8.4), but the resazurin indicator and growth of FN confirmed anaerobiosis throughout. Indeed the growth of FN was maximum at the lowest pH values. We assume that the reduced growth of FN at initial pH 5.5 in MES, compared with no buffer, may reflect the anaerobe's inability in the presence of buffer to elevate pH to permit optimum growth. This is most likely to be a FN-specific pH effect rather than a failure to achieve anaerobiosis: a greater than ten-fold increase in recoverable cfu/mL was still achieved in the presence of buffer.

We included a fixed molarity of each buffer to permit a crude assessment of the magnitude of pH change generated. No significant differences with respect to growth rate in MES or MOPS were identified. Using both buffers permitted an extended pH range encompassing that of the normal vagina and the more alkaline conditions found in BV. We chose the single sampling point at 25h as preliminary experiments (results not shown) indicated that growth of LB in the basal medium was maximal at 20 - 25h without evidence of a rapid decline in viability thereafter. Although we included Hb in an attempt to mimic menses, the Hb concentration used was the highest compatible with filter sterilization; heat sterilization may have altered the properties of the medium and reduced reproducibility. Although this concentration of hemoglobin is presumably lower than that achieved in the vagina mid-menses, it did adversely affect the growth of LJ.

Typically 60–95% of healthy women harbor LB in the vagina at counts of 10^7–10^9 cfu/gm secretions. LC and LJ predominate in the normal vagina and an LC-predominant flora may represent a stable flora [5]; the importance of LG is less clear. LB are thought to protect the female genital tract primarily by maintaining a low pH through metabolism of oestrogen-stimulated glycogen in shed epithelial cells. Certainly, LB are capable of generating and surviving very low pH environments *in vitro* [2] and most vaginal lactic acid is of the D-lactate isomer and therefore of bacterial and not human origin [3]. LB may also inhibit opportunist bacteria through the production of bacteriocins, immune stimulation and *via* nutrient and stearic competition. There is also substantial interest in the role of hydrogen peroxide (H_2O_2) production by LB. The lowest prevalence of BV is seen in those colonized both vaginally and rectally by H_2O_2-producing LB; colonization at either site is more protective than colonization at neither [1].

The normal vagina has a pH of 3.5 – 6.0 [4] and is anaerobic, with pO_2 and pCO_2 lower and higher, respectively, than atmospheric levels; menstruation increases pH [9,12] and oxygen tension [8]. Studies of LB characteristics that may protect the vagina should address these conditions. Although we used just one strain each of the three major species – and could not achieve the lowest pH of the healthy vagina with our buffers - the differences in growth and acidification profiles across the pH range suggest that this simple model could be used to evaluate other LB characteristics deemed important for vaginal health. For example, our results indicate that the LC strain used may survive the alkaline and anaerobic conditions of incipient BV whilst still acidifying. In contrast, the LJ, whilst offering substantial acidification at lower pH values, may succumb as the pH of the vagina increases. By identifying strain or species differences this model could facilitate selection of strains – or combinations of strains – with probiotic potential.

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