SimUrine: A Novel, Fully Defined Artificial Urinary Media for Enhanced Microbiological Research of Urinary Bacteria

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Key Words
Artificial urine, Urobiome, Urinary tract infection, Microbiome research, Bacterial cultivation, Microbial ecology

Funding

NWO ENW-XL OCENW.XL21.XL21.088

Disclosures
AJW discloses compensated membership on the following scientific advisory boards: Astek, Cerillo, Pathnostics, and Urobiome Therapeutics. All other authors have no disclosures.

Acknowledgments
We wish to thank Jacob Hogins and Larry Reitzer (UT-Dallas) for their suggested modifications to the SimUrine formulation, to Kathrin Tomasek for performing the osmolarity tests of our media, to Ayla Kwant for the conductivity and turbidity testing, and to Peter Dijkstra and Julien Es Sayed for their help testing viscosity and other physicochemical parameters.

Author Contributions
Conceptualization: AJW
Funding Acquisition: MdeV, AJW
Project Administration: MdeV, AJW
Methodology: PGM, BIC
Data Acquisition: PGM, BIC, MV, MHK
Analysis: PGM, BIC
Writing Original Draft: PGM
Review and Edit: PGM, BIC, MV, MHK, MdeV, AJW

ABSTRACT

Urinary tract infections represent one of the most prevalent bacterial diseases, yet current diagnostic and research methodologies are hampered by inadequate culture media that fail to replicate the bladder biochemical environment. Conventional artificial urine formulations contain undefined components, lack essential nutrients, or inadequately support urinary microbiome (urobiome) growth. To address these limitations, we developed SimUrine, a fully defined synthetic urine medium that aims to replicate human bladder chemistry while supporting diverse microbial growth requirements.

SimUrine was systematically developed through iterative optimization of multi-purpose artificial urine, incorporating defined concentrations of carbon sources, vitamins, trace elements, and amino acids within physiologically relevant ranges. The modular design enables component substitution without complete reformulation, facilitating customization for culturomics, antimicrobial susceptibility testing, and microbial ecology studies, while reducing batch-to-batch variability associated with authentic urine.

Performance evaluation demonstrated SimUrine's capability to support growth of fastidious urobiome members, including *Lactobacillus* species, *Aerococcus urinae*, and *Corynebacterium riegelii*, which fail to proliferate in conventional minimal media. Physicochemical characterization confirmed that SimUrine formulation exhibits properties within normal human urine ranges for density, conductivity, osmolarity, and viscosity, ensuring physiological relevance.

Clinical applications revealed reduced antibiotic susceptibility compared to standard media, suggesting more accurate representation of *in vivo* conditions. Co-culture experiments using *Escherichia coli* and *Enterococcus faecalis* demonstrated previously unobserved microbial interactions, highlighting SimUrine's utility for investigating urobiome dynamics.

SimUrine represents a significant advancement in urobiome research methodology, providing a standardized, reproducible platform for investigating urobiome under physiologically relevant conditions, potentially improving fundamental understanding and clinical diagnostic approaches.

IMPORTANCE

Urinary tract infections affect millions globally, yet current research and diagnostic methods rely on inadequate culture media that fail to replicate the bladder's unique biochemical environment. This fundamental limitation has hindered accurate UTI research and potentially compromised clinical treatment decisions. SimUrine addresses this critical gap as the first fully defined synthetic urine medium that mimics human bladder chemistry while supporting growth of diverse urinary microbes. The breakthrough enables cultivation of urobiome organisms in a minimal medium that resembles natural conditions, revealing novel microbial interactions that influence urinary health. Crucially, SimUrine demonstrates different antimicrobial susceptibility patterns compared to standard clinical media, suggesting current testing protocols may inaccurately predict treatment outcomes. This standardized, reproducible platform eliminates the variability of authentic urine samples while maintaining physiological relevance, potentially transforming urobiome research methodology and improving clinical diagnostic accuracy for urinary tract infections worldwide.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial diseases worldwide. They are particularly common in females; approximately 50-60% will experience at least one UTI in their lifetime (Al Lawati et al., 2024). The diagnosis of UTIs can be challenging due to the variable presentation of symptoms including pyuria, dysuria, and fever, which are not present in all cases. Symptom inconsistency has provoked debates within the medical community regarding standardized diagnostic criteria (Werneburg et al., 2024). Another significant challenge in UTI diagnosis is the discrepancy between these symptoms and the bacterial counts enumerated from standard urine culture technique – a methodology that often produces false negative results, particularly overlooking fastidious and anaerobic bacteria (Hilt et al., 2014). Newer diagnostic techniques with heightened sensitivity have increased the detection rates of bacteria among symptomatic individuals (Haley et al., 2023; Hilt et al., 2014; Moreland et al., 2025a). However, our understanding of these detected bacteria is limited, especially concerning the role of hard-to-cultivate species of the urinary microbiome (urobiome) in the context of UTIs (Moreland et al., 2025b; Price et al., 2016).

These limitations stem partly from the lack of culture media that accurately replicate the bladder's metabolic environment. Commercially available media, fortified with general nutrients, fail to replicate the nutritional composition and complexity of human urine (Reitzer & Zimmern, 2019). Using authentic urine samples introduces significant donor-to-donor variability, compromising experimental reproducibility (Asscher et al., 1966; Sreedevi et al., 2024). Another strategy is to use pooled urine, which has been theorized to reduce variation. However, a recent report showed that pooled urine is artificially optimized and not average (Hogins et al., 2022). Thus, without a standardized urine medium, comparing experimental results between experiments and different laboratories remains difficult.

Unfortunately, current formulations aiming to replicate bladder-like conditions either contain undefined components, do not encompass the complete range of essential nutrients, or are inadequate to support growth of the broad spectrum of urinary microbes (Brooks & Keevil, 1997; Zandbergen et al., 2021). Salt-based media formulations have been developed to mimic the

electrolyte composition of urine; nevertheless, these fail to sustain microbial growth due to their lack of or limited carbon content and absence of other vital nutrients (Chutipongtanate & Thongboonkerd, 2010; Sarigul et al., 2019). To achieve a standardized synthetic urine medium, it is essential to recreate the conditions that members of the urobiome encounter in their natural habitat (Sreedevi et al., 2024), incorporating carbon sources and vital nutrients necessary for the growth of diverse urinary microbes, including species considered to be pathogenic or commensal.

The objective of this study is to develop a fully defined medium that accurately replicates the chemical composition of urine in the human bladder and accommodates various experimental applications, including culturomics, co-evolutionary research, and synthetic communities. To achieve this end, we modified multi-purpose artificial urine (MP-AU) (Sarigul et al., 2019), incorporating defined concentrations of carbon sources, vitamins and salts, with the aim to maintain medium concentrations within the ranges reported for human urine. To determine the efficacy of our formulations, the experimental media were tested for their ability to support the growth of a diverse set of target microbes.

The resultant medium, termed SimUrine, alleviates the inconsistencies associated with using actual urine and ensures more uniform outcomes in urobiome investigations. SimUrine also exhibits physicochemical properties within the normal range of human urine including density, conductivity, refractive index, and viscosity – making for a physiologically relevant *in vitro* artificial medium.

SimUrine's composition is structured modularly, allowing for the substitution of individual components to accommodate varying research requirements without requiring full medium reconstruction. The modular design also enables facile, prolonged storage and replacement of components upon their expiration date. SimUrine supports a wider range of species than conventional minimal media while sustaining antibiotic susceptibility testing and displaying comparable or enhanced microbial survival rates, including for many fastidious urobiome members.

145 In summary, SimUrine addresses the significant constraints of existing artificial urine media by
146 providing a fully defined, customizable, nutrient-optimized medium for diverse research
147 applications, enhancing our comprehension of urinary microbial viability and interactions in
148 closely replicated bladder environments.

149 # METHODOLOGY: EXPERIMENTAL DESIGN AND MATERIALS

150 ## Bacterial Strains

151 Urinary bacterial isolates used in this study were sourced from strain collections of the Wolfe lab
152 collection at Loyola University Chicago or the de Vos lab at the University of Groningen
153 (**Supplementary Table 1**).

154 ## Reference Media

155 Our initial experiments utilized NYCIII medium as either a reference or to start cultures (as
156 indicated in each case), due to the fastidious growth requirements of *Lactobacillus* species.
157 However, to ensure simplicity and reproducibility, we conducted subsequent experiments using
158 Brain Heart Infusion (BHI) medium, except for the antibiotic susceptibility testing, for which
159 Mueller-Hinton Broth (MHB) was used to culture reference strains.
160

161 ## Optimization of MP-AU for Bacterial Growth

162 To assess bacterial viability in MP-AU (Sarigul et al., 2019), we evaluated *Escherichia coli*
163 (UMB1180) growth and survival in a modified version (mMP-AU), where $K_2C_2O_4.H_2O$ was
164 replaced by $NH_4C_2O_4.H_2O$. For this aim, we first grew the bacteria in NYCIII medium (Geaman
165 et al., 2024) for 48 hours at 37°C in 5% CO_2 and diluted to OD = 1. The culture was centrifuged,
166 washed three times using PBS 1X and resuspended in either 1 ml of mMP-AU or M9 salts. A 500
167 µL aliquot of this resuspension was inoculated into 5 ml of mMP-AU or M9 salts and incubated
168 at 37°C for 4 days in 5% CO_2 with continuous orbital agitation at 200 rpm. Samples were taken
169 every day, and serial dilutions were plated on BHI agar for enumeration of colony-forming units
170 (CFU/mL) to determine survival.
171

172 To determine if an enriched version of mMP-AU could support the growth of fastidious species,
173 we tested urinary isolates of *Lactobacillus jensenii* (UBM8651) and *Aerococcus urinae*
174 (UMB5254, aka type strain ATCC 51268) in mMP-AU supplemented with various glucose
175 concentrations. To evaluate additional nutritional requirements, mMP-AU was fortified with an
176 equimolar carbon source (1% each) mix (glucose, galactose, maltose, sucrose, fructose, mannitol,
177 and sorbitol), a vitamin mixture (thiamin 5.0 mg/L, riboflavin 5.0 mg/L, niacin 5mg/L, pantothenic
178 acid 5.0 mg/L, pyridoxine hydrochloride 10.0 mg/L, biotin 2.0 mg/L, and 4-aminobenzoic acid 5.0

179 mg/L) and casamino acids. Bacterial cultures were evaluated daily for survival, enumerating
180 CFU/mL on Colistin Nalidixic Acid (CNA) agar plates under 5% CO_2.
181
182 To assess the impact of residual nutrients from rich media on bacterial growth in nutrient-limited
183 conditions, strains of *E. coli* (UMB3190, aka ATCC 10798) and *Klebsiella pneumoniae*
184 (UMB9987) were first grown overnight in BHI. These precultures then were diluted 1:100 into
185 either mMP-AU or M9 salts and cultured under our standard conditions: 48 hours at 5% CO_2, 37°C
186 with orbital agitation of 200 rpm.
187

188 ## SimUrine Formulation Optimization

189 Several versions of SimUrine were tested and the final formulation is described in **Supplementary**
190 **Protocols 1** and **Supplementary Table 2**. Other versions are included for reference in
191 **Supplementary Protocols 2.** These formulations were designed to mimic the nutrient-limited
192 bladder environment through progressive enrichment of mMP-AU and by tuning reagent
193 concentrations to stay within urine ranges, while designing a modular protocol to enable facility,
194 prolonged storage, and component replacement. Different formulations were tested for growth of
195 selected isolates, previously cultured on BHI agar plates.
196
197 The first version of SimUrine (**SimUrine.v1**) was formulated as follows: mMP-AU supplemented
198 by adding N-acetylglucosamine, L-threonine, and L-serine (to mimic mucin composition without
199 the turbidity or contamination problems associated with direct use of mucins) and lactic acid,
200 pyruvic acid, acetic acid, manganese sulfate, and L-cysteine (to protect facultative species and
201 improve fastidious species growth). Bacterial growth was assessed for two consecutive periods of
202 48 hours.
203
204 Based on the performance of **SimUrine.v1**, and with the aim to extend use of the medium to a
205 wider range of microbes, a second version of SimUrine was designed (**SimUrine.v2**). Inspired by
206 the work on bacterial communities from the gut (Shetty et al., 2022), we incorporated vitamins
207 and trace elements into this formulation.
208

