bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Differential vaginal *Lactobacillus* species metabolism of glucose, L and D-lactate by $^{13}$C-nuclear magnetic**
2 **resonance spectroscopy**

3 Emmanuel Amabebe[a], e.amabebe@sheffield.ac.uk

4 Dilly O. Anumba[a], d.o.c.anumba@sheffield.ac.uk

5 Steven Reynolds[b*], steven.reynolds@sheffield.ac.uk

6 [a]Department of Oncology and Metabolism, University of Sheffield, Level 4, Jessop Wing, Tree Root Walk,
7 Sheffield S10 2SF, UK.

8 [b]Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Royal Hallamshire
9 hospital, Glossop Road, Sheffield S10 2JF, UK

10 ***Corresponding author***

11 Dr Steven Reynolds, BSc (Hons), PhD
12 Academic Unit of Radiology, Department of Infection, Immunity and Cardiovascular Disease, University of
13 Sheffield, Royal Hallamshire hospital, Glossop Road, Sheffield S10 2JF, UK. +44 114 215 9596,
14 steven.reynolds@sheffield.ac.uk

15

16 **Acknowledgements:**
17 We are grateful to the University of Sheffield, UK, for providing support and the 9.4T MRS spectrometer with
18 which this study was performed.

19
20
21
22
23
24
25
26
27

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

28 **Abstract**

29 **Introduction**

30 Cervicovaginal dysbiosis can lead to infection-associated spontaneous preterm birth.

31 **Objective**

32 To determine whether vaginal *Lactobacillus* species, *L. crispatus* and *L. jensenii,* differentially metabolise glucose,
33 L- and/or D-lactate to propagate their survival/dominance.

34 **Methods**

35 Bacteria were incubated anaerobically for 24h at 37°C, with $^{13}C_u$-glucose, $^{13}C_3$-D-lactate or $^{13}C_3$-L-lactate
36 (singularly or combined) for 24h. $^{13}$C-spectra were acquired using a 9.4T NMR spectrometer.

37 **Results**

38 *L. crispatus* and *L. jensenii* (n=6 each) metabolised $^{13}$C-glucose to $^{13}$C-lactate and $^{13}$C-acetate. *L. jensenii*
39 converted more $^{13}C_3$-D- or $^{13}C_3$-L-lactate to $^{13}$C-acetate than *L. crispatus*, $p<0.001$.

40 **Conclusion**

41 Conversion of glucose and lactate to acetate by *L. jensenii* compared to *L. crispatus*, suggests a possibly important
42 pathomechanism of dysbiosis and infection-associated spontaneous preterm birth.

43 **Keywords**: Preterm birth, vaginal lactobacilli, lactate, acetate, $^{13}$C-NMR.

44
45
46
47
48
49
50
51
52
53

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

54 **Introduction**

55 In conjunction with the host vaginal habitat, the vaginal bacteria produce unique metabolic by-products. In a
56 healthy vagina, lactobacilli are the predominant species and have been linked to increased likelihood of term
57 delivery (Amabebe and Anumba, 2018; Stafford *et al.*, 2017). The four major vaginal lactobacilli (*L. crispatus*, *L.*
58 *jensenii*, *L. iners* and *L. gasseri*) differentially produce L- and D-lactic acid that lowers the pH of the
59 cervicovaginal space, creating unfavourable conditions for other invading species. D-lactic acid is believed to be
60 more potent than L-lactic acid in relation to protection against colonisation by potentially pathogenic organisms
61 within the vagina and the accompanying inflammatory response (Witkin *et al.*, 2013). Anaerobes are also
62 endogenous to the vagina and are associated with infection and preterm birth (PTB, i.e. delivery <37 weeks of
63 gestation) especially when lactobacilli are deficient (Amabebe and Anumba, 2018).

64 Metabolism by cells can be tracked by $^{13}$C-Nuclear Magnetic Resonance (NMR) to examine specific pathways
65 (Buescher *et al.*, 2015). The use of $^{13}$C labelled substrates means that they are metabolised identically to those
66 found naturally, with the cell viability maintained throughout the experiment. Additionally, strategic placement
67 of the $^{13}$C label (Buescher *et al.*, 2015) allows the activity of single or multiple metabolic pathways to be identified,
68 even if the end product is the same (Bruntz *et al.*, 2017).

