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Short communication

Lactobacillus crispatus represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity

Joana Castro [a, b, 1], Ana Paula Martins [a, 1], Maria Elisa Rodrigues [a], Nuno Cerca [a, *]

[a] Centre of Biological Engineering (CEB), Laboratory of Research in Biofilms Rosário Oliveira (LIBRO), University of Minho, Campus de Gualtar, Braga, Portugal
[b] Instituto de Ciências Biomédicas Abel Salazar (ICBAS), University of Porto, Porto, Portugal

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ABSTRACT

Using a chemically-defined medium simulating genital tract secretions, we have shown that pre-adhering *Lactobacillus crispatus* to Hela epithelial cells reduced cytotoxicity caused by *Gardnerella vaginalis*. This effect was associated to the expression of vaginolysin and was specific to *L. crispatus* interference, as other vaginal facultative anaerobes had no protective effect.

Lactobacillus crispatus is an important urogenital species that is routinely found in the vagina of healthy women [1,2], contributing to the maintenance of normal vaginal microbiota, while its absence has been associated with a range of vaginal abnormalities, especially bacterial vaginosis (BV) [3,4]. BV is the leading vaginal disorder in women of reproductive age worldwide and it has been associated with serious public health consequences, including pelvic inflammatory disease [5], acquisition and transmission of the HIV virus [6] and preterm birth [7]. It has been proposed that *Gardnerella vaginalis,* a facultative anaerobe, is the pathogen responsible for the initiation of BV [8,9]. This bacteria is capable of adhering to vaginal cells, establish biofilms and induce cytotoxicity on vaginal epithelial cells [10,11]. However, despite being the most prevalent and virulent species found in BV, *G. vaginalis* can also be a part of the vaginal microbiota in healthy women [12,13]. Genetic differences among *G. vaginalis* strains may underlie the diverse pathological features and outcomes that have been associated with this species, raising the possibility that distinct pathogenic and non-pathogenic strains or even subspecies exist [14–18]. The *G. vaginalis* diversity might represent a critical turning point in clarifying ecological interactions and virulence factors contributing to symptoms and sequelae of BV [19]. Therefore, understanding the interactions between beneficial lactobacilli and *G. vaginalis* is of extreme importance to help unravel the pathogenesis and progression of this condition.

This study aimed to investigate whether the BV-positive and BV-negative *G. vaginalis* strains differ in their abilities to interact with a cervical epithelial (HeLa) cell monolayer pre-treated with *L. crispatus*, using a functional cytotoxicity model, which represents a significant improvement over our previous study [10], since we used a medium simulating genital tract secretions (mGTS) [20] and epithelial cells were previously covered with *L. crispatus*, mimicking a normal vaginal ecosystem. First, bacterial suspensions of three BV-positive G. vaginalis strains (UM067, NCBI accession number: KP996675.1; UM121, NCBI: KP996681.1; UM241, NCBI: KP996683.1), three BV-negative G. vaginalis strains (UM016, NCBI: KP996686.1; UM085, NCBI: KP996679.1; UM131, NCBI: KP996676.1) and *L. crispatus* EX533959VC06 were grown in mGTS for 48 h at 37 °C with 10% CO_2 (Shel Lab, Cornelius Oregon, USA) [20]. For the cytotoxicity assays, *L. crispatus* suspension, adjusted to

* Corresponding author. Centre of Biological Engineering (CEB) , Laboratory of Research in Biofilms Rosário Oliveira (LIBRO) , University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail address: nunocerca@ceb.uminho.pt (N. Cerca).
[1] J. C. and A. P. M. contributed equally to this work.

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1×10^7 cfu/mL, was added to a monolayer of HeLa cells for 3 h. Afterwards, blind bacterial suspensions, adjusted to the same concentration, were added to a HeLa cells monolayer pre-adhered with *L. crispatus* for 3 h. Additional controls were conducted to analyze whether *L. crispatus* effects reflect a specie-dependent response or whether other vaginal associated species can exhibit similar effects. Thus, herein, we included the following bacterial species as controls: *Corynebacterium tuberculostearicum* UM137Ct2 (NCBI: KT805279); Staphylococcus warnerii UM224Sw (NCBI: KT923488); and Nosocomiicoccus ampullae UM121Na (NCBI: KT805272), due to their low cytotoxicity and adhesion levels similar to *L. crispatus* [21]. Cytotoxicity was scored on a 0 to 5 scale [11]. Numeric scores were assigned as follows: 0, no difference between the test and the control; 1, 25% of the cells were rounded; 2, 25–50% of the cells were rounded; 3, 50% of the cells were rounded with partial monolayer disruption; 4, 50% cells were rounded, with extensive disruption of the monolayer; and 5, complete disruption or absence of the monolayer.