209 Given the biological relevance of iron, we assessed the impact of hemin supplementation in our
210 formulation with two concentrations: one as described in **SimUrine.v3** and one tenfold greater.
211 The need of uric acid for bacterial growth also was evaluated by growing strains in the presence
212 and absence of this compound.
213
214 To reduce spontaneous precipitate formation, improve medium stability, and keep uric acid in our
215 formulation, the reagent order was modified in **SimUrine.v4**: now uric acid was added after urea
216 and not before. We also improved buffering capacity of the medium with the addition of 4-(2-
217 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and to better mimic the nutrient profile
218 of urine, we added non-ionic surfactant Tween80 and extra peptides (Reitzer & Zimmern, 2019;
219 Schmied et al., 2021). Given the role of cysteine as a key energy and carbon source in various
220 environments (Göbbels et al., 2021), we also assessed the use of its dimeric form cystine, by testing
221 a version of **SimUrine.v.4** with cysteine replaced by cystine.
222
223 To minimize precipitate formation during storage, and reduce chelation of trace elements, sodium
224 bicarbonate and 3-(N-morpholino) propanesulfonic acid (MOPS) were added as buffering agents
225 instead of HEPES, resulting in **SimUrine.v5**. In contrast to earlier formulations that included
226 autoclaving of the final medium, this new formulation was sterilized by filtration with a 0.2 µm
227 filter.
228
229 To optimize conditions for *Lactobacillus* species growth, while considering the biological
230 relevance of the following nutrients, **SimUrine.v5** was enriched with glucose, $FeSO_4$, L-valine, L-
231 threonine, and L-tryptophan (Kwoji et al., 2022; Liu et al., 2020; Ramoneda et al., 2023; Saguir &
232 De Nadra, 2007; Veith et al., 2015). $NaHCO_3$ was added and pH reduced to increase stability.
233 Three different concentrations of urea were evaluated. Based on these experiments, a final version,
234 **SimUrine.v6** version was adopted.
235

236 SimUrine Evaluation

237 To assess the performance of the different SimUrine versions, pure bacterial isolates were first
238 grown overnight in a 20 ml glass tube containing 4 ml of the corresponding version of SimUrine
239 at standard conditions (37°C, 5% CO_2 with orbital agitation at 200 rpm). Overnight cultures were

240 diluted 1:100 into the noted SimUrine versions, and growth was monitored in 96-well flat-bottom
241 plates under standard conditions (37°C, 5% CO_2 and orbital agitation at 200 rpm) for 24, 48 or 72
242 hours (depending on the species). Optical density (OD) was assessed every ten minutes using
243 Biotek (Winooski, VT, USA) or Cerillo Alto (Charlottesville, VA, USA) plate readers (indicated
244 with each result). To confirm species identity, colonies were obtained from the cultures at the end
245 of each growth curve and identified using mass spectrometry on a Bruker MALDI-TOF (Billerica,
246 MA, USA).
247

248 ## Physicochemical Properties of SimUrine.v6

249 To characterize **SimUrine.v6** for broader applications, physicochemical parameters (density,
250 refractive index, viscosity, conductivity, and osmolarity) were determined from two independent
251 batches prepared two weeks apart, each measured in technical triplicate at 20°C. Density was
252 determined using a volumetric flask and an analytical balance, refractive index was measured
253 using a KERN Abbe ORT 1RS refractometer (Kern & Sohn, Balingen, Germany)**,** viscosity using
254 an MCR 702e rheometer (Anton Paar, Breda, The Netherlands), conductivity using a portable
255 conductivity meter (Mettler-Toledo, Columbus, OH, USA) and osmolarity using an Omo1
256 osmometer (Advance instruments, Norwood, MA, USA).
257

258 ## Evaluation of SimUrine.v6 in Microbial Ecology-Relevant Experiments

259 To compare the performance of **SimUrine.v6** for urobiome-related assays, antibiotic susceptibility
260 tests were performed on *E. coli* (UPEC20). A 1:19 ratio of Trimethoprim (TMP) and
261 sulfamethoxazole (SMX) was tested at a starting concentration of 32 µg/mL TMP and 608 µg/mL
262 SMX, with serial two-fold dilutions. Susceptibility to kanamycin was also tested, starting at 128
263 µg/mL. MHB was used as the reference medium. In all assays, the initial inoculum was ~ 2.5 x
264 10^5 CFU/mL. Optical density was measured in a 96-well plate using a Cerillo Alto reader (Cerillo,
265 Charlottesville, VA) at 37°C and 5% CO_2.
266

267 ## Co-culture of *E. coli* and *E. faecalis* Strains

268 Reference strains of *E. coli* (ATCC25922) and *E. faecalis* (ATCC29212) were obtained from the
269 American Type Culture Collection (ATCC). Prior to experimental use, both strains were sub-

270 cultured onto Trypticase Soy Agar (TSA) supplemented with 5% sheep blood and incubated at
271 37°C in a 5% CO_2 atmosphere for bacterial propagation and viability assessment. Overnight
272 cultures of both bacterial strains were prepared in NYCIII medium and SimUrine.v6 medium, with
273 a minimum of five biological replicates per strain. Cultures were incubated at 37°C with orbital
274 shaking at 200 rpm. Following overnight incubation, cultures were harvested by centrifugation at
275 3,000 × g for 10 minutes at room temperature, supernatants were discarded, and bacterial pellets
276 were resuspended in fresh medium (NYCIII or SimUrine.v6, respectively). Bacterial cell density
277 was standardized using optical density measurements at 600 nm (OD_{600}), with all cultures adjusted
278 to an initial OD_{600} of 0.05. For growth kinetic measurements, 80 µL of standardized bacterial
279 suspension was combined with 720 µL of the appropriate growth medium (NYCIII or
280 SimUrine.v6) in individual wells of a dual-well system (Cerillo co-culture duet system,
281 Charlottesville, VA). Control wells contained 800 µL of sterile medium without bacterial
282 inoculum.
283
284 Growth kinetics were monitored over 24 hours using a Cerillo Alto microplate reader with
285 continuous orbital shaking at 200 rpm at 37°C. Optical density measurements (OD_{600}) were
286 recorded at 30-minute intervals, and final readings at 24 hours were used for subsequent data
287 analysis. Growth curves and standard error calculations were performed using MATLAB-R2025a
288 (The MathWorks, Inc., 2025).
289

290 SimUrine.v6 Protocol for 500 mL of Media (Abbreviated):

291 Preparation:
292 Follow this abbreviated protocol in a sterile environment using aseptic technique to ensure
293 reproducibility. All stock solutions must be prepared and either filtered (0.2 µm) or autoclaved
294 separately before making the medium (see **Supplementary Protocol 1 and Supplementary Table**
295 **2, for details and reagents**).
296
297 Once stock solutions are prepared and sterilized, dissolve the module reagents sequentially as
298 indicated into 200 mL of dH_2O, stirring at 40°C: starting with the **mMP-AU** module, followed by
299 the **Amino acids** module, the **Carbon sources** module, and the **Others** module. While the medium
300 is warm, add 500 µL of Tween80, 1.05 g of MOPS and 7.5 g of urea. Continue adding the stock

solutions sequentially, check and adjust pH to 6 with HCl or NaOH, add freshly prepared uric acid solution, and finally L-serine, as indicated in the protocol. Check pH and adjust if needed. Complete volume to 500 mL with H_2O. Filter-sterilize the media using a 0.2 µm filter. To avoid precipitation, avoid storage at temperatures lower than 4°C. To extend lifespan, protect from light.

RESULTS

Optimization of mMP-AU for Bacterial Survival

To compare viability of *E. coli* (UMB1180) in minimal salt bases, we monitored survival over 8 days. Survival was marginally better in mMP-AU compared to M9 salts across the whole experiment (**Fig.1**). This trend was maintained through day 8, indicating that mMP-AU supports bacterial viability at least as effectively as M9.

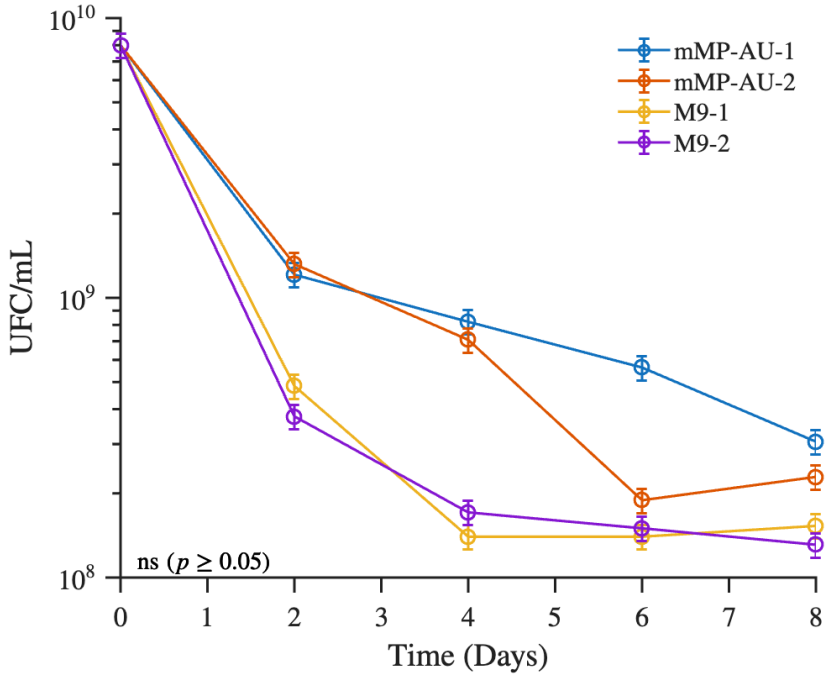

Fig.1 | Survival of *E. coli* (UMB1180) in either mMP-AU or M9 salts. Two technical replicates were conducted for each condition, with each replicate averaging two biological replicates. The y-axis displays logarithmic value of CFU/mL of *E. coli,* and the x-axis represents time in days, standard deviation is displayed for each point. These results suggest that both salt bases similarly support limited survival of urobiome species.

Glucose Supplementation Influences Survival of Urinary Isolates

To evaluate the contribution of a carbon source to survival of urobiome species, we incorporated glucose to the minimal salt bases. For mMP-AU and M9 salts, glucose supplementation had a

contrasting effect on survival of a urinary isolate of *Lactobacillus jensenii* (UBM8651). For mMP-AU, a glucose concentration above 2% reduced survival, whereas elevated glucose concentrations had little effect on survival in M9 salts (**Supplementary Figure 1A**); however, survival on either salt base did not extend beyond one day. For *Aerococcus urinae* (UBM5254), increased glucose concentration was directly associated with enhanced survival when using mMP-AU as the salt base. In contrast to *L. jensenii* (UBM8651), *A. urinae* (UBM5254) survived for a second day, but only in glucose-supplemented M9 salts (**Supplementary Figure 1B**).

As elevated glucose concentrations reduced *L. jensenii* (UMB8651) survival in mMP-AU, we tested the impact of various carbon sources and nutrients on UMB8651 growth in modified versions of mMP-AU. Growth was primarily supported by amino acids and vitamins, while other supplements failed to fully sustain bacterial proliferation (**Supplementary Figure 2**).

