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The *Enterococcus* secretome inhibits the growth of *Mycobacterium tuberculosis* complex mycobacteria

Wafaa Achache^{1,2}, Jean Louis Mege^{1,2}, Mustapha Fellag^{1,2} and Michel Drancourt^{1,2,*}

Abstract

Enterococcus mundtii, a commensal intestinal bacterium, was demonstrated to inhibit the growth of some *Mycobacterium tuberculosis* complex (MTC) species that cause tuberculosis in humans and mammals. To further explore this preliminary observation, we cross-investigated five *E. mundtii* strains and seven MTC strains representative of four MTC species using a standardized quantitative agar well diffusion assay. All five *E. mundtii* strains, calibrated at 10 MacFarland, inhibited the growth of all *M. tuberculosis* strains with various susceptibility profiles, but no inhibition was observed with lower inoculums. Further, eight *E. mundtii* freeze-dried cell-free culture supernatants (CFCS) inhibited the growth of *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium canettii*, the most susceptible MTC species (inhibition diameter 25±1 mm), proportionally to CFCS protein concentrations. The data reported here indicate that the *E. mundtii* secretome inhibited growth of all MTC species of medical interest, which broadens previously reported data. In the gut, the *E. mundtii* secretome may modulate the expression of tuberculosis, exhibiting an anti-tuberculosis effect, with some protective roles in human and animal health.

DATA SUMMARY

The authors confirm that all data and protocols are provided in this article. All strains investigated in this study were obtained from The Rickettsiae Unit Strain Collection (CSUR), under the international collection number: WDCM 875.

INTRODUCTION

Tuberculosis is a deadly infection in mammals and humans, transmissible by the transcutaneous, digestive and especially respiratory tract routes, caused by any of the 13 closely related species of mycobacteria forming the *Mycobacterium tuberculosis* complex (MTC) [1]. Among these species, *Mycobacterium tuberculosis*, *Mycobacterium bovis* and its derivative, *M. bovis* Bacillus bilié de Calmette-Guérin (BCG), are the most frequently encountered species in clinical microbiology laboratories worldwide [2], and *Mycobacterium africanum* and *Mycobacterium canettii* are mainly documented in patients exposed in the Horn of Africa [3, 4]. Of particular concern are cases of tuberculosis caused by MTC isolates resistant to first-line rifampicin-based anti-tuberculosis combinations, stimulating efforts to evaluate new drugs, as well as the reintroduction of old drugs, such as those used for decades as anti-leprosy drugs [5, 6].

Over the last decade, several lines of observation have indicated reciprocal interactions between the gut microbiota and MTC mycobacteria [7–9]. MTC mycobacteria can be found in intestinal tissues and faeces in the case of intestinal tuberculosis, and in the faeces in the case of pulmonary tuberculosis [10]. Accordingly, excreted faeces could be used as surrogate specimens on which to base the detection of *M. tuberculosis* by PCR and culture [11]. Experimental models incorporating either *M. canettii* or *M. tuberculosis* have confirmed lymphatic and pulmonary tuberculosis after an oral route of transmission of these MTC pathogens [12]. Finally, these experimental data agree with medical observations made during the so-called Lübeck disaster in Germany in 1929–1933, where 251 neonates were vaccinated by the oral route with a BCG vaccine contaminated with *M. tuberculosis*, 173 neonates developed tuberculosis and 72 died [13]. Increasingly, gut microbiota distortions are being implicated in the pathogenesis of several infectious and non-infectious diseases. Therefore, the role of gut microbiota in modulating the natural history of tuberculosis is being increasingly considered, yet is not entirely understood [14]. A few recent studies have disclosed

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Author affiliations: ¹Aix-Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France; ²IHU Méditerranée Infection, Marseille, France.

***Correspondence:** Michel Drancourt, michel.drancourt@univ-amu.fr

Keywords: *Enterococcus mundtii*; *Mycobacterium tuberculosis*; anti-tuberculosis activity; inhibition spectrum; tuberculosis.

Abbreviations: BCG, *M. bovis* Bacillus bilié de Calmette-Guérin; CFCS, cell-free culture supernatant; DPBS, Dulbecco's PBS; MRS broth, De Man, Rogosa, and Sharpe broth; MTC, *Mycobacterium tuberculosis* complex.

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significant differences in gut microbiota composition between tuberculosis and non-tuberculosis patients: in tuberculosis patients, pathogenic species of *Actinobacteria* and *Proteobacteria* were significantly more abundant, while faeces collected from apparently healthy individuals were enriched in *Bacteroidetes* and *Firmicutes* [15, 16].

Bacteria of the genus *Enterococcus* are among *Firmicutes* commensals of the gastrointestinal tract [17] known to produce antimicrobial peptides [18–20]. In fact, *Enterococcus* species produce a wide range of diverse antimicrobial peptides, often more than one per strain, some of which are atypical and distinct from known antimicrobial peptides [21]. Enterococci have received increased attention in recent years, due to their applications in medical treatments and their consideration as beneficial organisms for health [17, 22].