Furthermore, we also quantified the expression levels of vaginolysin (*vly*) and sialidase (*sld*) transcripts in three different conditions: *G. vaginalis* planktonic cells (pre-infection) (*i*); after *G. vaginalis* infection on a monolayer of HeLa cells (post-infection in HeLa) (*ii*); and after *G. vaginalis* infection on a monolayer of HeLa cells pre-treated with *L. crispatus* (post-infection in HeLa with Lc) *(iii)*. Briefly, total RNA of these three different conditions was extracted using an ExtractME RNA Bacteria & Yeast kit (Blirt S.A., Poland) with minor changes, as optimized before [22]. Quantitative PCR (qPCR) was prepared by mixing together 5 μL of iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA), 2 μL of 1:100 diluted cDNA, 0.5 μL of 5 μM Forward and Reverse primes and water up to 10 μL. Primer sequences for target genes are listed in our previous studies, as follows: *16sRNA* (Fw and Rv) [23]; *vly* (Fw2 and Rv2) and *sld* (Fw1 and Rv1) [10]. Normalized gene expression was determined by using the delta C_t method ($E^{\Delta Ct}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) - C_t (target gene) and E stands for the reaction efficiency experimentally determined. At least three biologic replicates of each condition were performed. The data were analyzed using the *t*-test or A-NOVA with the statistical software package GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). *P*-values of less than 0.05 were considered significant.

Our results highlighted that BV-positive *G. vaginalis* strains were able to induce more extensive damages on the HeLa monolayer than BV-negative strains (Fig. 1a), supporting our previous data [10]. Remarkably, when *L. crispatus* was pre-adhered to HeLa, cytotoxicity effect of all *G. vaginalis* was significantly reduced ($p < 0.05$). A possible explanation for this fact could be that *L. crispatus* is blocking *G. vaginalis* adherence [24], indicating that competitive exclusion of this species could be a key role protecting the vagina from invading pathogens [25].

Trying to unravel whether this response was *L. crispatus*-specific, we pre-coated the HeLa monolayer with *C. tuberculostearicum*, *N. ampullae* or *S. warnerii*. Curiously, we verified that the selected bacterial species were not able to reduce the cytopathogenic alterations caused by *G. vaginalis* on the epithelial monolayer (Fig. 1b). This supports the specific role of *L. crispatus* in reducing *G. vaginalis* cytotoxicity. Recent studies have shown that cytoprotective effect of *L. crispatus* seems to be related with stimulation of immune response [26], reduction of cell apoptosis [27], or changes on the physical properties of the plasma membrane in HeLa cells [28].

The different cytotoxic activity between BV-negative and BV-positive isolates could be due to a pre-forming toxin produced by *G. vaginalis*, vaginolysin, which is able to induce cell death and is thus a virulence factor [29]. Furthermore, *G. vaginalis* virulence has also been associated to sialidase [30]. This enzyme is known to facilitate the destruction of the protective mucus layer on the vaginal epithelium [30]. Therefore, to compare the cytotoxicity effect and expression levels of *vly* and *sld* transcripts between the two groups of strains, we used a BV-positive *G. vaginalis* UM241 and a BV-negative *G. vaginalis* UM131 strain, which carry both genes of interest (Castro et al., 2015). Our results revealed differences in the expression of both genes, being the transcript levels of *vly* (Fig. 2a) higher when compared to the transcript levels of *sld* (Fig. 2b), similar to what was verified in our previous report [10].