Effect of Residual Nutrients from the Inoculum on Bacterial Growth

Strains of *E. coli* (UBM3190) and *K. pneumoniae* (UBM9987) pre-grown on the rich medium BHI and then transferred to mMP-AU or M9 salts without supplementation reached between one-tenth and one-third of their maximum OD when grown in BHI, indicating that residual nutrients from the rich medium supported partial growth (**Fig.2A and Fig.2B**).

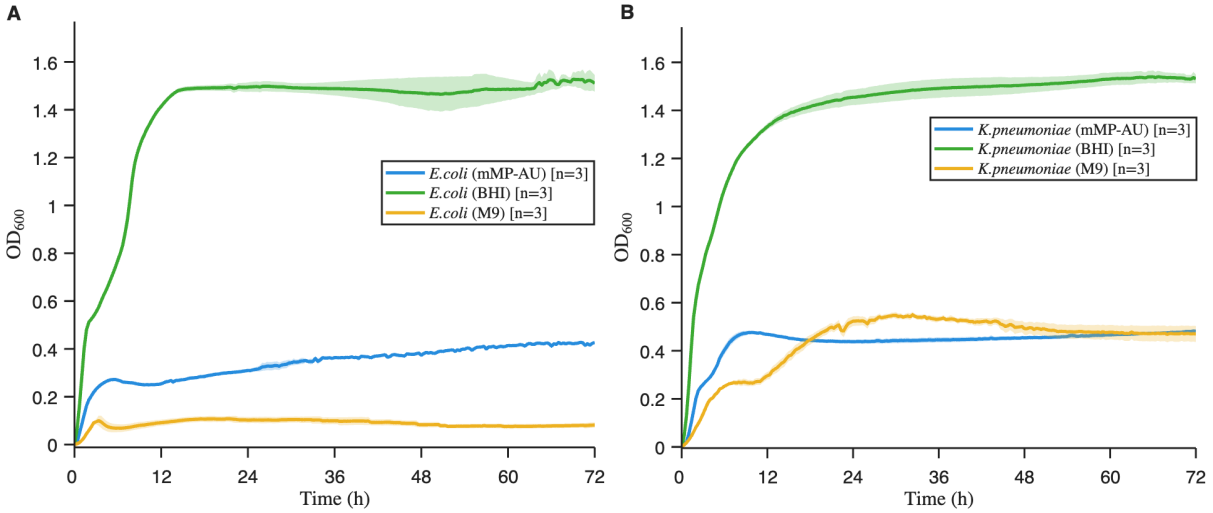

Fig.2 | Effect of trace nutrients from complex media on bacterial growth. Overnight cultures of *E. coli* (UMB3190) (A) and *Klebsiella pneumoniae* (UMB9987) (B) were diluted 1:100 into BHI, M9 or mMP-AU and incubated for 48 hours. Optical density at 600 nm (OD_{600}) is shown on the y-axes; time (hours) on the x-axes. Solid lines represent mean values and shading standard error of the mean.

SimUrine Formulation Optimization

SimUrine.v1. This simplest version of SimUrine supported the discrete growth of the facultative anaerobic bacterial species *E. coli* (UBM3190), *Actinotignum schaalii* (UBM13319), *A. urinae* (UBM5254), *Klebsiella pneumoniae* (UBM9987) and *Streptococcus anginosus* (UBM8616), while failing to support the growth of *Enterococcus faecalis* (**Fig.3**).

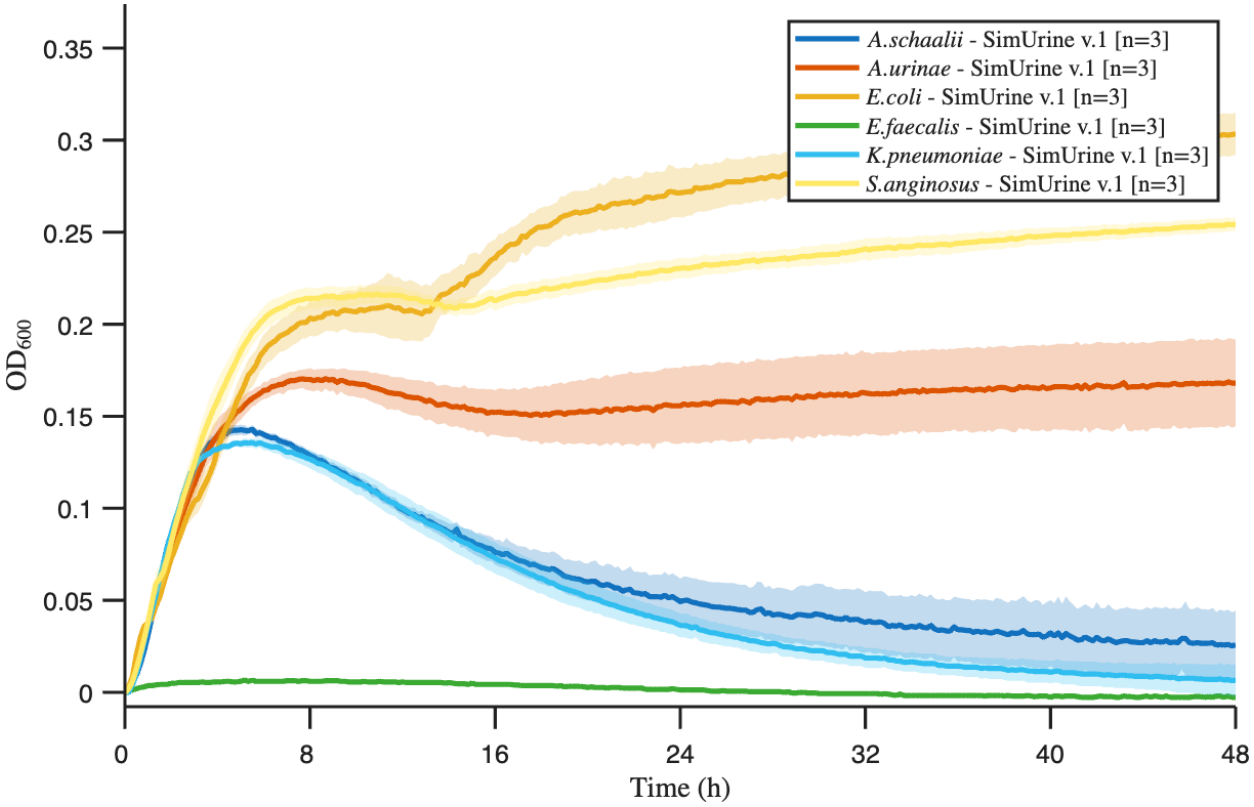

Fig.3 | Growth in SimUrine.v1. Cultures of different bacterial isolates over 48 hours. OD_{600} is shown on the y-axis; time (hours) on the x-axis. Solid lines represent mean values and shading standard error of the mean.

SimUrine.v2. Adding vitamins and trace elements to our formulation markedly enhanced the growth of *E. coli* (UBM3190), with OD_{600} values increasing from 0.2-0.3 (**Fig.3**) to 0.7, which exceeded growth in BHI (**Fig.4A**). *Klebsiella pneumoniae* (UBM9987) also showed improved growth compared to SimUrine.v.1 (**Fig.3 and Fig.4B**); however, unlike *E. coli* (UBM3190), *K. pneumoniae* did not surpass its growth in BHI. Despite enhanced bacterial growth with this version, a precipitate formed in SimUrine.v2 after 24 hours of preparation (**Supplementary Figure 3**).

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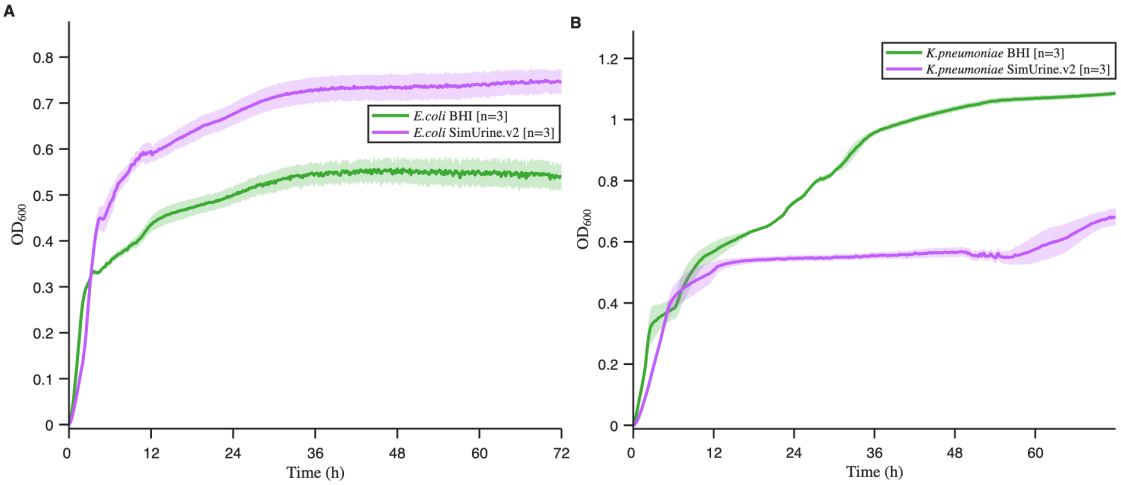
363



364

365

366 **Fig.4 | Growth in SimUrine.v2**. Cultures of *E. coli* (UBM3190) (A) and *K. pneumoniae*
367 (UMB9987) (B) in SimUrine.v2. Optical density at 600 nm (OD_{600}) is shown on the y-axis; time
368 (hours) on the x-axis. Parallel cultures in BHI (green lines) were used as a reference. Solid lines
369 represent mean values and shading standard error.

370

371 ***SimUrine.v3.*** Hemin supplementation (either 1X or 10X) to produce SimUrine.v3 did not
372 substantially improve *E. coli* CFT073 growth (compared to SimUrine.v2 growth) but had a marked
373 effect on the growth characteristics of *S. anginosus* (UBM8616) (**Fig.5A and Fig.5B**). Based on
374 these results, we adapted our formulation, reducing the concentration of hemin from 10X to 5X,
375 while evaluating the importance of uric acid for bacterial growth. These modifications revealed
376 that uric acid was essential for reaching higher OD_{600} values and enhancing exponential growth,
377 while hemin 5X performed well (**Supplementary Figure 4)**. Hemin incorporation defined
378 SimUrine.v.3.

