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Short communication

An *in vitro* model of catheter-associated urinary tract infections to investigate the role of uncommon bacteria on the *Escherichia coli* microbial consortium

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ABSTRACT

Uncommon bacteria, such as *Delftia tusurhatensis* have been isolated from CAUTIs in combination with well-established pathogenic bacteria such as *Escherichia coli*. Nonetheless, the reason why *E. coli* coexists with other bacteria instead of outcompeting and completely eliminating them is unknown. As such, a flow cell reactor simulating the hydrodynamic conditions found in CAUTIs (shear rate of 15 s^{-1}) was used to characterize the microbial physiology of *E. coli* and *D. tsuruhatensis* individually and in consortium, in terms of the growth kinetics and substrate uptake. Single-species biofilms showed that up to 48 h the cultivable cell counts significantly increased for both species ($p < 0.05$). When in dual-species biofilm, *E. coli* outnumbered *D. tsuruhatensis* up to 16 h and then *D. tsuruhatensis* gained a fitness advantage. However, the assessment of the spatial distribution of the dual-species biofilm by LNA/2′OMe-FISH revealed that *E. coli* and *D. tsuruhatensis* coexist and tend to co-aggregate over time, which suggests that both bacteria are able to cooperate synergistically. Substrate uptake measurements revealed that *D. tsuruhatensis* metabolized citric acid more rapidly, presumably leaving more uric acid available in the medium to be used by *E. coli*. In conclusion, *E. coli* and uncommon bacteria seem to cooperate, when sharing the same environment under dynamic conditions, leading to the persistence of both bacteria in a stable microbial community.

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1. Introduction

In hospitals and nursing homes, there is a regular occurrence of infections, of which about 9% are attributed to catheter-associated urinary tract infections (CAUTIs) [1]. Urinary catheters are medical devices used in patients to control the urine drain due to incontinence problems or post-operative urine retention [2]. Unfortunately, most patients experience long-term catheterization, which is frequently associated with polymicrobial infections [3–6]. In fact, with time, microorganisms end up forming polymicrobial biofilms on the surface of urinary catheters [7–9].

A biofilm is a community of microorganisms adhered to a biotic or abiotic surface, which is enclosed in an extracellular polymeric substances (EPSs) matrix. Compared to planktonic microorganisms, they have an altered phenotype associated with a reduced growth rate, a high tolerance to antimicrobial agents and to the host immune system, and an altered expression of specific genes [10–13]. Typically, microbial biofilms display a coordinated and cooperative behavior [14], where the concentration of individual populations is adjusted according to the conditions found in the environment [15].

Studies that combine *E. coli* with other pathogenic bacteria, have already been addressed in context of CAUTIs (e.g. [25]). Recently, uncommon bacteria such as *Delftia tusurhatensis* have been isolated and identified in nosocomial infections involving polymicrobial

Abbreviations: EPS, extracellular polymeric substance; FISH, fluorescence *in situ* hybridization; CLSM, confocal laser scanning microscopy; LNA, locked nucleic acid; 2′OMe, 2′-O-Methyl-RNA; CAUTIs, catheter-associated urinary tract infections; TSA, tryptic soy agar; CFUs, colony forming units; O.D., optical density; AUM, artificial urine medium; Υ, shear strain rate; τ_w, shear stress; μ, fluid viscosity; Q, flow rate; Re, Reynolds number; ρ, fluid density; W, fitness value; m, Malthusian parameter.

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biofilms [7,16], including CAUTIs [7]. The pathogenic potential of these uncommon bacteria is undefined. Nonetheless, this type of bacteria appears in catheter-associated biofilms in combination with well-established pathogenic bacteria (e.g. *Escherichia. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) [7]. The role of uncommon bacteria is underestimated probably due to the absence of commercial media and kits to detect these bacteria in hospitals. But, as reported in previous studies [15,17], *E. coli* and uncommon bacteria are good biofilm producers on abiotic surfaces (e.g. silicone, polystyrene), and when in co-culture they are able to form a stable microbial consortia, where both bacteria coexist, even when inoculated at different proportions. Also, the analysis of ecological interactions between *E. coli* and uncommon bacteria has revealed that these bacteria tend to interact either synergistically or, at least, display a neutralism behavior. These previous studies have only described the possible synergistic interactions assessing bacterial adhesion, biofilm formation and overall antibiotic resistance of mixed-species consortia in static conditions. Nonetheless, knowledge about the microbial physiology of *E. coli* and *D. tsuruhatensis* under dynamic conditions, especially when concerning nutritional requirements, remains unknown. Hence, a pressing need exists for research directed toward understanding microbial interactions that drive CAUTIs biofilm communities involving uncommon and pathogenic bacteria under dynamic conditions.