Recently, we reported the *in vitro* inhibitory effect of *Enterococcus mundtii* against MTC species [23]. Here, we further investigated the experimental anti-tuberculosis activity of different *Enterococcus* species to characterize and explore the inhibition spectrum of the *Enterococcus* secretome against some MTC representative species.

METHODS

Mycobacterium strains

All bacterial strains investigated were obtained from the CSUR (The Rickettsiae Unit Strain Collection) of the University-Hospital Institute laboratory (IHU-Méditerranée Infection, Marseille, France). The identification of all strains was confirmed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) MS [24]. All manipulations of live MTC mycobacteria were performed in the NSB3 laboratory. Seven MTC strains, including four *M. tuberculosis* (strain S10, CSUR Q3640 lineage 4.8; strain S14, CSUR Q3641 lineage 4.8; strain S15, CSUR Q3643 lineage 3.1; and strain S16, CSUR Q3644 lineage 4.6), *M. canettii* (CSUR Q3751), *M. africanum* (CSUR 2018–2-B6) and *M. bovis* (CSUR Q0209), were inoculated in Middlebrook 7H10 agar medium (Becton Dickinson) containing 10% oleic acid–albumin–dextrose–catalase (OADC; Becton Dickinson) for 15 days at 37°C in an aerobic atmosphere enriched with 5% CO₂. Mycobacterial colonies were resuspended in 1 ml of Dulbecco's PBS (DPBS; Thermo Fisher Scientific), vigorously mixed on a vortex mixer for 10 min, homogenized with glass beads (Sigma-Aldrich) to remove clumps and finally calibrated at 1 MacFarland using optical densitometry.

Enterococcus strains

A total of 27 *Enterococcus* species were investigated in this study. These strains were grown on Columbia agar solid medium, supplemented with 5% sheep blood (COS; bioMérieux) for 24 h in an aerobic atmosphere enriched with 5% CO₂ at 37°C. *E. mundtii* colonies were suspended in 1 ml of DPBS, vortexed for 2 min and adjusted to 10 McFarland equivalent of 3×10⁹ c.f.u. ml⁻¹ according to serial dilution methodology (Table 1).

E. mundtii cell-free culture supernatant

E. mundtii strains were grown in De Man, Rogosa, and Sharpe broth (MRS broth; Sigma-Aldrich) prepared as follows: 1 litre of MRS broth supplemented with 1 ml of Tween 80 (Sigma-Aldrich) was autoclaved at 121°C for 15 min. A 500 µl volume of *E. mundtii* suspension inoculated into 100 ml of autoclaved MRS broth was incubated at 30°C for 18 h under aerobic conditions. Then, *E. mundtii* cultures were centrifuged for 15 min at 3200 g at 4°C and the supernatant was filtered using a 0.22 µm filter (Carl Roth) in order to obtain a cell-free culture supernatant (CFCS). The CFCS was adjusted to pH 6.5 using an NaOH solution to eliminate any pH-dependent bias, and 20 ml of CFCS was lyophilized for 24 h at –80°C. Lyophilized CFCS was resuspended in 2 ml of DPBS, then diluted with DPBS at 1:2, 1:5, 1:8 and 1:10. CFCS dilutions were inoculated on COS medium for 24 h at 37°C in an aerobic atmosphere enriched with 5% CO₂ to ensure their sterility. To determine the nature of inhibitory factors, *E. mundtii* CFCS was incubated with proteinase K (1 mg ml⁻¹) at 56°C for 2 h, prior to inhibition testing as above. Also, heat resistance was assessed by incubating the *E. mundtii* CFCS for 15 min at 100°C, prior to inhibition testing as above. Protein concentration was quantified by the Qubit assay (Bio-Rad). Non-inoculated MRS broth was used as a negative control throughout the experimental process.

Agar well diffusion assay

E. mundtii anti-tuberculosis activity was evaluated by the agar well diffusion assay. Initially, a mycobacterial suspension was inoculated on Middlebrook 7H10 agar for 24 h at 37°C in an aerobic atmosphere enriched with 5% CO₂. Two wells were prepared in the centre of the 7H10 agar medium Petri dish: 100 µl of *E. mundtii* suspension was inoculated in a first well, and 100 µl of DPBS was inoculated in the other well as a negative control. Then, the anti-tuberculosis activity of *E. mundtii* CFCS was evaluated using 100 µl of CFCS in a first well, while 100 µl of MRS broth was used as a negative control in the second well. Petri dishes were incubated at 37°C for 15 days under an aerobic condition enriched with 5% CO₂. The 1:2, 1:5, 1:8 and 1:10 CFCS dilutions were tested respectively and the diameter of MTC growth inhibition area around each well was measured in millimetres focusing restrictively on the inhibition areas. The *E. mundtii* CSURP2005 strain and its CFCS diluted at 1:5 were used to standardize the agar well diffusion assay conditions against other strains of MTC, including *M. canettii*, *M. bovis* and *M. africanum*; all manipulations were performed in triplicate (Fig. 1).