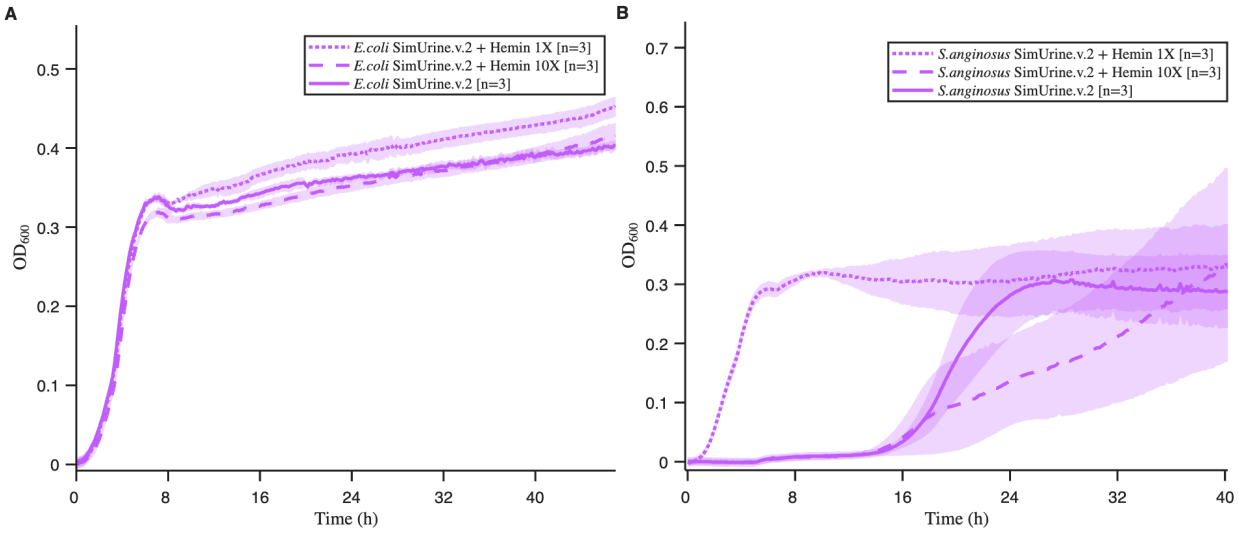
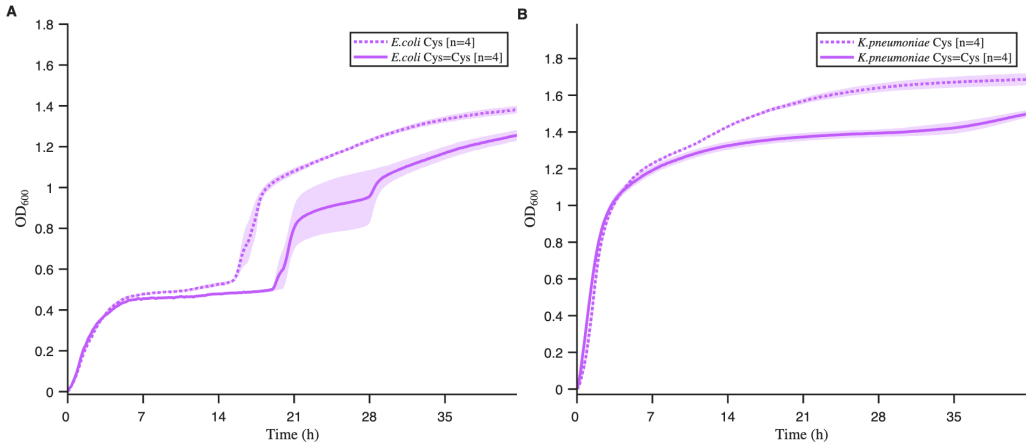


Fig.5 | Growth in SimUrine.v2 and hemin supplemented versions. Cultures of *E. coli* (UBM3190) (A) and *S. anginosus* (UMB8616) (B) in SimUrine.v2 (solid lines) and SimUrine.v.2 supplemented with hemin 1X (dotted lines) and hemin 10X (dashed lines). Optical density at 600 nm (OD_{600}) is shown on the y-axes; time (hours) on the x-axes. Lines represent mean values and shading standard error of the mean.

SimUrine.v4 represented a major improvement of SimUrine formulation. We incorporated amino acids within urine-relevant concentration ranges (Schmied et al., 2021), changed the order of hemin and uric acid supplementation, and introduced both HEPES and Tween80. We used this formulation to test if bacteria could grow with cysteine or its dimer, cystine. For *E. coli* (UBM3190) and *K. pneumoniae* (UBM9987), SimUrine.v4 resulted in vastly improved OD_{600} (compare **Fig.6A and Fig.6B** to **Fig.5A and Fig.5B)** and resulted in substantial growth of *E. faecalis* and *C. riegelii* (**Fig.6C and Fig.6D**). In all cases, cysteine performed better than cystine.



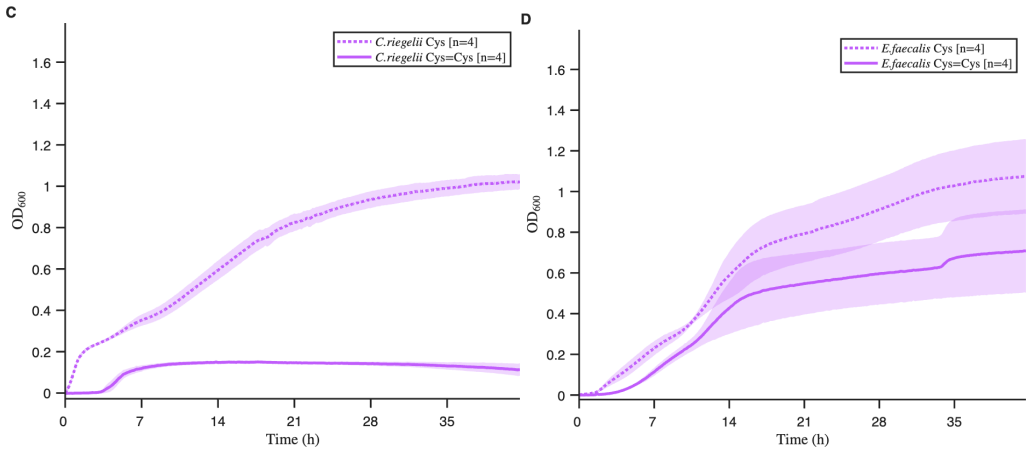


Fig.6 | Growth in SimUrine.v4. Cultures of *E. coli* (UBM3190) (A), *K. pneumoniae* (UBM9987) (B), *C. riegelii* (UBM12267) (C) and *E. faecalis* (UBM3193) (D) in cysteine (dotted lines) or cystine (solid lines). Optical density at 600 nm (OD_{600}) is shown on the y-axes; time (hours) on the x-axes. Lines represent mean values and shading standard error of the mean.

SimUrine.v5. We increased long term formulation stability without altering growth characteristics by replacing HEPES with MOPS, incorporating sodium bicarbonate, and by filter-sterilizing the medium (data not shown).

SimUrine.v6. Supplementation of SimUrine.v5 with glucose, $FeSO_4$, L-valine, L-threonine, and L-tryptophan supported the growth of a larger number of microbes than previous versions, including *Lactobacillus* species, *E. faecalis, Proteus mirabilis, Prevotella bucallis,* among others (**Supplementary Table 1**). Urea concentration did not substantially affect the growth of *E. faecalis* (UBM3193) (**Fig.7A**), but the higher urea concentrations improved the growth of *P. mirabilis* (UBM7310) (**Fig.7B**). The new formulation reduced growth of *E. faecalis* compared to SimUrine.v4 (**Fig.6D**). Despite the reduced growth, the higher urea concentration was kept in the final formulation to better reflect physiological urine levels, as bacterial viability was not compromised.

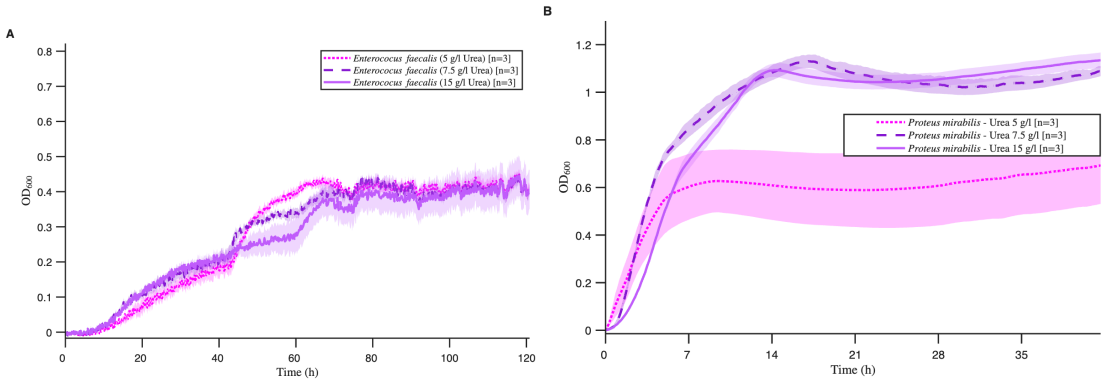


Fig.7 | Effect of urea in SimUrineV6. *E. faecalis* (UBM7540) (A), and *P. mirabilis* (strain 83) (B) in SimUrineV6 with 3 different concentrations of urea. Optical density at 600 nm (OD_{600}) is shown on the y-axes; time (hours) on the x-axes. Lines represent mean values and shading standard error.

Use of SimUrine.v6 in biologically relevant urobiome microbial experiments

We evaluated the use of SimUrine.v6 in two ecology-relevant contexts: antibiotic susceptibility testing and a bacterial interaction assay. *E. coli* (UPEC20) exhibited reduced susceptibility to both TMP-SMX and kanamycin in SimUrine.v6 (**Fig.8A and Fig.9A**), compared to that observed in the richer, complex reference medium, MHB (**Fig.8B and Fig.9B**).

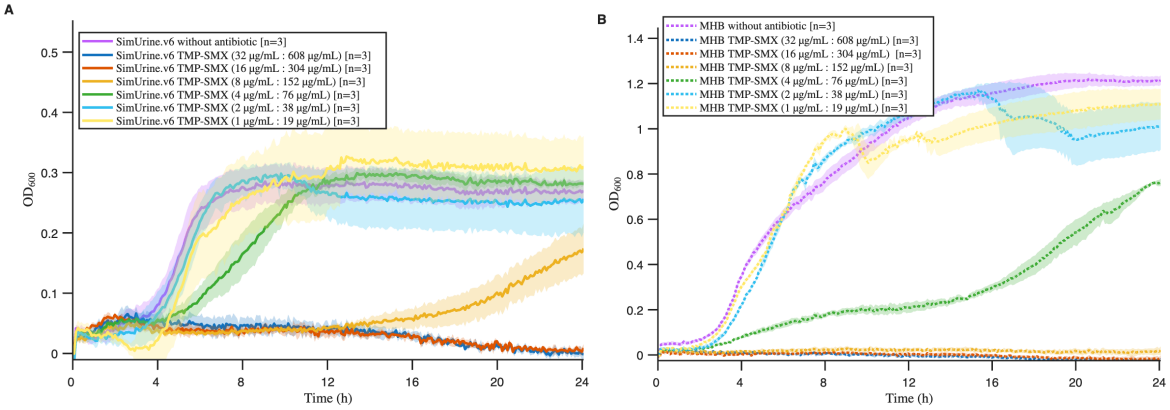


Fig.8 | TMP-SMX susceptibility of *E. coli* (UPEC20**)**. Cultures of *E. coli* in SimUrine.v6 (A) and MHB (B). Initial antibiotic concentration TMP-SMX (Trimethoprim 32 µg/mL: Sulfamethoxazole 608 µg/mL) was serially diluted by half each time, to a final concentration of TMP-SMX 1µg/mL:19 µg/mL. Cultures in media without antibiotic were used as control. Optical density at 600 nm (OD_{600}) is shown on the y-axis; time (hours) on the x-axis. Lines represent mean values and shading standard error of the mean.