69 Although, lactobacilli are known to thrive in the acidic condition of the vagina (pH < 4.5), the mechanism
70 underpinning how lactobacilli, and other anaerobes, interact with lactic acid remains unresolved (Amabebe and
71 Anumba, 2018). In this short communication, we report on the use of $^{13}$C-NMR to examine how different
72 *Lactobacillus* species (*L. crispatus* and *L. jensenii*) differentially metabolise glucose, L- and/or D-lactate to
73 propagate their survival and dominance.

74 **Material and Methods**

75 *Bacterial culture*

76 One strain each of two common vaginal *Lactobacillus* species *L. crispatus* (ATCC 33820) and *L. jensenii* (ATCC
77 25258) were cultured in De Man, Rogosa and Sharpe broth (MRS, Oxoid CM0359, Thermo Scientific,
78 Bassingstoke, UK), under anaerobic (80% $N_2$, 10% $CO_2$, 10% $H_2$) condition at 37°C for 24h. After this time, 50
79 µl of bacteria in broth was transferred to 400 µl of fresh broth and then subcultured with the addition of 50 µl 100
80 mM $^{13}C_u$-glucose, $^{13}C_3$-D-lactate, $^{13}C_3$-L-lactate (or 25 µl each when $^{13}C_u$-glucose and $^{13}C_3$-D/L-lactate were
81 combined) for a further 24 hours (all sourced from Sigma Aldrich, Gillingham, UK). Six separate incubation were
82 performed for each species. Post incubation, samples were stored at -80°C until $^{13}$C-NMR scanning. Broth only

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

83 (non-inoculated medium) and bacterial samples without $^{13}C$-labelled substrates were used as controls. The amount
84 of bacterial colony forming units (CFU)/µl were estimated at time of $^{13}C$-substrate addition using a Helber
85 counting chamber with Thoma ruling (area = 1/400 $mm^2$, depth = 0.02 mm; Hawksley Z30000).

86 *NMR acquisition and processing*

87 $^{13}C$-NMR samples were prepared containing 430 µl bacterial sample in a 5 mm NMR tube (Norell, Morganton,
88 NC, USA) with 20 µl $D_2O$ (Sigma Aldrich) and 10 µl of 200 mM $^{13}C$-urea (chemical shift and concentration
89 reference, Sigma Aldrich) and 15 µl of penicillin/streptomycin (tube concentration ~105 units/ml penicillin and
90 ~105 µg/ml streptomycin, Sigma Aldrich).

91 Spectra were acquired by a 9.4T Bruker AVANCE III NMR spectrometer (Bruker BioSpin GmbH, Karlsruhe,
92 Germany) using a $^{13}C\{^{1}H\}$ inverse-gated pulse sequence (Spectral Width = 239 ppm, Number of acquisitions =
93 4096, Acquisition Time = 0.5 s, Delay Time = 2 s, Time domain points = 24036, flip angle = 16°) at room
94 temperature (21°C). Each acquired spectrum was apodised with a 5 Hz exponential line broadening function,
95 phase and baseline corrected using Bruker Topspin v3.4 software and referenced to the urea signal at a frequency
96 offset $\delta = 165.5$ ppm.

97 *Metabolite integration and normalisation*

98 Spectrum integrals were determined using a custom peak fitting algorithm (Matlab R2018b, Mathworks, Natick,
99 MA, USA) to identify and separate overlapping peaks where singlet and doublet peaks were present at a particular
100 chemical shift (Fig. 1a: $^{13}C_3$-lactate, 22.1 – 23.05 ppm; $^{13}C_3$-acetate, 23.2 – 26.5 ppm). Doublet peaks were
101 summed to give an integral for lactate or acetate formed from $^{13}C_u$-glucose metabolism (Fig. 1b).

102 Metabolite integrals were normalised by the concentration of live bacteria present in the incubated samples. This
103 was estimated by measuring the lactate integral, $Lac_{Broth}$, from the conversion of broth glucose (i.e. not $^{13}C$-labelled)
104 after 48 hours of incubation (24h prior to $^{13}C$-substrate addition plus a further 24h incubation post $^{13}C$ addition)
105 and was not observed in broth only samples. For each incubation experiment all integrals were divided by $Lac_{Broth}$.