Table 1. List of *Enterococcus* species investigated in this study from CSUR, under the international collection number: WDCM 875

CSUR international collection no. WDCM 875			
Strain	CSUR number	Strain	CSUR number
<i>E. mundtii</i>	CSURP724	<i>E. pallens</i>	CSURP1924
<i>E. mundtii</i>	CSURQ1712	<i>E. pallens</i>	CSURP1925
<i>E. mundtii</i>	CSURP7988	<i>E. phoeniculicola</i>	CSURP1828
<i>E. mundtii</i>	CSURP5399	<i>E. phoeniculicola</i>	CSURP7585
<i>E. mundtii</i>	CSURP2005	<i>E. pseudoavium</i>	CSURP1213
<i>E. avium</i>	CSURQ4946	<i>E. pseudoavium</i>	CSURP2652
<i>E. avium</i>	CSURQ5146	<i>E. saccharolyticus</i>	CSURP1919
<i>E. avium</i>	CSURQ5145	<i>E. saccharolyticus</i>	CSURP1024
<i>E. durans</i>	CSURP8822	<i>E. sanguinicola</i>	CSURP1723
<i>E. durans</i>	CSURQ2511	<i>E. thailandicus</i>	CSURP3312
<i>E. durans</i>	CSURP8400	<i>E. timonensis</i>	CSURP1024
<i>E. casseliflavus</i>	CSURQ3866	<i>E. cecorum</i>	CSURQ4894
<i>E. casseliflavus</i>	CSURQ5159	<i>E. malodoratus</i>	CSURQ5750
<i>E. faecalis</i>	CSURP6215	<i>E. raffinosus</i>	CSURQ3178
<i>E. faecalis</i>	CSURP6305	<i>E. burkinafasonensis</i>	CSURQ0835
<i>E. faecium</i>	CSURP3600	<i>E. devriesei</i>	CSURP0494
<i>E. faecium</i>	CSURQ4911	<i>E. diestrammenae</i>	CSURP0303
<i>E. gallinarum</i>	CSURP4099	<i>E. eurekensis</i>	CSURP2018
<i>E. gallinarum</i>	CSURQ4127	<i>E. gilvus</i>	CSURP5799
<i>E. hirae</i>	CSURQ3681	<i>E. massiliensis</i>	CSURP7858
<i>E. hirae</i>	CSURP2034	<i>E. ovatus</i>	CSURP1924
<i>E. dispar</i>	CSURP0234	<i>E. ovatus</i>	CSURP588
<i>E. dispar</i>	CSURP2030	<i>E. mediterraneensis</i>	CSURP2034
<i>E. mediterraneensis</i>	CSURP2034	-	-

MTC, *Mycobacterium tuberculosis* complex.

Statistical analyses

Data were entered into Microsoft Excel for Office to calculate the mean of inhibition diameters. Statistical analysis was done using GraphPad software (v8.0). A one-way ANOVA test was used to compare the mean of the inhibition diameters of *E. mundtii* strains and to compare the susceptibility of mycobacteria to enterococci. Differences were considered statistically significant at $P < 0.05$.

RESULTS

E. mundtii suspension anti-tuberculosis activity

The agar well diffusion assay indicated that the five *E. mundtii* strains evaluated here inhibited the growth of all *M. tuberculosis* strains: a clear inhibition area was observed around the well containing the 10 MacFarland inoculum of *E. mundtii* suspension after a 15 day inoculation, but not with the 8 MacFarland or 5 MacFarland suspension, suggesting that the effect observed here was dependent on the *E. mundtii* inoculum in the absence of any growth inhibition zone around the DPBS negative control (Fig. 2a). Comparing the inhibition zone diameters resulting from cross-investigation of the five *E. mundtii* strains and the four *M. tuberculosis* strains (S10, S14, S15, S16) revealed that *E. mundtii* strains had a significantly different inhibitory effect as follows: *E. mundtii* CSURQ1712 and *E. mundtii* CSURP7988 between two strains *M. tuberculosis* S10 and *M. tuberculosis* S15 ($P < 0.05$) (Fig. 2bE and bF), *E. mundtii* CSURP724 between *M. tuberculosis* S10 and *M. tuberculosis* S14 ($P < 0.05$) (Fig. 2bG), *E. mundtii* CSURP5399 between *M. tuberculosis* S10 and all other *M. tuberculosis* strains tested ($P < 0.05$) (Fig. 2bH), and finally