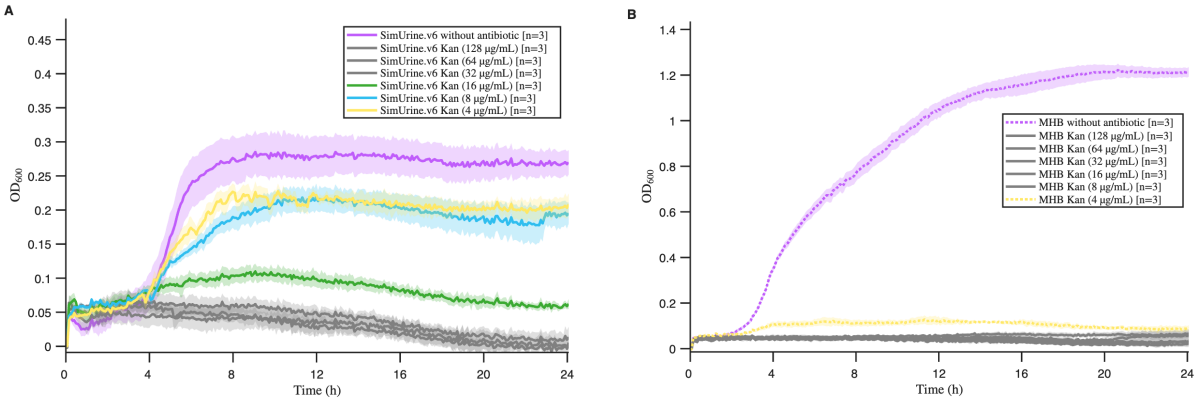


Figure 9 | Kanamycin susceptibility of *E. coli* (UPEC20**)**. Cultures of *E. coli* in SimUrine.v6 (A) and MHB (B) in decreasing concentrations of Kanamycin. Initial antibiotic concentration Kan (128 µg/mL) was serially diluted by half each time, to a final concentration of Kan (4µg/mL). Gray curves indicate no growth for both media; colored curves indicate growth. Cultures in media without antibiotic were used as control. Optical density at 600 nm (OD_{600}) is shown on the y-axis; time (hours) on the x-axis. Lines represent mean values and shading standard error of the mean.

Growth characteristics of *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) also differed in SimUrine.v6 versus the rich, complex NYCIII. In NYCIII, entry of *E. coli* into exponential growth was rapid and there was little difference in the growth characteristics of *E. coli* cultured alone and in co-culture with *E. faecalis* (**Fig.10A**). In SimUrine.v6, entry of *E. coli* into exponential growth was similarly slower when grown either alone or in co-culture with *E. faecalis*, but entry of *E. coli* into stationary phase was more abrupt and occurred at a lower OD when co-cultured with *E. faecalis* (**Fig.11A**). In NYCIII, entry of *E. faecalis* into exponential growth was similarly rapid when grown alone or in co-culture with *E. coli*, while entry into stationary phase occurred at a slightly lower OD when co-cultured with *E. coli* than when grown alone (**Fig.10B**). In SimUrine.v6, *E. faecalis* grown alone experienced a long lag but when co-cultured with *E. coli* failed to grow (**Fig.11B**).

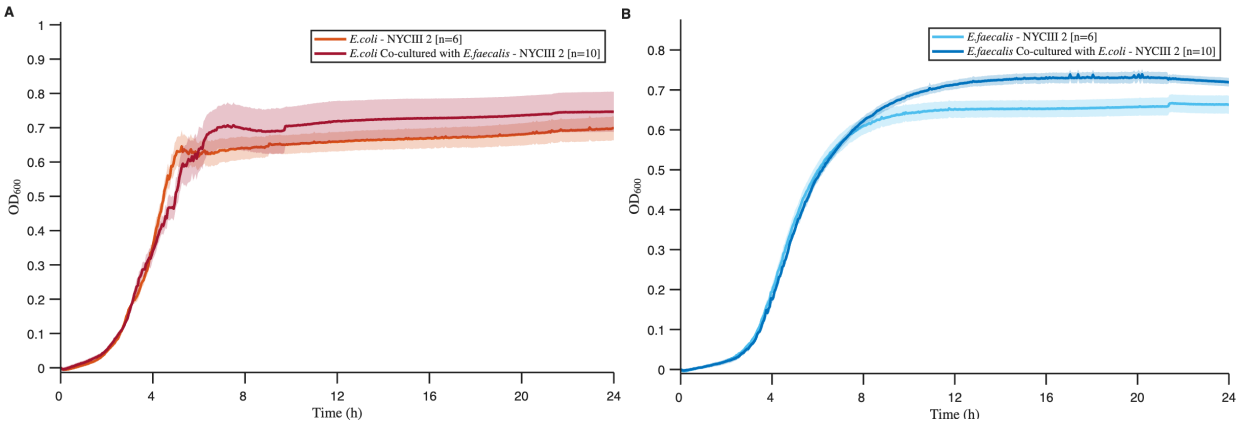


Fig.10: **Bacterial growth curve in NYCIII medium of *E. coli* (ATCC 25922) (A) and *E. faecalis* (ATCC 29212) in dual system.** Optical density at 600 nm (OD_{600}) is shown on the y-axis; time (hours) on the x-axis. Lines represent mean values and shading standard error of the mean. The orange-colored line in panel A represents individual *E. coli* cultures whereas the light blue line in panel B represents individual *E. faecalis* cultures. The dark red line in panel A represents *E. coli* co-cultured with *E. faecalis* in opposing duet wells whereas the dark blue line in panel B represents *E. faecalis* co-cultured with *E. coli* in opposing duet wells.

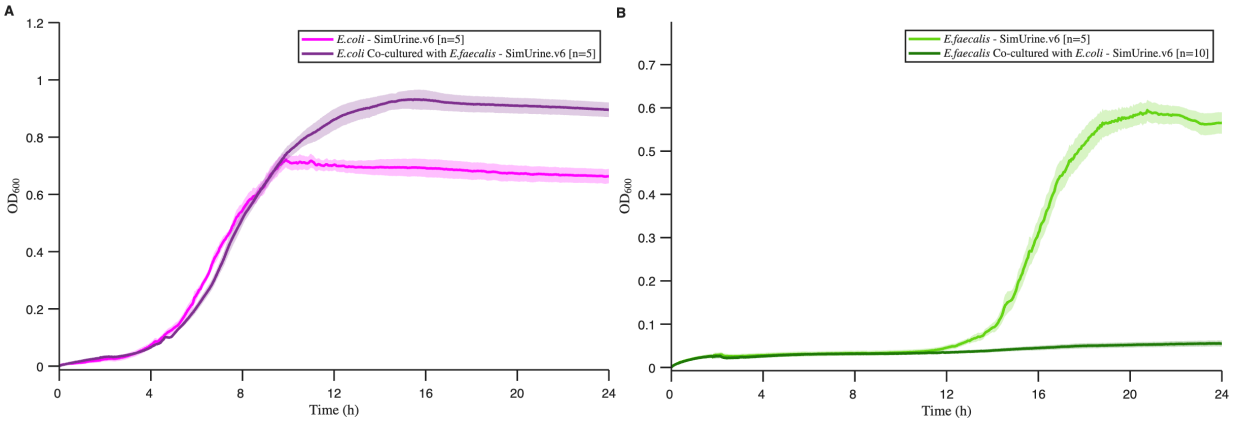


Fig.11: Bacterial growth curve in SimUrine.v6 medium of *E. coli* (ATCC 25922) (A) and *E. faecalis* (ATCC 29212) in dual system. Optical density at 600 nm (OD_{600}) is shown on the y-axis; time (hours) on the x-axis. Lines represent mean values and shading standard error of the mean. The magenta line in panel A represents individual *E. coli* cultures whereas the light green line in panel B represents individual *E. faecalis* cultures. The plum-colored line in panel A represents *E. coli* co-cultured with *E.faecalis* whereas the dark green line in panel B represents *E. faecalis* co-cultured with *E. coli* in opposing duet wells.

SimUrine.v6 physicochemical parameters

SimUrine.v6 exhibited physicochemical properties within the normal range of human urine (as described in the literature (**Table 1**), including low viscosity, making it suitable for microfluidic applications.

473 **Table 1**

Parameter (20°C)	SimUrineV6	diH_2O	Urine (Reference)
Density (g/mL)	1.0095 (+/- 0.0035)	0.9869	1.005-1.030 (Armstrong et al., 1998)
Conductivity (mS/cm)	16.9	0	1-34 (~22) (Fazil Marickar, 2010; Putnam & McDonnell Douglas Astronautics Company-West., 1971)
Refractive index	1.3404	1.333	1.34 (Yadav et al., 2020)
Viscosity (mPa·s)	1.1080	1.000	1.05 -1.2 (Inman et al., 2013)
Osmolarity mOsm/kg	671.25 (+/- 3.78)	0	500 - 850 (Perrier et al., 2015)

474

475 **DISCUSSION**

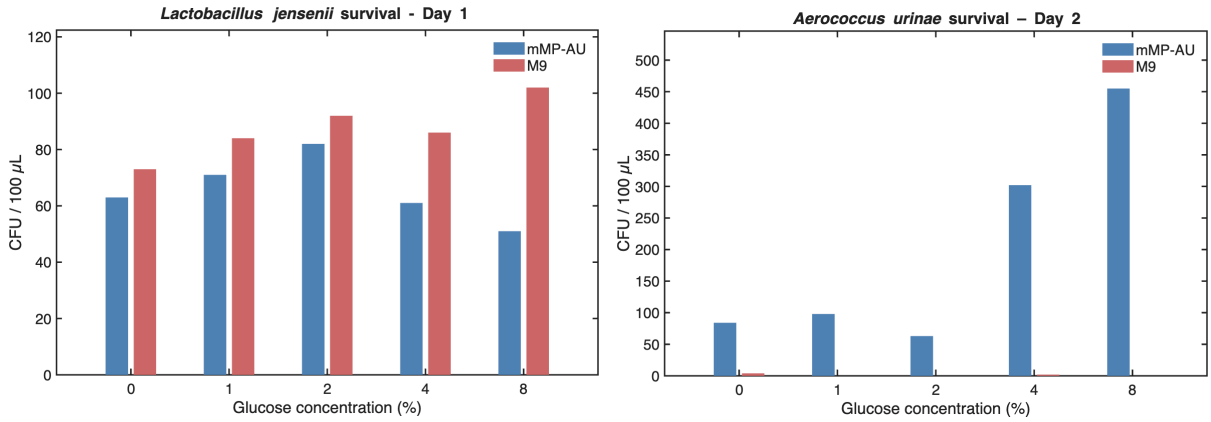
476 Conventional culture media for human urobiome studies utilize standardized formulations that fail
477 to reflect the urinary tract's natural biochemical environment. This discrepancy stems primarily
478 from the use of undefined reagents that create complex, proprietary formulations that are difficult
479 to replicate across laboratories. The human bladder maintains distinct physiological parameters
480 including specific pH, osmolarity, and metabolite concentrations that differ substantially from
481 commercial media compositions. We developed SimUrine, a fully chemically defined medium
482 where each component was selected to either match the documented human biochemical
483 parameters or support urobiome growth at minimal effective concentrations.
484
485 Following urobiome discovery, researchers have attempted to isolate, culture and characterize
486 these microbes, using artificial urine media or enriched formulations (e.g., NYCIII medium) to
487 maximize isolate recovery. While these approaches have enabled the isolation and study of novel
488 strains, current media formulations have limited our understanding of their behavior under native
489 conditions.
490
491 SimUrine development integrates current knowledge of urine composition, artificial urine media,
492 and cultivation strategies used for other microbial communities, such as the gut microbiome
493 (Shetty et al., 2022). By adapting the established MP-AU medium, we successfully cultured
494 bacteria important for bladder health and disease while providing an open, defined, and modifiable
495 medium formulation. This medium permits the behavioral study of many fastidious and anaerobic
496 bacteria species that previously failed to survive under previous strategies.
497
498 Development of SimUrine followed a systematic approach, beginning with a basic salt base (MP-
499 AU) that was subsequently enriched with multiple carbon sources to identify the most relevant
500 biochemical requirements for bacterial growth. Our initial version (SimUrine.v1) incorporated
501 nutrients accessible to the urobiome within the bladder environment. While N-acetylglucosamine,
502 L-threonine, and L-serine may not be components of excreted urine, the urobiome has access to
503 mucins and epithelial cells within the bladder lining, from where these nutrients can be
504 metabolized (Neugent et al., 2023). This formulation yielded improved growth for the tested
505 bacteria (**Fig.3**).