Interestingly, our data also revealed that after post-infection by

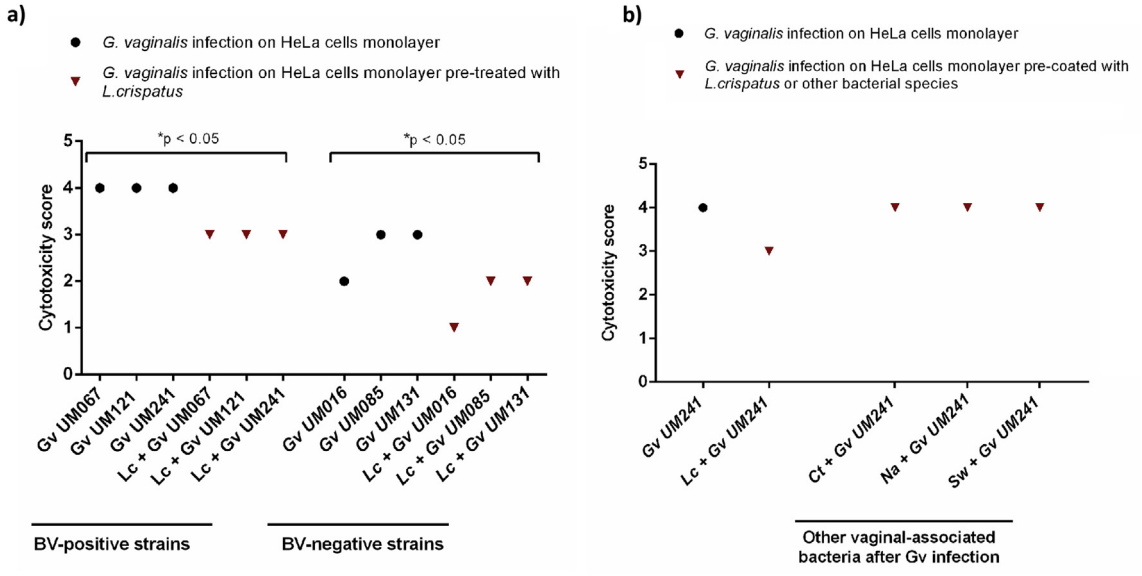

Fig. 1. Differential cytotoxicity profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strains) and without BV (BV-negative strains). **a)** Cytotoxicity score of BV-positive and BV-negative Gv strains, in two different conditions: Gv infection on a monolayer of HeLa cells and on a monolayer pre-coated with *L. crispatus* (Lc); **b)** cytotoxicity score after Gv infection on a monolayer of HeLa cells pre-coated with Lc or with other vaginal-associated species: *C. tuberculostearicum* (Ct), *N. ampullae* (Na) and *S. warnerii* (Sw). *Values are significantly different between the 2 groups of Gv strains under the same conditions (one-way ANOVA, $p < 0.05$).

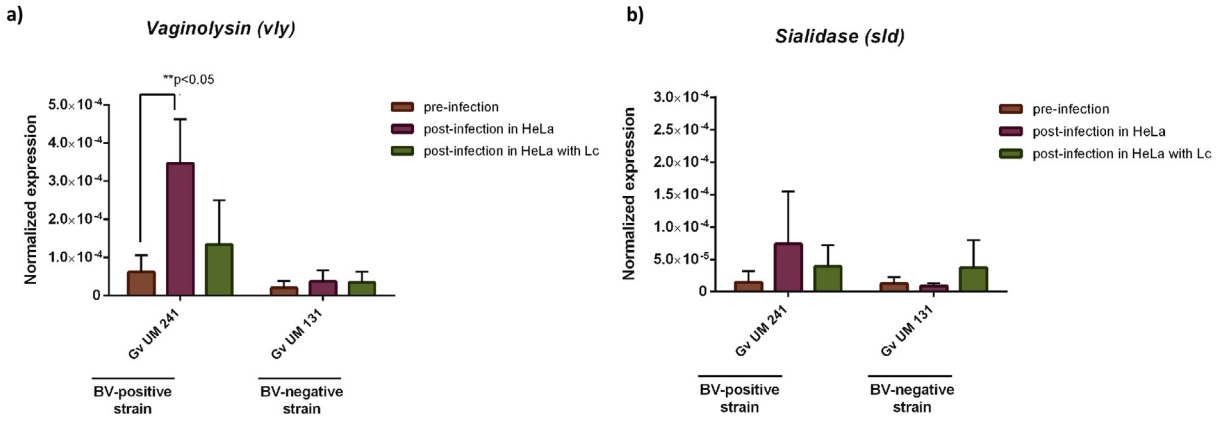

Fig. 2. Differential transcriptomic profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strain) and without BV (BV-negative strain). **a)** Expression of vaginolysin (*vly*) and; **b)** sialidase (*sld*) genes by Gv isolates. Transcript levels within Gv planktonic cells (pre-infection), after Gv infection on a monolayer of HeLa cells (post-infection in HeLa) and after Gv infection on a monolayer of HeLa pre-treated with *L. crispatus* (post-infection in HeLa with Lc) were quantified. Results are expressed as normalized expression in relation to 16S rRNA and represented as mean ± SEM. **A specific condition was significantly different in terms of gene expression between a BV-positive and a BV-negative strain (T-test, $p < 0.05$).

a BV-positive strain, transcript levels of both genes were significantly higher than in pre-infection (Fig. 2a and b). Remarkably, pre-coating the HeLa monolayer with *L. crispatus* caused a repression of expression in 2.58-fold and 1.89-fold for *vly* and *sld* transcripts, respectively. Regarding the BV-negative strain, the same tendency was observed for *vly* expression between the post- and pre-infection conditions (Fig. 2a). However, no differences were detected in *sld* expression (Fig. 2b). Surprisingly, our findings suggest that no direct association seems to exist between *vly* and *sld* expression by a BV-negative group and the presence of *L. crispatus,* despite the ability of this species to repress the cytotoxic activity of both *G. vaginalis* groups.