A flow cell system simulating the shear strain rate found in urinary catheters (15 s^{-1}) [18–20] was used and species behavior was then evaluated individually and in consortium, when exposed to artificial urine medium (AUM) flow and to the silicone material. The physiology of each bacterium was characterized in terms of the growth kinetics and the substrate's uptake (lactic acid, urea, citric acid, creatinine and uric acid) under dynamic conditions.

2. Material and methods

2.1. Strains and culture media

E. coli CECT 434 and *D. tsuruhatensis* BM90 were recovered from a frozen stock (−80 °C), streaked on Tryptic Soy Agar (TSA) (Merk, Germany) and grown overnight at 37 °C. For the inocula preparation, each bacterium was inoculated in 250 ml of AUM and cultures were placed in an incubator (AGITORB 200, Aralab, Portugal) during 16–18 h at 37 °C and 150 rpm. AUM was prepared as previously described [21], but as yeast extract and peptone are a mixture of polypeptides and amino acids, they were not added to the medium to enable the measurement of substrate's uptake. Then, cell concentration was assessed by optical density (O.D.) at 620 nm and the inocula were diluted in AUM in order to obtain a final concentration of 10^8 CFU.ml^{-1}. Each diluted inoculum was used to inoculate the reactor system during 1 h at a flow rate (Q) of 0.5 mlmin^{-1}.

2.2. Determination of bacterial growth rates

The growth rate for each bacterium was determined in AUM (without yeast extract and peptone) in a batch culture as described in a study performed by Azevedo et al. [17].

2.3. Flow cell reactor setup

The reactor system used in this study (Fig. 1) consists of a vertical flow cell, water bath at 37 °C, peristaltic (B1) and centrifugal pumps (B2), vessel containing the nutrients, recirculating silicone tubes and a waste vessel. The flow cell used is a rectangular Perspex column with 10 apertures in removable rectangular pieces of Perspex where silicone coupons were placed. All specifications of the flow cell reactor and on the assembling of the system are provided in Supplementary material (Supplementary Method 1).

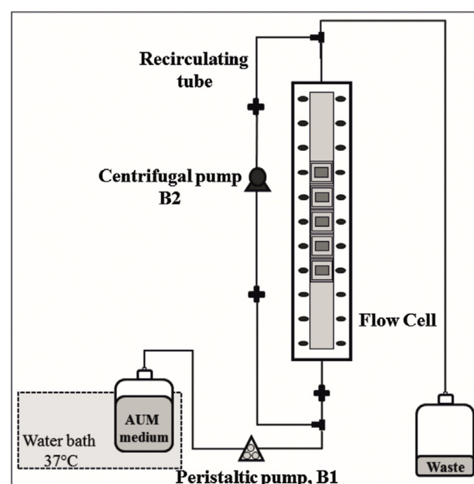

Fig. 1. Schematic representation of the flow cell reactor. Pumps B1 and B2 controlled the flow rate of AUM (0.5 ml min^{-1}) and the velocity of recirculating fluid (300 ml min^{-1}), respectively.

While the dynamic system used here has geometric features distinct from urinary catheters, important aspects involved in CAUTIs were simulated such as the composition of the AUM, which was reported as suitable to mimic the urine in a wide range of microbial studies [21–25]. The temperature of AUM was kept at 37 °C, corresponding to the human body temperature. In addition, the flow rate was adjusted to mimic a natural urine flow (see calculations in Supplementary Method 1); it should be noticed that within the human body the catheter will be subjected to an intermittent flow, with periods from absent to high flow rates. As previous experiments were performed under static conditions [15,17], the dynamic system used in present study allowed to simulate the periods of high urine flow rate. Also, this system only allows a correct simulation of an intraluminal colonization, as flow is restricted to the catheter lumen.

After defining the system settings, biofilms were formed and analyzed as described in Supplementary Method 1. The fitness values (W) and Malthusian parameters (m) were determined as previously described [15,17].

2.4. Statistical analysis

For each parameter, the average and standard deviation were calculated. Results were compared using ANOVA by applying Levene's test of homogeneity of variance and the Tukey multiple-comparisons test, using the SPSS software. Statistical tests were carried out at a significance level of 0.05.