506 To enable growth of a broader range of microbes, we selected nutrients and vitamins identified
507 from successful gut microbiome cultivation studies. SimUrine.v2 included common vitamins and
508 trace elements, resulting in enhanced growth of *E. coli* (UBM3190) and *K. pneumoniae*
509 (UBM9987) (**Fig.4**), although it did not support the optimal growth of other species of interest,
510 such as *E. faecalis* and *A. urinae*, among others. Subsequent testing revealed that hemin
511 incorporation and expanded amino acid availability had considerable effects on bacterial growth,
512 as demonstrated in SimUrine.v.4 (**Fig.5, Supplementary Figure 4**).
513
514 The inclusion of Tween80, an important medium surfactant, along with improved buffering
515 capacity through HEPES addition, extended media utility to more fastidious organisms such as *C.*
516 *riegelii* (**Fig.6**). However, *Lactobacillus* species remained uncultured with this formulation. Final
517 optimization involved replacing HEPES with MOPS, adding sodium bicarbonate, implementing
518 filter sterilization rather than autoclaving, and supplementing with $FeSO_4$, which enabled
519 *Lactobacillus* to grow in glass tubes (**Supplementary Table 1**). We hypothesize that HEPES
520 combined with phosphate buffer provides insufficient buffering capacity under 5% CO_2
521 incubation, potentially contributing to trace element chelation that affects fastidious bacterial
522 growth. In contrast, MOPS maintains pH stability and reduces trace element chelation, while
523 $FeSO_4$ enhances iron bioavailability. Whereas we could culture *Lactobacillus* species in glass
524 tubes, we have not yet been able to obtain reliable growth curves using 96-well plates. Increasing
525 the diameter and volume of the wells may enable reliable growth curve generation.
526
527 Beyond the logical importance of using a medium that better replicates actual urine composition,
528 our antibiotic susceptibility and co-culture results demonstrate the feasibility of utilizing SimUrine
529 in clinical investigations. Using this fully defined medium, for the tested strain (**Fig.8**), we detected
530 lower antibiotic susceptibility than standard values for *E.coli* (Lewis II et al., 2023), even when
531 using *in vivo*-like TMP-SMX proportions (U.S. Food and Drug Administration, 2011), potentially
532 reflecting susceptibilities closer to those encountered under native bladder conditions.
533 Additionally, SimUrine enabled observation of previously unknown interactions between
534 urobiome community members, highlighting the importance of physiologically relevant culture
535 conditions for understanding clinical microbiology.
536

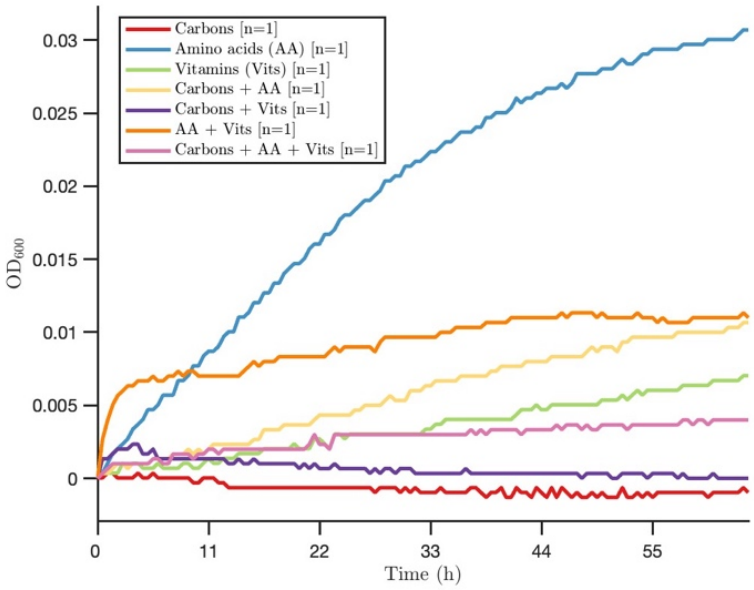
537 SimUrine enables the use of physiological urea concentrations without completely suppressing
538 growth of urea-susceptible bacteria (**Fig.7**). Urea incorporation proved beneficial for species such
539 as *P. mirabilis* (**Fig.7B**). Indeed, urea is noted to be the most abundant organic molecule in human
540 urine at concentration ranges between 9.3-23.3g/L (Putnam & McDonnell Douglas Astronautics
541 Company-West, 1971). At 15g/L, SimUrine's urea concentration allows for more accurate
542 representation of a natural bladder environment while maintaining broad bacterial compatibility.
543
544 SimUrine represents a fully defined culture medium that supports growth of diverse bacterial
545 species and clinical isolates while remaining adaptable to specific research and clinical
546 requirements. The medium's complete chemical definition ensures reproducibility across
547 laboratories, while its modular design allows targeted modifications without compromising
548 stability. Previous artificial urine compositions, such as those formulated by Brooks and Keevil or
549 Zandbergen, utilize protein hydrolysates that provide wide coverage of necessary amino acids, but
550 introduce unnecessary variability via undefined compositions (Brooks & Keevil, 1997;
551 Zandbergen et al., 2021). With defined nutrient compositions, the dietary behaviors of tested
552 bacteria can be fully characterized with SimUrine.
553
554 The clinical implications of this work extend beyond improved pathogen recovery and
555 characterization. By providing culture conditions that more accurately reflect the native bladder
556 environment, SimUrine enables more clinically relevant assessment of antimicrobial susceptibility
557 patterns (**Fig. 8 and Fig.9**) and microbial interactions (**Fig.11**) that may be not observed when
558 using rich media (**Fig.10**) (Lewis II et al., 2023). We acknowledge that the *E. faecalis* suppression
559 in co-culture (**Fig.11B**) needs mechanistic investigation, which exceeds the purpose of this work.
560
561 This enhanced physiological relevance may lead to improved therapeutic decision-making and
562 better understanding of urobiome dynamics in health and disease. The defined nature of SimUrine
563 medium, and its physicochemical properties, makes it ideal for use in the study of the urobiome,
564 using the duet system, microfluidic devices, organoid-on-a-chip devices, or simple flasks.
565
566 SimUrine cannot fully simulate several aspects of human urine. Certainly, it would be nearly
567 impossible to account for the thousands of biomolecules that can be isolated in human urine, the

568 majority of which are found in near trace amounts. However, even the most minute quantities of
569 certain compounds may have physiological relevance when studying urobiome behaviors.
570 SimUrine excludes hormones, xenobiotics, chromogens, cellular debris, mucoproteins, and
571 macromolecules (DNA, collagen) due to instability, undefined composition, wide physiological
572 ranges, or cost. However, SimUrine attempts to account for the vital contribution of
573 glycosaminoglycans in the bladder environment towards bacterial survival with the inclusion of
574 N-acetylglucosamine, L-threonine, and L-serine, all glycosaminoglycan degradation metabolites.
575 It will be important to account for these missing variables when attempting to simulate bladder
576 environments. Incorporating cell cultures such as urothelial cell lines or organoids may attempt to
577 address some of these deficits.
578
579 Future applications of SimUrine include standardized urobiome research protocols, probiotic
580 efficacy testing under physiologically relevant conditions, and development of personalized
581 therapeutic approaches based on individual urine chemistry profiles. The medium's defined
582 composition also facilitates mechanistic studies of host-microbe interactions within the urinary
583 tract ecosystem.

Supplementary Data



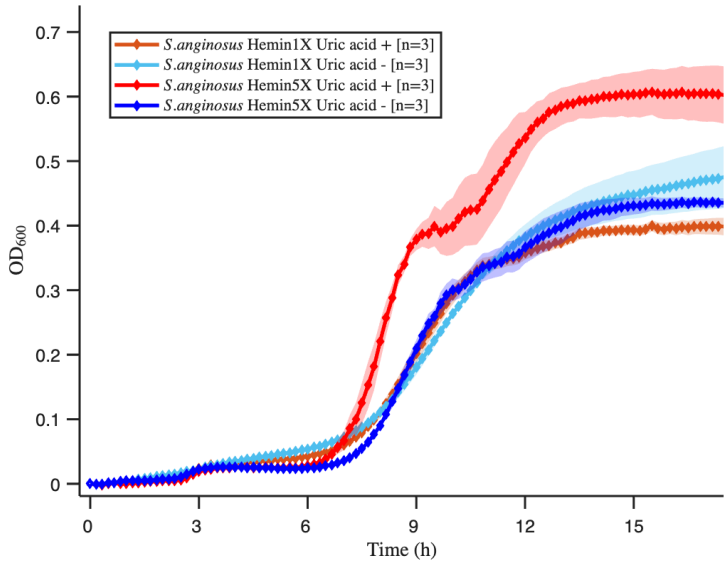
Supplementary Figure 1 | Effect of glucose supplementation on bacterial survival. (**A**) *Lactobacillus jensenii* (UMB8651) and (**B**) *Aerococcus urinae (*UMB5254*)* survival in mMP-AU or M9 salts supplemented with glucose. The y-axes display CFU/100 µL, and the x-axes represent glucose concentration.



Supplementary Figure 2 | Effect of nutrient supplementation on *Lactobacillus jensenii* growth. Growth curves of *L. jensenii* (UMB8651) cultured in mMP-AU supplemented with carbon sources, amino acid sources, vitamins, or their combinations. Optical density at 600 nm (OD_{600}) is shown on the y-axis; time (hours) on the x-axis

Supplementary Figure 3: **SimUrine.v1**. Left image shows the medium as it looked just out of the autoclave; right image, after 24 hours. Precipitate was formed within 24 hours.

605



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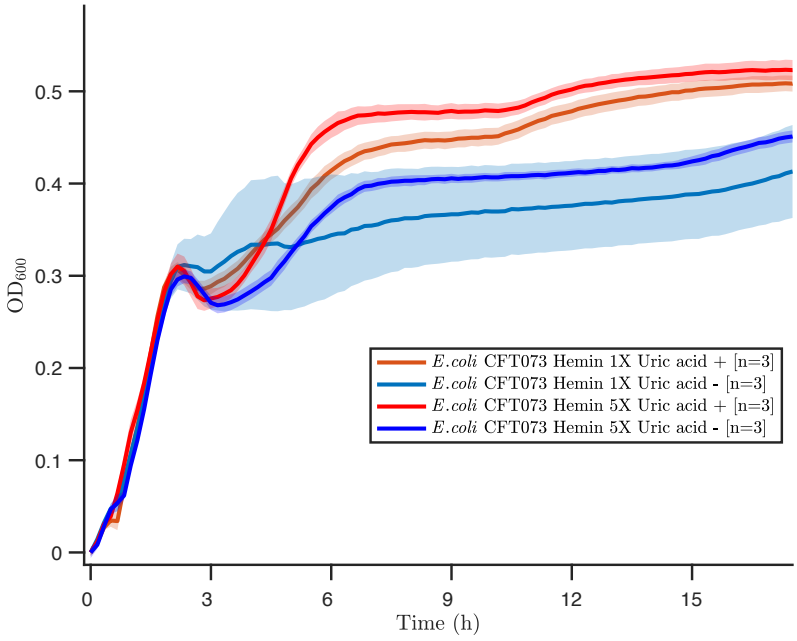