Taking in consideration our novel findings and our previous observations [10,24] we underline the importance of *L. crispatus,* since it seems to possess some factors that can trigger protective mechanisms against BV-positive *G. vaginalis* strains [25]. A limitation of our study was the use of only a representative *Lactobacillus* species, correlated with healthy vaginal microflora, and we did not explore how other lactobacilli would interact with either *G. vaginalis* or other bacterial species found in BV. However, there is no doubt that a refined genomic characterization of the *G. vaginalis* strains might allow a better knowledge of the molecular mechanisms behind the different patterns of cytotoxicity.

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References

[1] R.F. Lamont, J.D. Sobel, R.A. Akins, S.S. Hassan, T. Chaiworapongsa, J.P. Kusanovic, et al., The vaginal microbiome: new information about genital tract flora using molecular based techniques, BJOG 118 (2011) 533–549. https://doi.org/10.1111/j.1471-0528.2010.02840.x.

[2] J. Ravel, P. Gajer, Z. Abdo, G.M. Schneider, S.S. Koenig, S.L. McCulle, et al., Vaginal microbiome of reproductive-age women, Proc. Natl. Acad. Sci. U. S. A. 108 (Suppl 1) (2011) 4680–4687. https://doi.org/10.1073/pnas.1002611107.

[3] J.M. Macklaim, A.D. Fernandes, J.M. Di Bella, J.A. Hammond, G. Reid, G.B. Gloor, Comparative meta-RNA-seq of the vaginal microbiota and differential expression by *Lactobacillus iners* in health and dysbiosis, Microbiome 1 (2013) 12. https://doi.org/10.1186/2049-2618-1-12.

[4] H. Verstraelen, R. Verhelst, G. Claeys, E. De Backer, M. Temmerman, M. Vaneechoutte, Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora, BMC Microbiol. 9 (2009) 116. https://doi.org/10.1186/1471-2180-9-116.

[5] H. Sharma, R. Tal, N.A. Clark, J.H. Segars, Microbiota and pelvic inflammatory disease, Semin. Reprod. Med. 32 (2014) 43–49. https://doi.org/10.1055/s-0033-1361822.

[6] P. Mirmonsef, L. Krass, A. Landay, G.T. Spear, The role of bacterial vaginosis and trichomonas in HIV transmission across the female genital tract, Curr. HIV Res. 10 (2012) 202–210. PMCID: PMC3788616.

[7] A.S. Dingens, T.S. Fairfortune, S. Reed, C. Mitchell, Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study, BMC Pregnancy Childbirth 16 (2016) 278. https://doi.org/10.1186/s12884-016-1073-y.

[8] J.R. Schwebke, C.A. Muzny, W.E. Josey, Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model, J. Infect. Dis. 210 (2014) 338–343. https://doi.org/10.1093/infdis/jiu089.

[9] A. Machado, N. Cerca, Influence of Biofilm Formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis, J. Infect. Dis. 212 (2015) 1856–1861. https://doi.org/10.1093/infdis/jiv338.

[10] J. Castro, P. Alves, C. Sousa, T. Cereija, A. Franca, K.K. Jefferson, et al., Using an *in-vitro* biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis* isolates, Sci. Rep. 5 (2015) 11640. https://doi.org/10.1038/srep11640.

[11] J.L. Patterson, A. Stull-Lane, P.H. Girerd, K.K. Jefferson, Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes, Microbiology 156 (2010) 392–399. https://doi.org/10.1099/mic.0.034280-0.

[12] A.A. Aroutcheva, J.A. Simoes, K. Behbakht, S. Faro, *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems, Clin. Infect. Dis. 33 (2001) 1022–1027. https://doi.org/10.1086/323030.

[13] R.J. Hickey, L.J. Forney, *Gardnerella vaginalis* does not always cause bacterial vaginosis, J. Infect. Dis. 210 (2014) 1682–1683. https://doi.org/10.1093/infdis/jiu303.

[14] C.J. Yeoman, S. Yildirim, S.M. Thomas, A.S. Durkin, M. Torralba, G. Sutton, et al., Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential, PLoS One 5 (2010), e12411. https://doi.org/10.1371/journal.pone.0012411.