106 *Statistical analysis*

107 Statistical analysis was also performed using Matlab with D'Agostino-Pearson's tests for normality of data and a
108 Kruskal-Wallis with Bonferroni post-hoc test (KW-B) for comparison of multiple groups with $p < 0.05$ taken as
109 significant. Comparison between lactobacilli acetate and lactate integrals were performed using either Student's
110 t-test or Wilcoxon rank-sum test depending on the outcome of the normality test.

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

111 **Results and Discussion**

112 The average count (CFU) of each bacterial species before incubation with $^{13}$C-labelled substrates was: *L. crispatus*
113 = 4.4x10$^{7}$ ± 7.0x10$^{6}$ CFU/µl; and *L. jensenii* = 7.3x10$^{7}$ ± 1.1x10$^{7}$ CFU/µl (p = 0.1). The $^{13}$C-spectra showed
114 conversion of glucose (natural abundance and $^{13}$C-labelled) to lactate (22.8 ppm) and acetate (23.8 ppm) for both
115 species (n = 6 per species, Fig. 1). Higher quantities of lactate were produced by *L. jensenii* than *L. crispatus* for
116 all $^{13}C_u$-glucose containing incubations although these were not significant (Fig. 2a). The addition of $^{13}C_3$–L/D-
117 lactate to the incubation suppressed the conversion of $^{13}C_u$-glucose to lactate (Fig, 2a). *L. jensenii* showed
118 significantly higher conversion of both enantiomers of $^{13}C_3$-lactate to acetate (23.8 ppm) than *L. crispatus* (*L.*
119 *crispatus* vs *L. jensenii*: D-lactate to acetate: 0.26 ± 0.10 vs 1.71 ± 0.15, $p < 0.001$; L-lactate to acetate: 0.11 ±
120 0.04 vs 1.68 ± 0.14, $p < 0.001$, Fig. 2b). Six of the *L. jensenii* $^{13}$C spectra ($^{13}$C-glucose, n = 2; $^{13}$C-glucose/L-
121 lactate, n = 2; $^{13}$C-glucose/D-lactate, n = 1; $^{13}$C-L-lactate, n = 1) also showed peaks at 32.4 and 180.6 ppm
122 (assigned as succinate from HMBC spectra, data not shown).

123 Results show that *Lactobacillus* species that are present in the vagina differentially metabolise $^{13}$C-labelled
124 glucose and lactate to produce acetate and other metabolites. *L. jensenii* spectra showed that this species was able
125 to convert more D- or L-lactate to acetate than *L. crispatus*. D-lactate is believed to be a more potent anti-infection
126 and anti-inflammatory agent than L-lactate (van de Wijgert *et al.*, 2014; Witkin and Linhares, 2017; Witkin *et al.*,
127 2013) and other studies have shown that *L. crispatus* produces more D-lactate than *L. jensenii* (Amabebe and
128 Anumba, 2018; van de Wijgert *et al.*, 2014; Witkin and Linhares, 2017; Witkin *et al.*, 2013). This study observed
129 that *L. jensenii* converted a higher amount of $^{13}C_u$-glucose to lactate than *L. crispatus*. We did not ascertain if the
130 lactate produced was either the L- or D isomer. However, this could be done by either an NMR chiral shift reagent
131 (Zhang *et al.*, 2017a; Zhang *et al.*, 2017b) or absolute quantification of these isomers by enzyme-based
132 spectrophotometry as we have previously demonstrated (Amabebe *et al.*, 2019; Amabebe *et al.*, 2016a; Cavanagh
133 *et al.*, 2019).

134 Bacterial load (CFU) was not used for normalisation, as this did not correlate with $^{13}$C-lactate integrals observed
135 in the spectra (data not shown). Hence, the integrals in this study were normalised based on the assumption that
136 the observed lactate peak arising from non-$^{13}$C-labelled glucose present in the media (singlet peak between the
137 doublet for lactate at 22.8 ppm, Fig 2a) was proportional to the concentration of live bacteria. Justification for this
138 approach was made by the observation of significant correlations between the doublet peak $^{13}$C-lactate integrals,
139 ($^{13}$C-glucose to lactate metabolism), and singlet peak lactate integrals (broth glucose to lactate. Data not shown).