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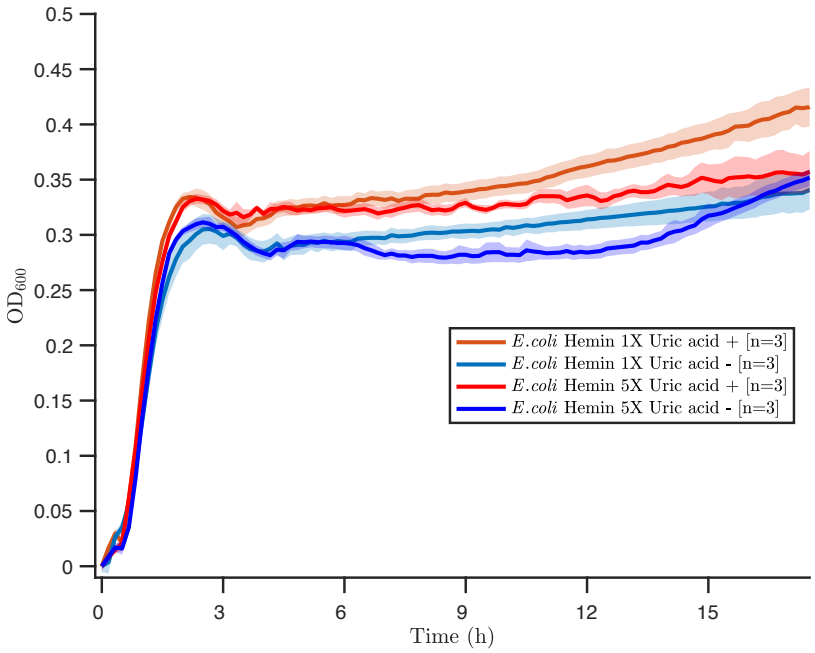

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Supplementary Figure 4: Cultures of *Streptococcus anginosus* (UBM 3192) (A), *E. coli* CFT073 (B) and *E. coli* (UBM3190) (C) in hemin-supplemented SimUrine.v3 in presence or absence of uric acid. Bold and light colors for high and low levels of hemin respectively, red tones for presence of uric acid and blue tones for absence. Optical density at 600 nm (OD600) is shown on the y-axis; time (hours) on the x-axis.

622 **Supplementary Table 1: Strains used on this publication**
623

Specie	Reference	Able to grow in SimUrine.V6 - 37°C
Escherichia coli	UMB1180	Not Tested
Lactobacillus jensenii	UBM8651	Only in tubes, 5 % CO_2
Aerococcus urinae	UMB5254 aka type strain ATCC 51268	Tubes and 96-well plates, 5% CO_2
Escherichia coli	UMB3190, aka ATCC 10798	Tubes and 96-well plates, 5% CO_2
Escherichia coli (UPEC20)	NCBI BioSample database. Accession SAMN041053058. National Library of Medicine, National Center for Biotechnology Information (RRID: SCR_004854).	Tubes and 96-well plates, 5% CO_2
Escherichia coli	ATCC 25922	Tubes and 96-well plates, 5% CO_2
Enterococcus faecalis	ATCC 29212	Tubes and 96-well plates, 5% CO_2
Klebsiella pneumoniae	UBM9987	Tubes and 96-well plates, 5% CO_2
Actinotignum schaalii	UBM13319	Only in glass tubes, 5 % CO_2
Streptococcus anginosus	UBM8616	Tubes and 96-well plates, 5% CO_2
Corynebacterium riegelii	UBM12267	Tubes and 96-well plates, 5% CO_2
Enterococcus faecalis	UBM3193	Tubes and 96-well plates, 5% CO_2
Enterococcus faecalis	UBM7540	Tubes and 96-well plates, 5% CO_2
Proteus mirabilis (strain83)	Local strain (Croxall et al., 2011)	Tubes and 96-well plates, 5% CO_2
Lactobacillus gasseri	UMB4205	Only in glass tubes, 5 % CO_2
Proteus mirabilis	UBM7310	Tubes and 96-well plates, 5% CO_2
Streptococcus anginosus	UBM3192	Tubes and 96-well plates, 5% CO_2
Lactobacillus gasseri	UBM245	Only in glass tubes, anaerobic

Lactobacillus jensenii	UBM387	Only in glass tubes, anaerobic
Prevotella buccalis	UBM536	Only in glass tubes, anaerobic

624

625 **Supplementary Protocols 1**

626 **SimUrine.v6**

627

628 Protocol for 500mL of media 1X.

629

630 Add 200 mL of H_2O to a flask and dissolve the following reagents one by one stirring and
631 keeping at 40°C.

632

Module	Reagent	g	g/L	Concentration (mM)
Core salts (mMP-AU)	Na_2SO_4	0.85	1.7	11.97
	NaCl	0.878	1.756	30.05
	Na_2HPO_4	0.3315	0.663	4.67
	NH_4Cl	0.75	1.5	28.04
	$NaH_2PO_4*H_2O$	1.29	2.58	18.67
	KCl	1.154	2.308	30.96
	Creatinine	0.44	0.88	7.78
	$NH_4SO_4*7H_2O$	0.014	0.028	0.114
	Potassium citrate monohydrated	0.513	1.026	3.16
Amino Acids	L-cysteine	0.25	0.5	4.17
	L-valine	0.25	0.5	4.27
	L-tryptophan	0.25	0.5	2.45
	L-threonine	0.75	1.5	12.59
Carbon Sources	n-acetyl-glucosamine	2.765	5.53	25
	D-glucose	0.1	0.2	1.11
	Lactic acid	150 µL	300 µL	4.02
	Pyruvic acid	8.92 µL	17.84 µL	0.253
	Acetic acid	8.92 µL	17.84 µL	0.312
Others	$FeSO_4$ (1mM)	500 µL	1 mL	0.001
	Ascorbic acid	0.05	0.1	0.568
	$NaHCO_3$	0.084	0.168	2

633

634 While media is hot, add 500 µL of Tween-80.
635 Add 1.05g of MOPS
636 Add 7.5 g of Urea
637
638
639
640
641 Add:

a) 0.5 mL of solution of trace elements 1 (Acid)
b) 0.5 mL of solution of trace elements 2 (Basic)
c) 5 mL of vitamin mix 1
d) 5 mL of vitamin mix 2
e) 2 mL of solution of $MgSO_4$ 108g/L (0.216 g).
f) 1 mL solution of $NH_4C_2O_4$ 13.5 g/L (0.0135 g).
g) 100 µL of solution of **$MnSO_4$** 0.8 g/L.
h) 5 mL of amino acids 100X solution.
i) 2500 µL of hemin solution 0.5 g/L (0.00125 g).

Check pH (it should be ~ pH= 6, adapt it to pH=6 with HCl)

j) 10 mL of uric acid solution 12.5 g/L (0.125 g).

Add 0.25 g of L-serine

Check pH (it should be ~ pH= 6, adapt it to pH=6 with HCl)

Complete volume to 500 mL with H_2O. Filter using 0.2 µm filter. pH should stay stable.

SOLUTIONS

The following solutions must be prepared and sterilized before using them.

a) Trace elements (Acid). (Filter using a 0.2 µm filter. Protect from light, 4°C or RT)

Reagent	mg	concentration
$CuCl_2 * 2H_2O$	1.70	0.1 mM
H_3BO_3	6.18	1 mM
$CoCl_2*6H_2O$	11.89	0.5 mM
$NiCl_2*6H_2O$	2.38	0.1 mM
$ZnCl_2$	6.82	0.5 mM
$FeCl_2*4H_2O$	149.11	7.5 mM

Dissolve in 100 mL of water and add 8.3 µL of HCl 37%.

b) Trace elements (Basic) (Filter using a 0.2 µm filter. 4°C or RT)

Reagent	mg	concentration
Na_2SeO_3	1.73	0.1 mM
$Na_2WO_4*2H_2O$	3.3	0.1 mM
$Na_2MoO_4*2H_2O$	2.42	0.1 mM

Dissolve in 100 mL of NaOH 10 mM

c) Vitamins 1 (Filter using a 0.2 µm filter. Protect from light, 4°C)

Reagent	mg
Biotin	2
Nicotinamide	20
Thiamine	20
Riboflavin	10

Dissolve in 100 mL of solution $CaCl_2$ (15.25 g/L)

d) Vitamins 2 (Filter using a 0.2 µm filter. Protect from light, 4°C)

Reagent	mg
p-aminobenzoic acid	10
Pantothenic acid	10
pyridoxamine	50
Cyanocobalamin	10
Folic acid	10

Dissolve in 100 mL of solution $CaCl_2$ (15.25 g/L)

e) $MgSO_4$ solution (Autoclave or filter using a 0.2 µm filter. RT or 4°C. Indef. Storage)

$MgSO_4 \cdot 7H_2O$ 5.4 g in 50 mL of H_2O

f) $NH_4C_2O_4$ solution (Autoclave or filter. Indef. Storage)

$NH_4C_2O_4 \cdot H_2O$ 1.35 g in 100 mL of H_2O

g) MnSO4 solution (Indef. Storage)

$MnSO_4$ 0.08 g in 100 mL of H_2O.

h) Amino acids 100X solution (100 mL)

Add 40 mL of H_2O and incorporate the water-soluble amino acids one by one. Dissolve individually the other amino acids in ~ 5mL of HCl 1M and add to the bottle. Complete volume to 100 mL with H_2O. To ensure solubility, keep solution at pH ~1.

Amino acid	Concentration mM	mg	Solubility
Arginine	0.13	226.40	H_2O
Histidine	0.86	884.40	H_2O
Isoleucine	0.075	98.30	**HCl**
Alanine	0.55	489.90	H_2O
Asparagine	0.094	124.20	H_2O
Aspartate	0.63	838.53	H_2O
Cystine	0.35	841.05	**HCl**
Glutamate	1.46	2148.00	H_2O
Glutamine*	0.53	774.50	H_2O
Glycine	4.27	3205.40	H_2O
Leucine	0.113	148.20	**HCl**
Lysine	0.27	394.70	**HCl**
Methionine	0.046	68.63	**HCl**
Phenylalanine	0.09	148.67	**HCl**
Proline	0.26	299.30	H_2O
Serine	0.28	294.20	H_2O

*Lifespan of Glutamine may be reduced over time, preparation of frozen aliquots of this amino acids 100X solution may be optimal**.**

i) Hemin solution (Autoclave, protect from light, 4°C).

Add 50 mg of Hemin in 1 mL of NaOH 1N, complete volume to 100 mL with H_2O.

j) Uric acid solution (Autoclave, 4 days at 4°C)

0.625 g in 5 mL NaOH 10 M, dissolve, and complete to 50 mL with H_2O.