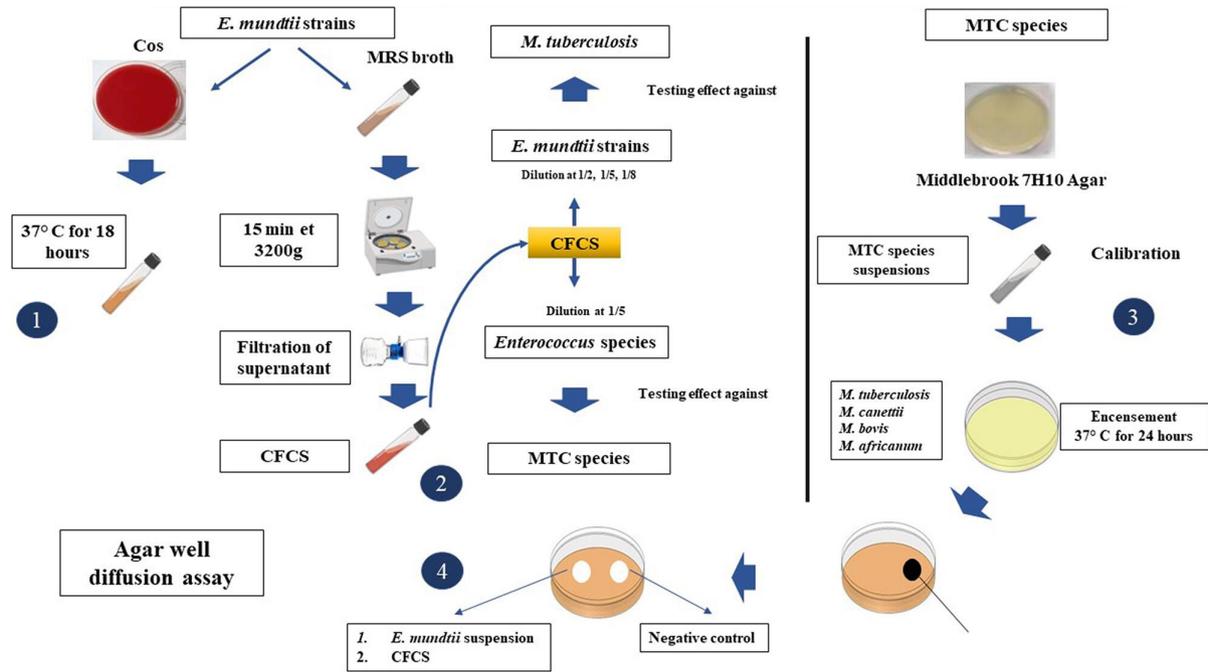


Fig. 1. Workflow for the agar well diffusion assay used in this study. 1. *E. mundtii* suspensions. 2. *Enterococcus* species supernatants. 3. MTC species suspensions. 4. Agar well diffusion assay.

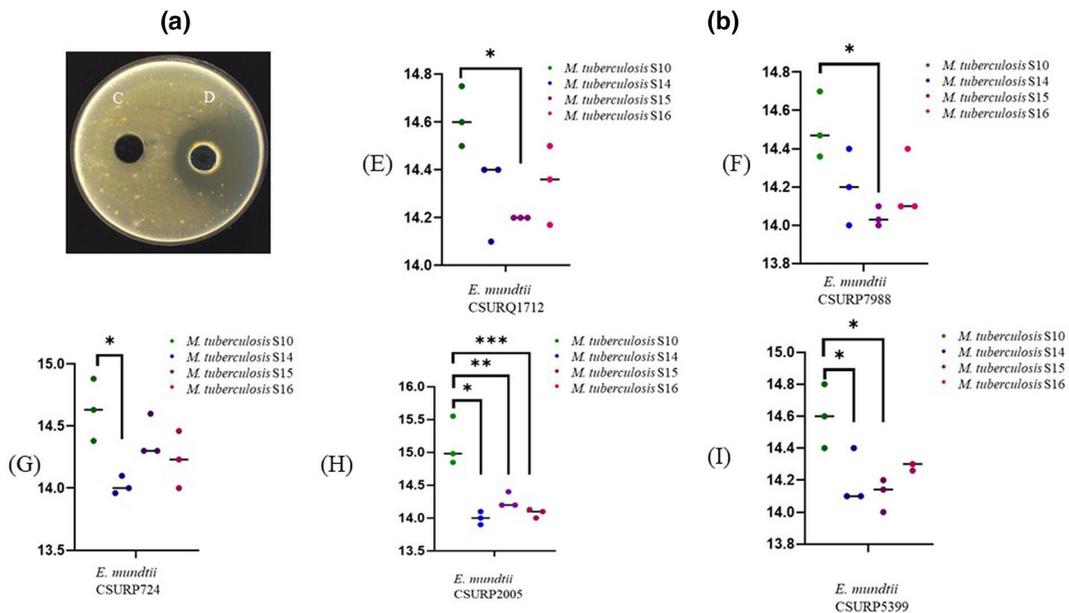


Fig. 2. *In vitro* activity of *E. mundtii* strains against four *M. tuberculosis* strains. (a) Antimicrobial assay of *E. mundtii* strain suspensions against *M. tuberculosis* strains using the agar well diffusion assay (C, DPBS as a negative control; D, *E. mundtii* suspension). (b) Comparison of the means of inhibition diameters (mm) of five *E. mundtii* strains including *E. mundtii* CSURQ1712 (E), *E. mundtii* CSURP7988 (F), *E. mundtii* CSURP724 (G), *E. mundtii* CSURP2005 (H) and *E. mundtii* CSURP5399 (I) and the susceptibility of *M. tuberculosis* strain S10, *M. tuberculosis* strain S14, *M. tuberculosis* strain S15 and *M. tuberculosis* strain S16 to *E. mundtii* strains using one-way ANOVA test. Statistical significance was defined as * $P < 0.05$.