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

140 Using this approach could potentially underestimate the quantity of live bacteria due to further metabolism of
141 lactate, e.g. to acetate. Considering acetate concentration is confounded by its presence in the broth, which then
142 overestimates the bacteria concentration (acetate integrals from broth only spectra would need to be measured to
143 account for this). Using CFU for normalisation requires careful assessment of the inhomogeneity in bacteria
144 distribution that can make this method inaccurate. Additionally, the viability of bacteria would need to be
145 determined, as some of the bacteria will be dead. Furthermore, bacteria that were metabolically active at the start
146 of the incubation would die over the duration of the incubation leading to an over correction of the integrals.

147 The conversion of glucose and lactate to acetate and succinate by *L. jensenii* compared to *L. crispatus*, suggests
148 that this may be an important pathomechanism of dysbiosis, altered vaginal pH, infection and infection-associated
149 spontaneous preterm birth. Elevated amounts of acetate and succinate in the vaginal space is associated with
150 increased pH and risk of infection such as bacterial vaginosis (Aldunate *et al.*, 2015; Ceccarani *et al.*, 2019), a
151 known risk factor of preterm birth and other poor reproductive outcomes (Amabebe and Anumba, 2018). Vaginal
152 bacterial communities dominated by *L. jensenii* usually have higher pH compared to when *L. crispatus*
153 predominates (Aldunate *et al.*, 2015). We have previously observed that symptomatic women at risk of preterm
154 birth show elevated cervicovaginal fluid acetate (Amabebe *et al.*, 2016a; Amabebe *et al.*, 2016b), which can be
155 combined with pro-inflammatory mediators to improve its performance as a predictive marker (Amabebe *et al.*,
156 2019).

157 These experiments were performed on bacteria that had been freeze thawed. As NMR is a non-destructive
158 technique, it would be possible to apply this method to live bacteria to sequentially acquire spectra and determine
159 the rate of conversion of substrates. Whilst it would be important to characterise the most common vaginal strains
160 of these species, alternative strains could also be characterised. Future studies will be expanded to other
161 *Lactobacillus* species and strains such as *L. iners* and *L. gasseri* and anaerobes including *Gardnerella*, *Bacteroides*
162 and *Mobiluncus*, as well as fungi (e.g. *Candida albicans*) that are known to exist within the vaginal milieu.

163 **Declarations**

164 **Funding:**

165 This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit
166 sectors. However, EA and DA are funded by National Institute for Health Research (NIHR, 17/63/26).

167 **Conflict of interest statement:**

168 The authors have no conflicts of interest to declare.

169 **Author contributions**

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

170 This work was conceived and conceptualised by EA and SR. EA and SR acquired and analysed the data with DA
171 supervising. All authors contributed to drafting and review of the manuscript, and approved the final version for
172 submission.

173

174 **References:**

175 Aldunate, M., Srbinovski, D., Hearps, A.C., Latham, C.F., Ramsland, P.A., Gugasyan, R., Cone, R.A. and
176 Tachedjian, G. (2015) Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids
177 produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Frontiers in Physiology* **6**.

178
179 Amabebe, E. and Anumba, D.O.C. (2018) The Vaginal Microenvironment: The Physiologic Role of Lactobacilli.
180 **5**.

181
182 Amabebe, E., Reynolds, S., He, X., Wood, R., Stern, V. and Anumba, D.O.C. (2019) Infection/inflammation-
183 associated preterm delivery within 14 days of presentation with symptoms of preterm labour: A multivariate
184 predictive model. *PLOS ONE* **14,** e0222455.

185
186 Amabebe, E., Reynolds, S., Stern, V., Stafford, G., Paley, M. and Anumba, D.O. (2016a) Cervicovaginal Fluid
187 Acetate: A Metabolite Marker of Preterm Birth in Symptomatic Pregnant Women. *Front Med (Lausanne)* **3,** 48.

188
189 Amabebe, E., Reynolds, S., Stern, V.L., Parker, J.L., Stafford, G.P., Paley, M.N. and Anumba, D.O. (2016b)
190 Identifying metabolite markers for preterm birth in cervicovaginal fluid by magnetic resonance spectroscopy.
191 *Metabolomics* **12,** 67.