3. Results and discussion

Microbial infections in catheterized patients are usually composed by a dominant pathogenic bacterium (e.g. *E. coli*) which might interact and coexist with other pathogens (e.g. *P. aeruginosa*, *E. faecalis* and *P. mirabilis*) [9,26,27], or even, with uncommon bacteria with a poorly understood role such as *D. tsuruhatensis* [7]. Hence, a polymicrobial community is established on the surface of an urinary catheter, in particular when the urinary catheter remains in the patient for several weeks or months [3–6]. Recently, some studies have studied the role of some uncommon bacteria in clinical infections and have highlighted their role in shaping the overall behavior of the microbial biofilm [15,17,28,29].

In present work, the physiology of *E. coli* and the uncommon bacteria *D. tsuruhatensis* under dynamic conditions similar to those found in catheterized urinary tract was evaluated, both in terms of

the microbial growth and the nutritional requirements, in order to better understand how these bacteria might persist in a microbial community and start disclosing the potential role of uncommon bacteria on *E. coli* behavior. In fact, despite uncommon bacteria appear at lesser extent in CAUTIs, the microbial interactions between uncommon bacteria and pathogenic bacteria are likely to occur.

3.1. Characterization of E. coli and D. tsuruhatensis single-species biofilm growth under dynamic conditions

First, the development of *E. coli* and *D. tsuruhatensis* single-species biofilm was assessed. As *E. coli* is one of the most frequently detected microorganisms in CAUTIs [30,31], it would be expectable that *E. coli* presents a higher ability to grow under dynamic conditions in AUM. However, this was not observed in terms of cultivable cell counts (Fig. 2a). Single-species biofilms showed that up to 48 h the cultivable cell counts significantly increased for both species ($p < 0.05$) with a notorious higher growth for *D. tsuruhatensis* biofilms. In fact, the assessment of the bacterial growth rate in AUM (without yeast extract and peptone) showed that *D. tsuruhatensis* grew faster (0.4879 h^{-1}) when compared to *E. coli* (0.2831 h^{-1}). After 48 h, both species stabilized with similar cultivable cell values (Log 6.24 $CFU.cm^{-2}$ for *E. coli* and Log 6.31 $CFU.cm^{-2}$ for *D. tsuruhatensis*, $p > 0.05$). These growth profiles were similar to those obtained in results presented in a previous study where biofilms of both bacteria were formed in silicone coupons in AUM under static conditions [15]. Nonetheless, the higher m values for single-species biofilms grown under static conditions indicated that, in these conditions, bacteria form more biofilm than under dynamic conditions (Fig. 2b).

Indeed, the assessment of the spatial distribution of biofilm populations by locked nucleic acid/2′-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2′OMe-FISH) in combination with Confocal laser scanning microscopy (CLSM) revealed that single-species biofilms in static conditions presented higher thickness values (21 μm for *E. coli* and 17 μm for *D. tsuruhatensis*) [15] compared to single-species biofilms formed under dynamic conditions (6 μm for both bacteria; Supplementary material – Fig. S1). It would be expectable that the removal of a substantial concentration of a carbon source (yeast extract) reduces the growth of a heterotroph. In fact, the data suggested that the absence of yeast extract and peptone and the hydrodynamic conditions that biofilms are subjected to when cultured in dynamic conditions, negatively affect the cell concentration. It is well established that the hydrodynamic conditions and the nutrients availability have a crucial impact in the adhesion process of the microorganisms, structure and behavior of microbial biofilms [32–37].

3.2. How uncommon bacterium might impact on E. coli population under dynamic conditions

To understand the potential role of *D. tsuruhatensis* on *E. coli* physiology, the fitness of *E. coli* and *D. tsuruhatensis* was determined and the spatial localization of the biofilm populations in *E. coli*/*D. tsuruhatensis* dual-species biofilm was analyzed (Fig. 3).