Table 2. Growth inhibition of four strains of *M. tuberculosis* by five strains of *E. mundtii* and DPBS buffer as a negative control

The measurement mean of growth inhibition is expressed in millimetres. Measurement of the inhibition area is in mm².

Strain	DPBS	<i>M. tuberculosis</i> strain S10	<i>M. tuberculosis</i> strain S14	<i>M. tuberculosis</i> strain S15	<i>M. tuberculosis</i> strain S16
<i>E. mundtii</i> CSURQ1712	0	14.6±1 mm (167 mm ²)	14.3±1 mm (160 mm ²)	14.2±1 mm (158 mm ²)	14.36±1 mm (161.9 mm ²)
<i>E. mundtii</i> CSURP724	0	14.63±1 mm (168.1 mm ²)	13.98±1 mm (153 mm ²)	14.36±1 mm (161.9 mm ²)	14.23±1 mm (159.3 mm ²)
<i>E. mundtii</i> CSURP7988	0	14.51±1 mm (165.3 mm ²)	14.2±1 mm (158 mm ²)	14.13±1 mm (156.8 mm ²)	14.2±1 mm (158 mm ²)
<i>E. mundtii</i> CSURP5399	0	14.6±1 mm (167 mm ²)	14.2±1 mm (158 mm ²)	14.14±1 mm (157 mm ²)	14.36±1 mm (161.9 mm ²)
<i>E. mundtii</i> CSURP2005	0	15.5±1 mm (188.6 mm ²)	14±1 mm (153.9 mm ²)	14.3±1 mm (160.6 mm ²)	14.23±1 mm (153.9 mm ²)

E. mundtii CSURP2005 between three strains *M. tuberculosis* S10, *M. tuberculosis* S14 and *M. tuberculosis* S15 (Fig. 2bI). Also, *M. tuberculosis* S10, which was more susceptible, exhibited the largest inhibition area (inhibition diameter 15.5±1 mm) (Table 2).

CFCS anti-tuberculosis activity

The protein concentration in each CFCS dilution of five *E. mundtii* strains (Table 3) indicated a maximum value for *E. mundtii* CSURP2005 (24.03 µg ml⁻¹) and a minimum value for *E. mundtii* CSURP7988 (21.2 µg ml⁻¹), while *E. mundtii* CSURQ1712, *E. mundtii* CSURP0724 and *E. mundtii* CSURP5399 showed values of 21.4, 22.5 and 22.3 µg ml⁻¹, respectively. When tested with *M. tuberculosis* strain S10, the five *E. mundtii* strains, CFCS diluted to 1:2, 1:5 and 1:8, showed anti-tuberculosis activity, while no zone of inhibition was observed with the 1:10 diluted CFCS and the negative control in MRS broth (Fig. 3a). An area of inhibition was observed around the well containing the CFCS, which varied with CFCS concentration; the 1:8 dilution showed the lowest inhibition activity (inhibition diameter 9±1 mm). Dilution less than 1:8 showed no effect against *M. tuberculosis* strains (Table 4). A significant difference was observed between the three dilutions of five CFCS ($P=0.001$) (Fig. 3b). The inhibition diameters observed here were proportional to the CFCS fraction and the protein content of each dilution (Fig. 3c). The *E. mundtii* CSURP2005 strain exhibiting the highest protein content and its 1:5 dilution with a more representative inhibition zone was chosen to standardize the agar well diffusion assay used in this study. Treating *E. mundtii* CFCS with proteinase K resulted in total disappearance of the inhibition zones, suggesting the protein nature of the inhibitory factors. Furthermore, anti-tuberculosis activity was not affected by 15 min of heating at 100 °C. Also, from a total of 27 *Enterococcus* species, eight showed anti-tuberculosis activity against four MTC species. More precisely, 15 strains showed anti-tuberculosis inhibitory effects, including five *E. mundtii* strains (CSURQ1712, CSURP724, CSURP7988, CSURP5399 and CSURP2005), two *E. avium* strains (CSURQ4946 and CSURQ5145), two *E. durans* (CSURP8822 and CSURQ2511), one *E. casseliflavus* strain (CSURQ3866), one *E. faecalis* strain (CSURP6215), one *E. faecium* strain (CSURP3600), one *E. hirae* strain (CSURQ3681), one *E. dispar* strain (CSURP0234) and one *E. devriesei* strain (CSURP0494), with variable susceptibility between strains (Fig. 4). There was no significant difference in the susceptibility of *M. tuberculosis*, *M. bovis* and *M. africanum*, but *M. canettii* exhibited the highest susceptibility, with an inhibition area diameter of 25±1 mm ($P=0.001$) (Fig. 5).