192
193 Bruntz, R.C., Lane, A.N., Higashi, R.M. and Fan, T.W. (2017) Exploring cancer metabolism using stable isotope-
194 resolved metabolomics (SIRM). *J Biol Chem* **292,** 11601-11609.

195
196 Buescher, J.M., Antoniewicz, M.R., Boros, L.G., Burgess, S.C., Brunengraber, H., Clish, C.B., DeBerardinis, R.J.,
197 Feron, O., Frezza, C., Ghesquiere, B., Gottlieb, E., Hiller, K., Jones, R.G., Kamphorst, J.J., Kibbey, R.G.,
198 Kimmelman, A.C., Locasale, J.W., Lunt, S.Y., Maddocks, O.D., Malloy, C., Metallo, C.M., Meuillet, E.J.,
199 Munger, J., Noh, K., Rabinowitz, J.D., Ralser, M., Sauer, U., Stephanopoulos, G., St-Pierre, J., Tennant, D.A.,
200 Wittmann, C., Vander Heiden, M.G., Vazquez, A., Vousden, K., Young, J.D., Zamboni, N. and Fendt, S.M. (2015)
201 A roadmap for interpreting (13)C metabolite labeling patterns from cells. *Curr Opin Biotechnol* **34,** 189-201.

202
203 Cavanagh, M., Amabebe, E. and Anumba, D.O.C. (2019) Differential Cytokine and Metabolite Production by
204 Cervicovaginal Epithelial Cells Infected with Lactobacillus crispatus and Ureaplasma urealyticum. *Anaerobe*,
205 102101.

206
207 Ceccarani, C., Foschi, C., Parolin, C., D'Antuono, A., Gaspari, V., Consolandi, C., Laghi, L., Camboni, T., Vitali,
208 B., Severgnini, M. and Marangoni, A. (2019) Diversity of vaginal microbiome and metabolome during genital
209 infections. *Scientific Reports* **9,** 14095.

210
211 Stafford, G.P., Parker, J.L., Amabebe, E., Kistler, J., Reynolds, S., Stern, V., Paley, M. and Anumba, D.O.C.
212 (2017) Spontaneous Preterm Birth Is Associated with Differential Expression of Vaginal Metabolites by
213 Lactobacilli-Dominated Microflora. *Front Physiol* **8,** 615.

214
215 van de Wijgert, J.H.H.M., Borgdorff, H., Verhelst, R., Crucitti, T., Francis, S., Verstraelen, H. and Jespers, V.
216 (2014) The Vaginal Microbiota: What Have We Learned after a Decade of Molecular Characterization? *PLOS*
217 *ONE* **9,** e105998.

218

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

219 Witkin, S. and Linhares, I. (2017) Why do lactobacilli dominate the human vaginal microbiota? *BJOG: An*
220 *International Journal of Obstetrics & Gynaecology* **124,** 606-611.