It was speculated that *D. tsuruhatensis* would dominate the microbial consortium since it grows faster compared with *E. coli*. However, the data only showed this behavior at 72 h with cultivable cell values reaching Log 5.27 $CFU.cm^{-2}$ for *E. coli* and Log 6.27 $CFU.cm^{-2}$ for *D. tsuruhatensis* ($p < 0.05$) (Fig. 3a). On the other hand, $W_{E.coli}$ results showed that *E. coli* presented a higher advantage up to 16 h ($W_{E.coli} > 1$ and $W_{D.tsuruhatensis} < 1$, with a $p < 0.05$). Then, the $W_{E.coli}$ decreased ($W_{E.coli} < 1$) and *D. tsuruhatensis* gained fitness advantages ($W_{D.tsuruhatensis} > 1$). However, for 48 h there are no statistically significant differences between $W_{E.coli}$ and $W_{D.tsuruhatensis}$ ($p > 0.05$). Part of these data might be corroborated with the previous study performed in silicone coupons under static conditions [15], where an advantage for *E. coli* when co-cultured with uncommon species was reported, especially for the early stages of biofilm development.

The assessment of the spatial distribution of biofilm populations by LNA/2′OMe-FISH in combination with CLSM revealed that *E. coli* and *D. tsuruhatensis* coexist and tend to co-aggregate over time (Fig. 3c). This spatial organization is also similar to a study performed in static conditions [15]. This suggests that the presence of flow and absence of yeast extract and peptone did not influence the spatial distribution of biofilm populations. This only affected the thickness of biofilms which was lower under dynamic conditions (6.38 μm at 72 h under dynamic conditions [Fig. 3cIII] and 14 μm at 48 h and 192 h under static conditions [15]).

Despite a slight decrease of $W_{E.coli}$ values for the last time points, this spatial organization typically means that both species are able to cooperate or interact synergistically [38], as previously reported [15]. In fact, as *E. coli* and *D. tsuruhatensis* might co-inhabit the same urinary catheter [7], it would be expectable that both species might cooperate and benefit from the presence of each other.

Two of the previous studies have suggested that the uncommon bacteria have a positive impact on *E. coli* fitness and under adverse conditions these bacteria seem to offer a protective effect over *E. coli*, increasing the tolerance of the overall microbial consortia to antibiotic agents [15,17]. Nonetheless, there are no studies reporting how substrate uptake is affected when both bacteria are present simultaneously. Thus, analysis of nutritional requirements of *E. coli* and *D. tsuruhatensis* when growing under dynamic conditions might explain why *E. coli* coexists with uncommon bacteria rather than alone.

3.3. Substrate uptake by E. coli and D. tsuruhatensis in single- and dual-species biofilms

The concentration profiles of lactic acid, uric acid, citric acid, urea and creatinine concentrations *E. coli*, *D. tsuruhatensis* and *E. coli*/*D. tsuruhatensis* are presented in Fig. 4. Both bacteria had preference for organic acids like lactic acid, uric acid (*E. coli* and *D. tsuruhatensis*) and citric acid (*D. tsuruhatensis*).

Concerning *E. coli* behavior, the results corroborated the observations of Lenski's long-term evolution experiment where *E. coli* was not able to use citric acid under aerobic conditions [39]. Instead, a higher uptake of lactic acid was observed up to 16 h by *E. coli* and *D. tsuruhatensis* which means that this substrate was clearly the primary carbon source for both bacteria when grown individually (Fig. 4a). Then, as the amount of lactic acid decreased substantially, *E. coli* started to uptake uric acid gradually until 72 h. On the other hand, *D. tsuruhatensis* consumed a higher amount of uric acid up to 48 h (Fig. 4b). At this point, *E. coli* was not able to uptake another substrate; but, *D. tsuruhatensis* was able to uptake the citric acid (Fig. 4c). When in consortium, *E. coli* and *D. tsuruhatensis* consumed lactic and citric acid more rapidly, whereas uric acid consumption was slower. Being *D. tsuruhatensis* the only species able to use citric acid, the increase on its consumption even when uric acid was still available, shows that *D. tsuruhatensis* changed its substrate preference due to the presence of *E. coli.*

4. Conclusions

The flow cell system allowed studying how *D. tsuruhatensis* influences the physiology of *E. coli* in conditions similar to those found in CAUTIs, providing data about the growth kinetics and the carbon sources that are preferentially consumed by each bacterium individually and in consortium. However, caution should be taken