DISCUSSION

The fact that *E. mundtii* inhibited the growth of some MTC species had been previously described in our laboratory [23], and the data reported here broadened such previous observations by cross-incorporating a set of *E. mundtii* strains and MTC species

Table 3. Dilution of the protein concentration (µg ml⁻¹) of the CFCS of the five *E. mundtii* strains investigated here

Strains	<i>E. mundtii</i> CSURP2005	<i>E. mundtii</i> CSURP5399	<i>E. mundtii</i> CSURP724	<i>E. mundtii</i> CSURP7988	<i>E. mundtii</i> CSURQ1712
Pure	24.03	22.3	22.5	21.2	21.4
Dilution 1:2	12.01	11	11.3	11	10.7
Dilution 1:5	5	4.4	5	4.24	4.28
Dilution 1:8	3	3	2.8	2.6	2.7
Dilution 1:10	2.4	2.23	2.2	2	2

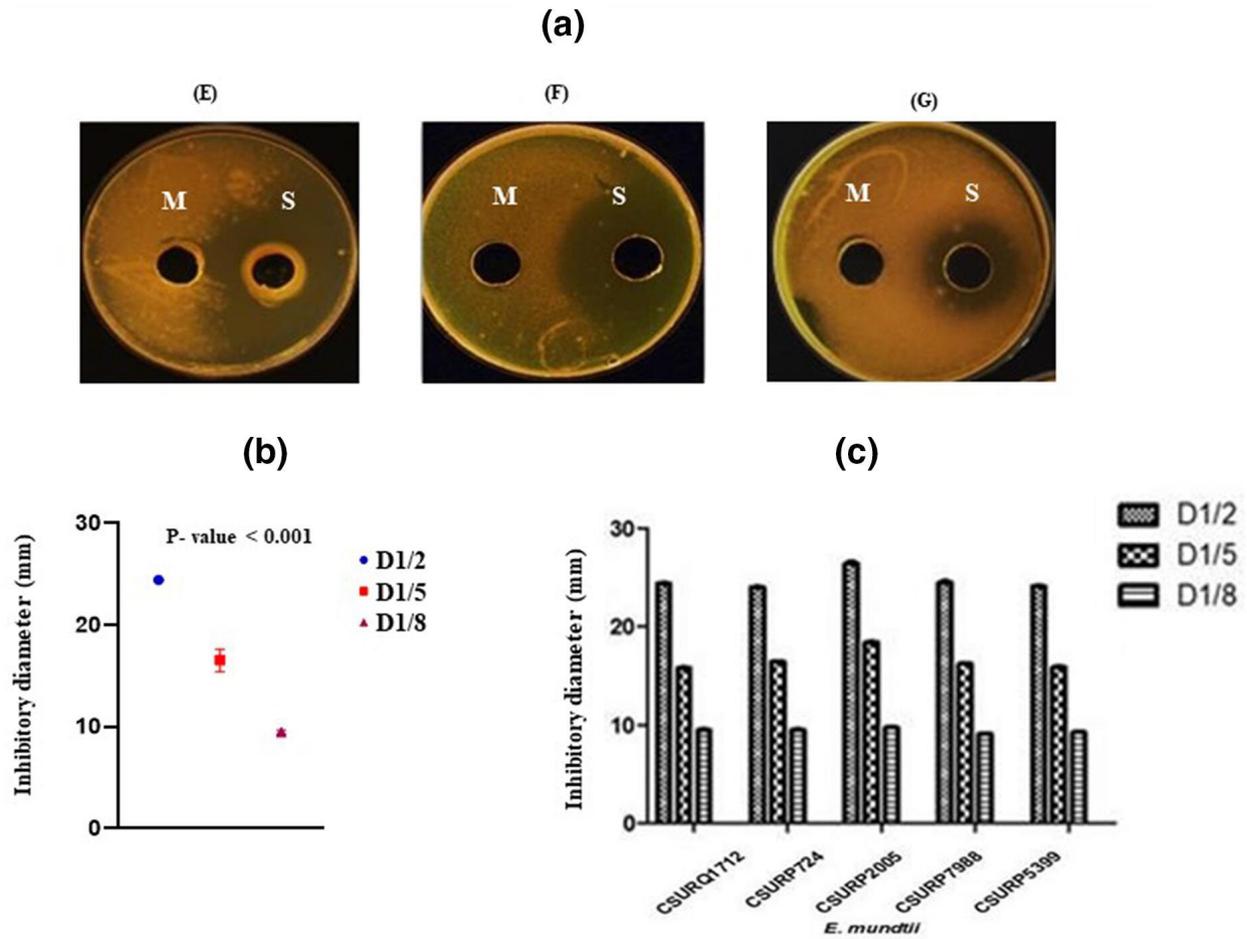


Fig. 3. *In vitro* activity of *E. mundtii* CSURP2005 CFCS against *M. tuberculosis* S10. (a) Antimicrobial assay of *E. mundtii* CSURP2005 CFCS dilution 1:2 (E), 1:5 (F) and 1:8 (G) against *M. tuberculosis* using agar well diffusion assay (M: MRS broth; S: *E. mundtii* CSURP2005 CFCS). (b) Comparison of the means of inhibition diameters (mm) of *E. mundtii* strain CFCS dilutions using the Kruskal–Wallis test (one-way ANOVA). (c) Inhibition diameters of MTC strains dependent on the dilution (D) of CFCS.