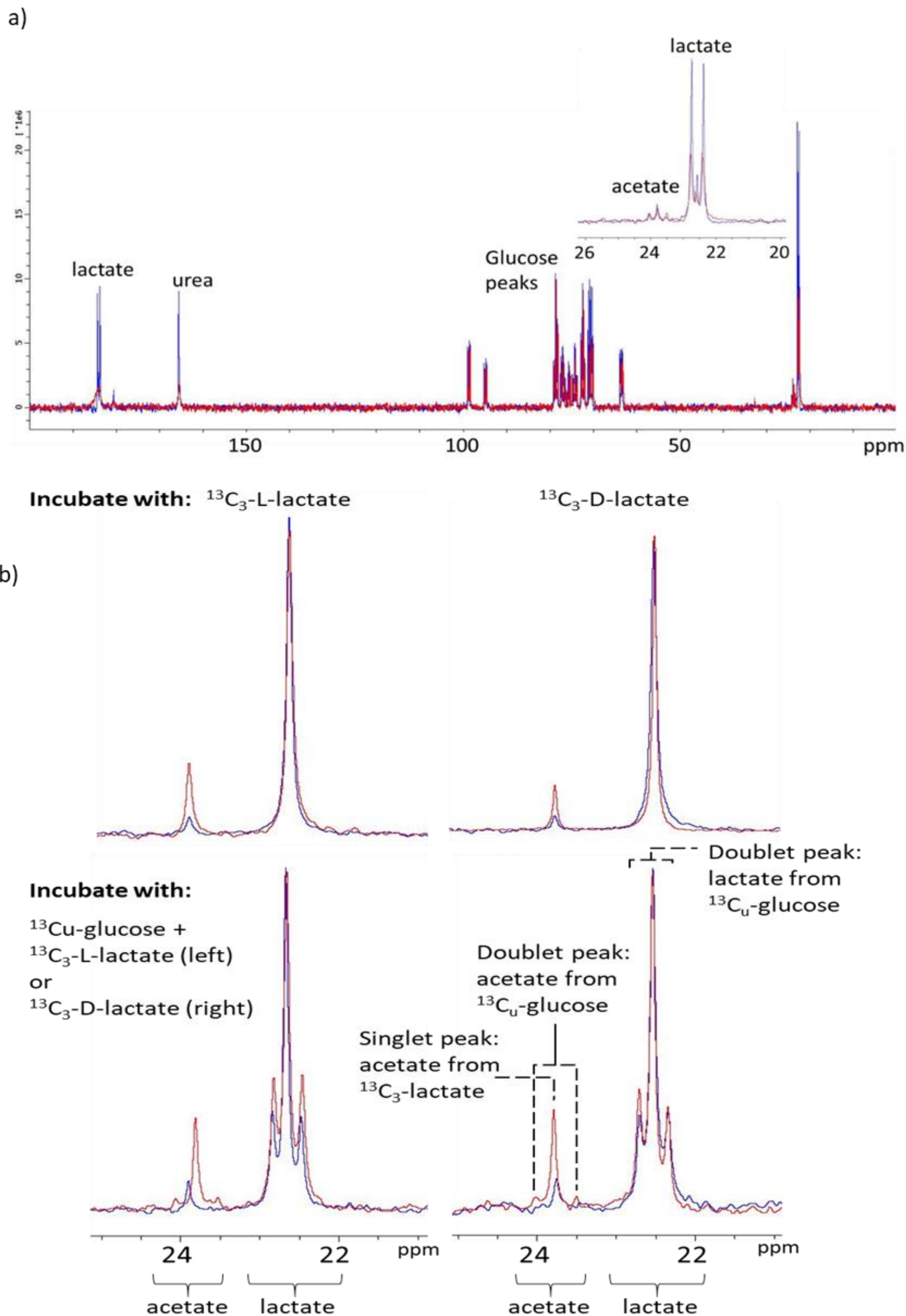
221
222 Witkin, S.S., Mendes-Soares, H., Linhares, I.M., Jayaram, A., Ledger, W.J. and Forney, L.J. (2013) Influence of
223 vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer:
224 implications for protection against upper genital tract infections. *mBio* **4,** e00460-13.

225
226 Zhang, L., Martins, A.F., Mai, Y., Zhao, P., Funk, A.M., Clavijo Jordan, M.V., Zhang, S., Chen, W., Wu, Y. and
227 Sherry, A.D. (2017a) Imaging Extracellular Lactate In Vitro and In Vivo Using CEST MRI and a Paramagnetic
228 Shift Reagent. *Chemistry* **23,** 1752-1756.

229
230 Zhang, L., Martins, A.F., Zhao, P., Tieu, M., Esteban-Gomez, D., McCandless, G.T., Platas-Iglesias, C. and
231 Sherry, A.D. (2017b) Enantiomeric Recognition of d- and l-Lactate by CEST with the Aid of a Paramagnetic Shift
232 Reagent. *J Am Chem Soc* **139,** 17431-17437.