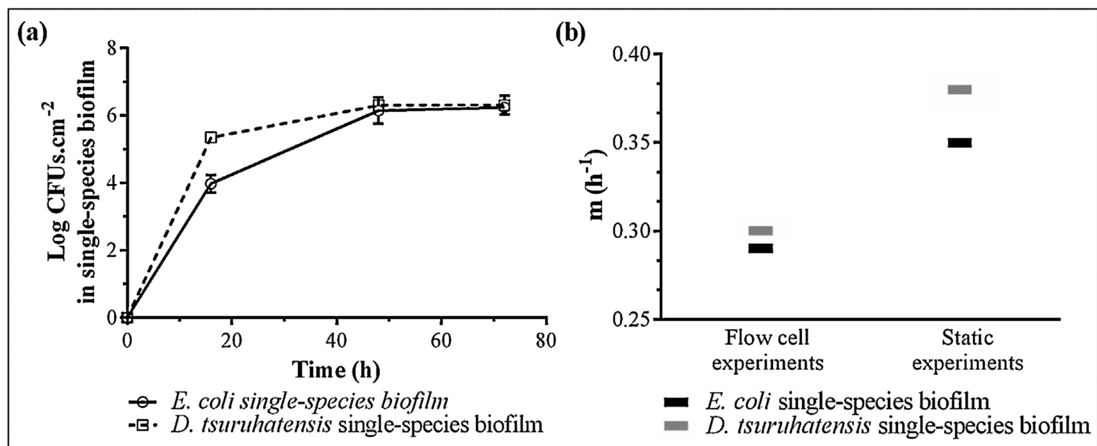

Fig. 2. Formation of *E. coli* and *D. tsuruhatensis* single-species biofilms. (a) Cultivable cell values for biofilms in silicone coupons under dynamic conditions; (b) Comparison of Malthusian parameter values for *E. coli* and *D. tsuruhatensis* single-species biofilms grown in silicone coupons under dynamic and under static conditions (results of static conditions were from [17]). The Malthusian parameters were determined between time 0 and 48 h, before the biofilm stabilization. Error bars represent standard deviations.