Table 4. Mean inhibition diameters (mm) of *E. mundtii* strains in CFCS dilutions, and MRS broth as a negative control, and measurement of the inhibition area (mm²)

Strains	MRS broth	<i>M. tuberculosis</i> strain S10 (dilution 1:2)	<i>M. tuberculosis</i> strain S10 (dilution 1:5)	<i>M. tuberculosis</i> strain S10 (dilution 1:8)	<i>M. tuberculosis</i> strain S10 (dilution 1:10)
<i>E. mundtii</i> CSURQ1712	0	24.39±1 mm (476 mm ²)	15.82±1 mm (197 mm ²)	9.51±1 mm (71 mm ²)	0
<i>E. mundtii</i> CSURP724	0	24.02±1 mm (452 mm ²)	16.43±1 mm (212 mm ²)	9.52±1 mm (71.1 mm ²)	0
<i>E. mundtii</i> CSURP2005	0	26.4±1 mm (547 mm ²)	18.42±1 mm (266 mm ²)	9.7±1 mm (73.8 mm ²)	0
<i>E. mundtii</i> CSURP7988	0	24.48±1 mm (470 mm ²)	16.21±1 mm (206 mm ²)	9.3±1 mm (68 mm ²)	0
<i>E. mundtii</i> CSURP5399	0	24.12±1 mm (457 mm ²)	15.8±1 mm (196 mm ²)	9.3±1 mm (68 mm ²)	0

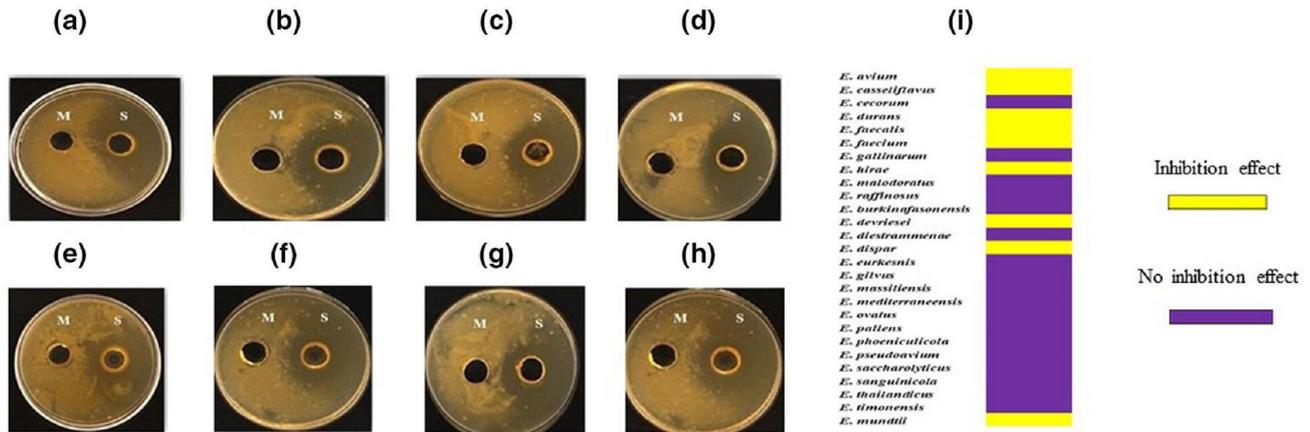


Fig. 4. *In vitro* activity of *Enterococcus* species CFCS against *M. tuberculosis*. (a) *E. avium* CSURQ4946. (b) *E. devriesei* CSURP0494. (c) *E. durans* CSURP8822. (d) *E. dispar* CSURP2034. (e) *E. hirae* CSURQ3681. (f) *E. casseliflavus* CSURQ5159. (g) *E. faecalis* CSURP6215. (h) *E. faecium* CSURP3600 (M: MRS broth; S: *Enterococcus* CFCS). (i) Heat map summarizing inhibitory (yellow) or non-inhibitory (violet) effect of the *Enterococcus* species against *M. tuberculosis*.

using a standardized agar well diffusion assay to compare cross-inhibition values. Accordingly, we observed that both *E. mundtii* suspensions and derived lyophilized culture supernatants inhibited the growth of *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. canettii*. This inhibition effect was reproducible and authenticated by the absence of zones of clearance. The inhibitory effect was proportional to the *E. mundtii* inoculum and the concentration of proteins in the derived lyophilized culture supernatant, while no zone of inhibition was observed with the 1:10 diluted CFCS considered as MIC, suggesting that inhibition of MTC growth was due to molecules excreted by *E. mundtii* in the culture broth, probably heat-stable peptides and proteins, most likely