233
234
235
236
237
238
239
240
241

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



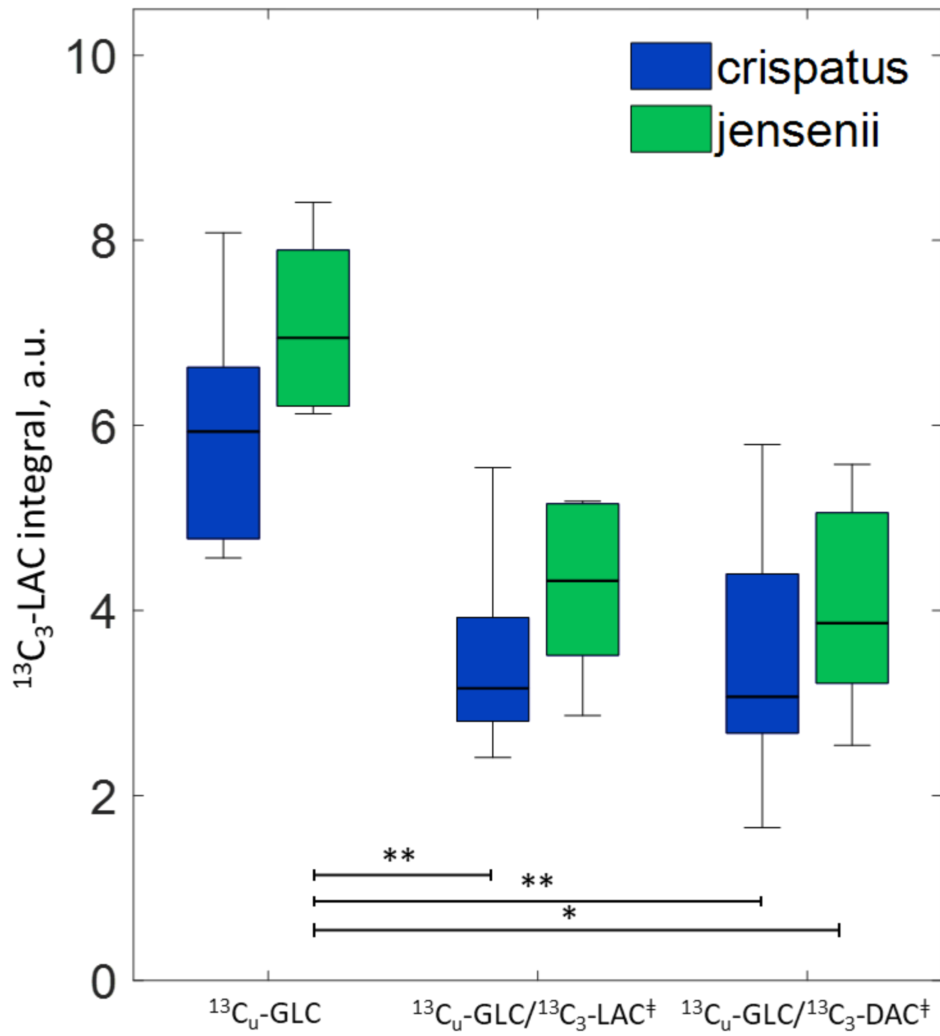

242
243 **Figure 1: a)** Overlaid $^{13}$C-NMR spectra of *L. crispatus* (blue) and *L. jensenii* (red) incubated with $^{13}C_u$-glucose.
244 Inset shows a zoomed region of the spectrum 20 – 26 ppm. **b)** *L. crispatus* (blue) and *L. jensenii* (red) incubated
245 with either: top, $^{13}C_3$-L-lactate (left) or $^{13}C_3$-D-lactate (right); bottom, $^{13}C_u$-glucose + $^{13}C_3$-L-lactate (left) or $^{13}C_u$-
246 glucose + $^{13}C_3$-D-lactate (right). Spectra scaled to lactate peak height. Doublet peaks of lactate and acetate arise
247 from the conversion of $^{13}C_u$-glucose. Singlet peaks arise from either singly labelled $^{13}C_3$-D/L-lactate or substrates
248 with natural abundance $^{13}$C (1.1%).

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

249

250

251 a)




252

253

254

b)