[15] M.D. Harwich Jr., M.G. Serrano, J.M. Fettweis, J.M. Alves, M.A. Reimers, G.A. Buck, et al., Genomic sequence analysis and characterization of *Sneathia amnii* sp. nov, BMC Genom. 13 (Suppl 8) (2012) S4. https://doi.org/10.1186/1471-2164-13-S8-S4.

[16] M. Janulaitiene, V. Paliulyte, S. Grinceviciene, J. Zakareviciene, A. Vladisauskiene, A. Marcinkute, et al., Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis, BMC Infect. Dis. 17 (2017) 394. https://doi.org/10.1186/s12879-017-2501-y.

[17] S.V. Balashov, E. Mordechai, M.E. Adelson, S.E. Gygax, Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR, J. Med. Microbiol. 63 (2014) 162–175. https://doi.org/10.1099/jmm.0.066407-0.

[18] J. Castro, N. Cerca, BV and non-BV associated *Gardnerella vaginalis* establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms, Anaerobe 36 (2015) 56–59. https://doi.org/10.1016/j.a

naerobe.2015.10.008.
[19] J.J. Schellenberg, M.H. Patterson, J.E. Hill, *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms, Res. Microbiol. S0923–2508 (2017) 30060–30068. https://doi.org/10.1016/j.resmic.2017.02.011.
[20] R.L. Stingley, H. Liu, L.B. Mullis, C.A. Elkins, M.E. Hart, *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) production and *Lactobacillus* species growth in a defined medium simulating vaginal secretions, J. Microbiol. Meth. 106 (2014) 57–66. https://doi.org/10.1016/j.mimet.2014.08.002.
[21] P. Alves, J. Castro, C. Sousa, T.B. Cereija, N. Cerca, *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model, J. Infect. Dis. 210 (2014) 593–596. https://doi.org/10.1093/infdis/jiu131.
[22] A. França, A.I. Freitas, A.F. Henriques, N. Cerca, Optimizing a qPCR Gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol, PLoS One 7 (2012), e37480. https://doi.org/10.1371/journal.pone.0037480.
[23] J. Castro, A. França, K.R. Bradwell, M.G. Serrano, K.K. Jefferson, N. Cerca, Comparative transcriptomic analysis of *Gardnerella vaginalis* biofilms vs. planktonic cultures using RNA-seq, NPJ Biofilms and Microbiomes 3 (2017) 3. https://doi.org/10.1038/s41522-017-0012-7.
[24] J. Castro, A. Henriques, A. Machado, M. Henriques, K.K. Jefferson, N. Cerca, Reciprocal interference between Lactobacillus spp. and Gardnerella vaginalis on initial adherence to epithelial cells, Int. J. Med. Sci. 10 (2013) 1193. https://doi.org/10.7150/ijms.6304.
[25] T. Ojala, M. Kankainen, J. Castro, N. Cerca, S. Edelman, B. Westerlund-Wikstrom, et al., Comparative genomics of *Lactobacillus crispatus* suggests novel mechanisms for the competitive exclusion of Gardnerella vaginalis, B BMC Genomics 15 (2014) 1070. https://doi.org/10.1186/1471-2164-15-1070.
[26] X.X. Niu, T. Li, X. Zhang, S.X. Wang, Z.H. Liu, *Lactobacillus crispatus* modulates vaginal epithelial cell innate response to Candida albicans, Chin. Med. J. 130 (2017) 273–279. https://doi.org/10.4103/0366-6999.198927.
[27] E. Motevaseli, M. Shirzad, S.M. Akrami, A.S. Mousavi, A. Mirsalehian, M.H. Modarressi, Normal and tumour cervical cells respond differently to vaginal lactobacilli, independent of pH and lactate, J. Med. Microbiol. 62 (2013) 1065–1072. https://doi.org/10.1099/jmm.0.057521-0.
[28] N. Calonghi, C. Parolin, G. Sartor, L. Verardi, B. Giordani, G. Frisco, et al., Interaction of vaginal *Lactobacillus* strains with HeLa cells plasma membrane, Benef. Microbes 8 (2017) 625–633. https://doi.org/10.3920/BM2016.0212.
[29] S.E. Gelber, J.L. Aguilar, K.L. Lewis, A.J. Ratner, Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from Gardnerella vaginalis, J. Bacteriol. 190 (2008) 3896–3903. https://doi.org/10.1128/JB.01965-07.
[30] L. Hardy, V. Jespers, M. Van den Bulck, J. Buyze, L. Mwambarangwe, V. Musengamana, et al., The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm, PLoS One 12 (2017), e0172522. https://doi.org/10.1371/journal.pone.0172522.