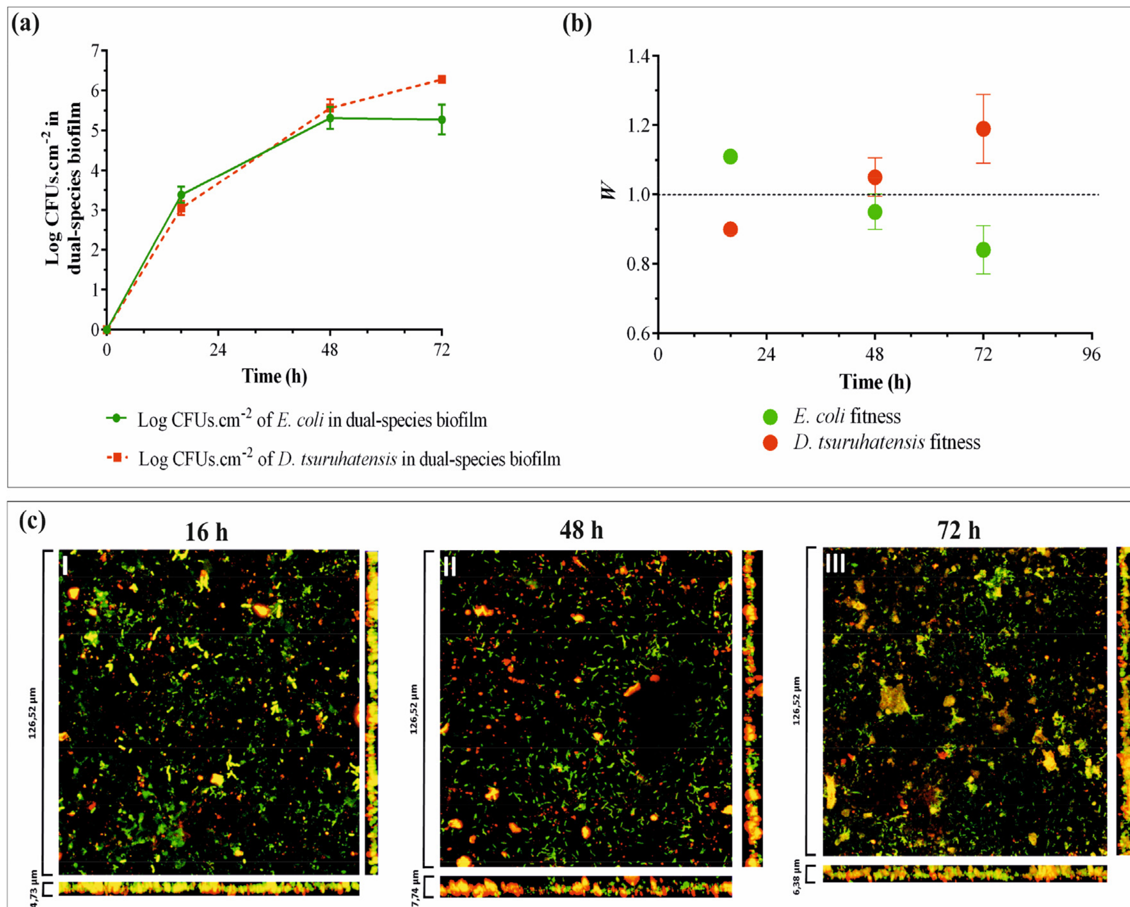

Fig. 3. Single- and dual-species biofilm growth in silicone material under dynamic conditions. (a) Cultivable cell values of each bacterium overtime; (b) Representation of the relative fitness of *E. coli* and *D. tsuruhatensis*. The dashed line represents a relative fitness of 1, which means that the species are equally fit within biofilms. Error bars represent standard deviations; (c) Images of LNA/2′OMe-FISH in combination with CLSM, showing the spatial organization of the *E. coli*/*D. tsuruhatensis* dual-species biofilm at (I) 16 h, (II) 48 h and (III) 72 h. The bottom and side images represent the transverse planes. Green cells correspond to *E. coli* and red cells correspond to *D. tsuruhatensis*.

when extrapolating these results to CAUTIs, since materials used in catheters production are very diverse and certainly have a great effect on bacterial adhesion and behavior. This system has been applied to non-coated silicon surfaces, but further studies should expand these tests to a broad range of other materials employed in catheters.

Under flow conditions, *E. coli* and *D. tsuruhatensis* are able to persist and survive within the biofilm community. While the fitness of each bacterial population presents only slight changes over time, results on substrate uptake seem to indicate that *E. coli* and *D. tsuruhatensis* cooperate metabolically in order to obtain the maximum nutritional benefit. Indeed, *D. tsuruhatensis* co-cultured with *E. coli*

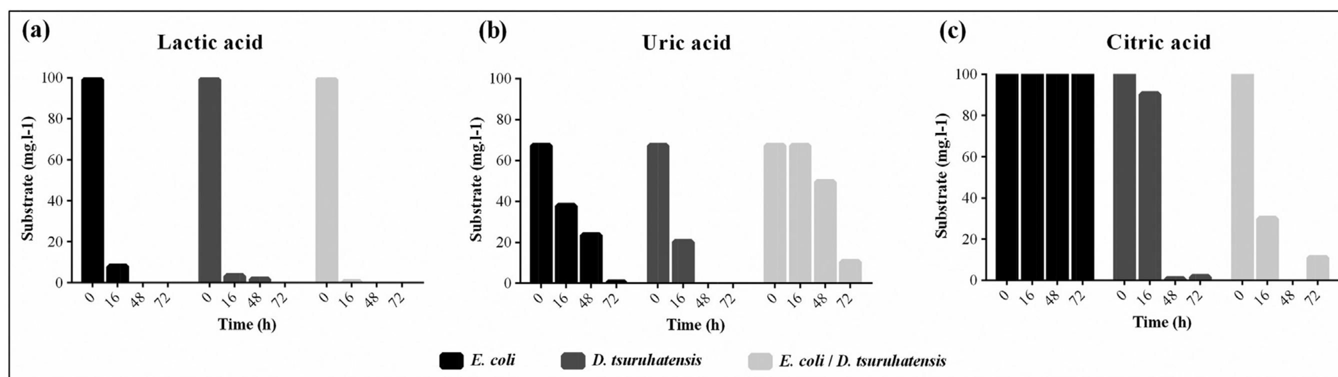

Fig. 4. The evolution of (a) lactic acid, (b) uric acid and (c) citric acid concentrations for *E. coli*, *D. tsuruhatensis* and *E. coli*/*D. tsuruhatensis*. Citric acid was not consumed by *E. coli*. Two other carbon sources present in artificial urine medium, creatinine and urea, were not consumed by *E. coli* and *D. tsuruhatensis*.

preferred the consumption of citric acid instead of uric acid, leaving more uric acid available to be used by *E. coli*. This hints at a cooperative interaction between *E. coli* and uncommon bacteria when these species share the same environment in order to guarantee their persistence and survival within microbial community.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.11.013.

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