710 **Supplementary Table 2: List of reagents utilized in this work.**
711

Correlative	Reagents	Code	Company
1	Acetic acid	A6283-1L	Sigma-Aldrich
2	Alanine	11442468	Thermo scientific Chemicals
3	Arginine	A5006-100G	Sigma-Aldrich
4	Asparagine	A0884-25G	Sigma-Aldrich
5	Aspartate	A9256-100G	Sigma-Aldrich
6	Biotin	B4501-100MG	Sigma-Aldrich
7	$CaCl_2$	C1016-100G	Sigma-Aldrich
8	Citric acid	C0759-100G	Sigma-Aldrich
9	$CoCl_2*6H_2O$	202185-25G	Sigma-Aldrich
10	Creatinine	228940500	Thermo scientific Chemicals
11	$CuCl_2 * 2H_2O$	10588502	Thermo scientific Chemicals
12	Cyanocobalamin	1.2459201	Sigma-Aldrich
13	Cystine	11414814	Thermo scientific Chemicals
14	$FeCl_2*4H_2O$	10348150	Thermo scientific Chemicals
15	Folic acid	F7876-10G	Sigma-Aldrich
16	Glutamate	10502751	Thermo scientific Chemicals
17	Glutamine	11482277	Thermo scientific Chemicals
18	Glycine	1.00590.1000	MERK
19	H_3BO_3	B0394-100G	Sigma-Aldrich
20	Hemin	10499291	Thermo scientific Chemicals
21	HEPES 1M	H3375-25G	Sigma-Aldrich
22	Histidine	H8000-5G	Sigma-Aldrich
23	Isoleucine	I2752-1G	Sigma-Aldrich
24	KCl	1.04936.1000	MERK
25	L-cysteine	A10435.18	Thermo scientific Chemicals
26	L-serine	S4500-1G	Sigma-Aldrich
27	L-threonine	1.0841101	Sigma-Aldrich
28	Lactic acid	10665302	Thermo scientific Chemicals
29	Leucine	L8000-25G	Sigma-Aldrich
30	Lysine	L5501-10MG	Sigma-Aldrich
31	Methionine	M9625-25G	Sigma-Aldrich
32	$MgSO_4*7H_2O$	447155000	Thermo scientific Chemicals
33	$MnSO_4*H_2O$	11478237	Thermo scientific Chemicals
34	n-acetyl-glucosamine	11438770	Thermo scientific Chemicals

35	Na_2HPO_4	1.06586.0500	Sigma-Aldrich
36	$Na_2MoO_4*2H_2O$	10164830	Thermo scientific Chemicals
37	Na_2SO_4	1.28211.1001	Sigma-Aldrich
38	$Na_2WO_4*2H_2O$	10117423	Thermo scientific Chemicals
39	NaCl	207790010	ACROS
40	NaH_2PO_4	1.06349.1000	Sigma-Aldrich
41	NaOH	S5881-1K	Sigma-Aldrich
42	Na_2SeO3	214485-5G	Sigma-Aldrich
43	$NH_4C_2O_4$	10054013	Thermo scientific Chemicals
44	NH_4Cl	A4514-500G	Sigma-Aldrich
45	$NH_4SO_4*7H_2O$	A5132-1K	Sigma-Aldrich
46	$NiCl_2*6H_2O$	654507-5G	Sigma-Aldrich
47	Nicotinamide	10447710	Thermo scientific Chemicals
48	p-aminobenzoic acid	A9878-5G	Sigma-Aldrich
49	Pantothenic acid	21210-5G-F	Sigma-Aldrich
50	Phenylalanine	11468840	Thermo scientific Chemicals
51	Proline	11439512	Thermo scientific Chemicals
52	pyridoxamine	10785052	Thermo scientific Chemicals
53	Pyruvic acid	10562721	Thermo scientific Chemicals
54	Riboflavin	10593871	Thermo scientific Chemicals
55	Serine	11489113	Thermo scientific Chemicals
56	Thiamine	10477540	Thermo scientific Chemicals
57	Tween80	P4780-500ML	Sigma-Aldrich
58	Urea	U5378-1K	Sigma-Aldrich
59	Uric acid	U2625-100G	Sigma-Aldrich
60	$ZnCl_2$	208086-5G	Sigma-Aldrich

712

713 **Supplementary Protocols 2**
714 **SimUrine.v1**
715
716 **Protocol for 500 ml of media 1X.**
717
718 Add 200 ml of H_2O to a bottle and add the following reagents one by one.
719

Reagent	g	g/L
Na_2SO_4	0.85	1.7
NaCl	0.878	1.756
Na_2HPO_4	0.3315	0.663
NH_4Cl	0.633	1.266
NaH_2PO_4	1.12	2.24
KCl	1.154	2.308
n-acetyl-glucosamine	2.765	5.53
L-threonine	0.75	1.5
$CaCl_2$•H2O	0.122	0.244

720
721 Dissolve individually in 5 ml of HCl 1M and add to the media.
722
723 0.25 g L-cysteine
724 0.25 g L-serine
725
726 Add: Lactic acid 75 µL, Pyruvic acid 4.46 µL, Acetic acid 4.46 µL
727
728 Dissolve individually in 20 ml of H_2O and add to the media:
729
730 Creatinine 0.44 g, $MgSO_4$*7H2O 0.54 g, NH_4SO_4*7H2O 0.014 g,
731 Potassium citrate 0.484 g
732
733 Add 60 ml of H_2O to complete ~350 ml.
734
735 Autoclave (adjust pH 6.5)
736
737 Add 10 ml of uric acid solution and 1 ml solution of $NH_4C_2O_4$ (See Supplementary protocol 1).
738
739 Add 7.5 g of urea, adjust pH to 6.5, complete with sterile water, and filter.

740 **SimUrine.v2**
741
742 **Protocol for 500 ml of media 1X.**
743
744 Add 200 ml of H_2O to a bottle and add the following reagents one by one.
745

Reagent	g	g/L
Na_2SO_4	0.85	1.7
NaCl	0.878	1.756
Na_2HPO_4	0.3315	0.663
NH_4Cl	0.633	1.266
NaH_2PO_4	1.12	2.24
KCl	1.154	2.308
n-acetyl-glucosamine	2.765	5.53
L-threonine	0.75	1.5

746
747 Dissolve individually in 5 ml of HCl 1M and add to the media.
748
749 0.25 g L-cysteine
750 0.25 g L-serine
751
752 Add: Lactic acid 75 µL, Pyruvic acid 4.46 µL, Acetic acid 4.46 µL
753
754 Dissolve individually in 20 ml of H_2O and add to the media:
755
756 Creatinine 0.44 g, $MgSO_4$*7H2O 0.54 g, NH_4SO_4*7H2O 0.014 g,
757 Potassium citrate 0.484 g
758
759 Add 60 ml of H_2O to complete ~350 ml.
760
761 Autoclave (adjust pH 6.5)
762
763 Add 5 ml of vitamin mix 1, 5 ml of vitamin mix 2*, 1 ml solution of $NH_4C_2O_4$, 0.5 ml of
764 solution of trace elements 1 (Acid), 0.5 ml of solution of trace elements 2 (Basic). (See
765 **Supplementary Protocol 1**).
766
767 Add 10 ml of uric acid solution (12.5 g/L).
768
769 Add 7.5 g of urea, adjust pH to 6.5, complete with sterile water, and filter.

770 **SimUrine.v3**
771
772 **Protocol for 500 ml of media 1X.**
773
774 Add 200 ml of H_2O to a bottle and add the following reagents one by one.
775

Reagent	g	g/L
Na_2SO_4	0.85	1.7
NaCl	0.878	1.756
Na_2HPO_4	0.3315	0.663
NH_4Cl	0.633	1.266
NaH_2PO_4	1.12	2.24
KCl	1.154	2.308
n-acetyl-glucosamine	2.765	5.53
L-threonine	0.75	1.5

776
777 Dissolve individually in 5 ml of HCl 1M and add to the media.
778
779 0.25 g L-cysteine
780 0.25 g L-serine
781
782 Add: Lactic acid 75 µL, Pyruvic acid 4.46 µL, Acetic acid 4.46 µL
783
784 Dissolve individually in 20 ml of H_2O and add to the media:
785
786 Creatinine 0.44 g, $MgSO_4$*7H2O 0.54 g, NH_4SO_4*7H2O 0.014 g
787 Potassium citrate 0.484 g
788
789 Add 60 ml of H_2O to complete ~350 ml.
790
791 Autoclave (adjust pH 6.5)
792
793 Add 5 ml of vitamin mix 1, 5 ml of vitamin mix 2*, 1 ml solution of $NH_4C_2O_4$, 0.5 ml of
794 solution of trace elements 1 (Acid), 0.5 ml of solution of trace elements 2 (Basic), 2500 µL of
795 hemin solution (See Supplementary protocol 1).
796
797 Add 10 ml of uric acid solution (12.5 g/L).
798
799 Add 7.5 g of urea, adjust pH to 6.5, complete with sterile water, and filter.
800
801
802

803 SimUrine.v4
804
805 **Protocol for 500 ml of media 1X.**
806
807 Add 200 ml of H_2O to a bottle and add the following reagents one by one.
808

Reagent	g	g/L
Na_2SO_4	0.85	1.7
NaCl	0.878	1.756
Na_2HPO_4	0.3315	0.663
NH_4Cl	0.633	1.266
NaH_2PO_4	1.12	2.24
KCl	1.154	2.308
n-acetyl-glucosamine	2.765	5.53
L-threonine	0.75	1.5
L-cysteine	0.25	0.5

809
810 Dissolve individually in 5 ml of HCl 1M and add to the media.
811
812 0.25 g L-cysteine
813 0.25 g L-serine
814
815 Add: Lactic acid 75 µL, Pyruvic acid 4.46 µL, Acetic acid 4.46 µL
816
817 Dissolve individually in 20 ml of H_2O and add to the media:
818
819 Creatinine 0.44 g, NH_4SO_4*7H2O 0.014 g
820 Potassium citrate 0.484 g
821
822 Add 60 ml of H_2O to complete ~350 ml.
823
824 Autoclave (adjust pH 6.5)
825
826 **After autoclaving, add:**
827
828 (Here and while media is hot, add Tween-80 1 ml per liter for *Lactobacillus* growth.)
829
830 a) 0.5 ml of solution of trace elements 1 (Acid)
831 b) 0.5 ml of solution of trace elements 2 (Basic)
832 c) 5 ml of vitamin mix 1
833 d) 5 ml of vitamin mix 2
834 e) 1 ml of solution of $MgSO_4$
835 f) 1 ml solution of $NH_4C_2O_4$.
836 g) 100 µL of solution of $MnSO_4$.

Adjust pH to 6.5 (~100µL of NaOH 10M)
(Check/adjust pH (6.5) and add 5 ml of HEPES 1M)

Add 7.5 g of **urea** and filter.

After filtering, add:

h) 5 ml of amino acids 10X solution.

i) 2500 µL of hemin solution. (check pH, add ~500 µL HCl 1M. Final pH 6.5)

j) 10 ml of uric acid solution.

Complete volume to 500 ml with autoclaved H_2O. pH should stay stable.

SimUrine.v5.

Protocol for 500 ml of media 1X.

Add 200 ml of H_2O to a bottle and add the following reagents one by one.

Reagent	g	g/L
Na_2SO_4	0.85	1.7
NaCl	0.878	1.756
Na_2HPO_4	0.3315	0.663
NH_4Cl	0.633	1.266
NaH_2PO_4	1.12	2.24
KCl	1.154	2.308
n-acetyl-glucosamine	2.765	5.53
L-threonine	0.75	1.5
L-cysteine	0.25	0.5

Dissolve individually in 5 ml of HCl 1M and add to the media.

0.25 g L-cysteine
0.25 g L-serine

Add: Lactic acid 75 µL, Pyruvic acid 4.46 µL, Acetic acid 4.46 µL

Dissolve individually in 20 ml of H_2O and add to the media:

Creatinine 0.44 g, NH_4SO_4*7H2O 0.014 g
Potassium citrate 0.484 g

Add 60 ml of H_2O to complete ~350 ml.

Autoclave (adjust pH 6.5)

After autoclaving, add:

(Here and while media is hot, add Tween-80 1 ml per liter for *Lactobacillus* growth.)

a) 0.5 ml of solution of trace elements 1 (Acid)
b) 0.5 ml of solution of trace elements 2 (Basic)
c) 5 ml of vitamin mix 1
d) 5 ml of vitamin mix 2
e) 1 ml of solution of $MgSO_4$

885 f) 1 ml solution of $NH_4C_2O_4$.
886 g) 100 µL of solution of $MnSO_4$.
887
888
889 Adjust pH to 6 (~100µL of NaOH 10M)
890 **(Check/adjust pH (6) and add MOPS 2.1 g/L)**
891
892 Add **2.5 g** of **urea** and filter.
893
894 **After filtering**, add:
895
896 k) 5 ml of amino acids 100X solution.
897
898 l) 2500 µL of hemin solution. (check pH, add ~500 µL HCl 1M. Final pH 6.5)
899
900 m) 10 ml of uric acid solution.
901
902 **Complete volume to 500 ml with autoclaved H_2O. pH should stay stable.**

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