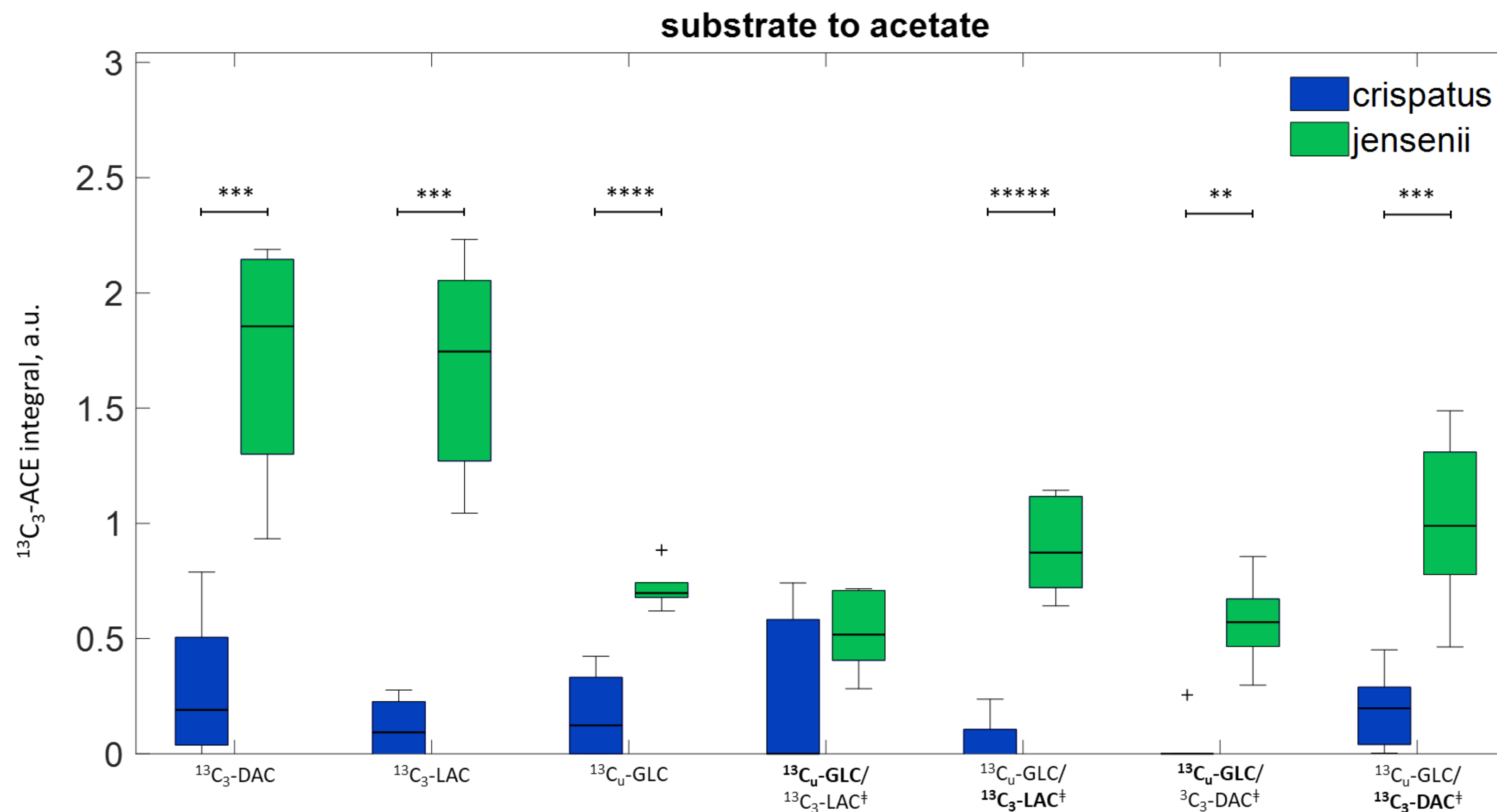



**Figure 2: a)** Normalised $^{13}C_3$-lactate integrals for the conversion of $^{13}C_u$-glucose (GLC), $^{13}C_u$-glucose + $^{13}C_3$-L-lactate (LAC) or $^{13}C_u$-glucose + $^{13}C_3$-D-lactate (DAC). ‡ For combination incubations, the source substrate that is converted to lactate is $^{13}C_u$-glucose (as detected from doublet peak in spectrum). Kruskal-Wallis with Bonferroni post-hoc test: *$p < 0.05$, **$p < 0.01$. **b)** Normalised $^{13}C_3$-acetate (ACE) integrations for the conversion of $^{13}C_u$-glucose (GLC), $^{13}C_u$-glucose + $^{13}C_3$-L-lactate (LAC) or $^{13}C_u$-glucose + $^{13}C_3$-D-lactate (DAC). ‡ For combination incubations, the source substrate that is converted to acetate is highlighted in bold. Boxplots show median, interquartile range (IQR) and whiskers (1.5xIQR). Wilcoxon rank sum: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ***** $p < 0.00001$.

bioRxiv preprint doi: https://doi.org/10.1101/2020.03.10.985580; this version posted March 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.