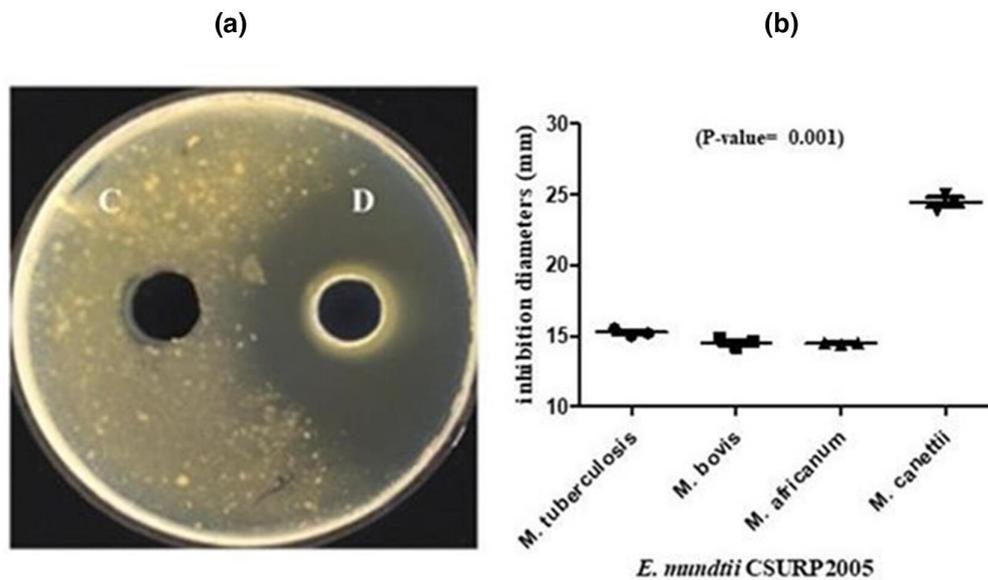


Fig. 5. *In vitro* activity of *E. mundtii* CURPS2005 CFCS against *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. canettii*. (a) Antimicrobial assay of *E. mundtii* CSURP2005 CFCS dilution 1:5 against *M. canettii* using the agar well diffusion assay. C: MRS broth; D: *E. mundtii* CSURP2005 CFCS diluted to 1:5. (b) Comparison of the means of *E. mundtii* CSURP2005 inhibition diameters (mm) against *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. canettii* using the Kruskal–Wallis test (one-way ANOVA on ranks).

bacteriocins, which are indeed heat-stable antimicrobial peptides produced extracellularly and synthesized by the ribosomal pathway, encoded by the plasmid or the chromosome [25, 26].

Indeed, *E. mundtii* was acknowledged to produce bacteriocins [26–29] with antibacterial activity against various isolates of Gram-positive genera. *E. mundtii* strain ATO6 and *E. mundtii* strain NFRI 7393 isolated from vegetables produced the bacteriocin mundticin, inhibiting growth of isolates of the genus *Listeria* [30] whereas *E. mundtii* strain QU 25 isolated from ovine faeces and *E. mundtii* strain CRL35 isolated from cheeses inhibited growth of various strains of *Pediococcus*, *Listeria*, *Lactobacillus* and *Leuconostoc* [27].

In the genus *Enterococcus*, anti-mycobacterial activities of bacteriocins have been reported. *Enterococcus italicus* strain BLN34 isolated from cow's milk inhibited growth of *Mycobacterium kansasii* [31], and *Enterococcus hirae* showed antagonistic effects against *Mycobacterium smegmatis* Mc155 [18]. Finally, *Enterococcus faecalis* has been characterized by its ability to inhibit the multiplication of *M. tuberculosis* [32].

As *E. mundtii* was detected in the gut microbiota in some individuals where *M. tuberculosis* was also detected, our data suggested that the *E. mundtii* secretome, probably bacteriocins, may modulate tuberculosis in the gut microbiota. The association between the gut microbiota and *M. tuberculosis* infection remains an avenue to be explored [33, 34]. Multiple metagenomic studies have indicated that the gut bacterial microbiota investigated in pulmonary tuberculosis patients significantly differed from that of tuberculosis-free, apparently healthy individuals [15, 35]. A decrease in the proportion of *Bacteroidetes* and *Firmicutes* has already been shown in the gut microbiota of tuberculosis patients compared to apparently healthy controls [34, 36]. Likewise, *Proteobacteria* and *Bacteroidetes* have been found to characterize the pulmonary microbiota in tuberculosis patients, while that collected in apparently healthy individuals was marked by the presence of *Firmicutes* bacteria [37].

Together, the findings of the present study support the need for additional research to investigate the potential contribution of enterococci to the control of mycobacteria.

Funding information

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Acknowledgements

The authors thanks Jeffrey Arrindel for his help in statistical analyses.

Author contributions

W.A., experimental design, analysis, interpretation and wrote the original draft of the manuscript. J.L.M. and M.F., contributed to data interpretation and wrote the original draft. J.L.M. and M.D. were responsible for project design, funding, and critically reviewing the manuscript. All authors declare that they have read and approved the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Peer review history

VERSION 2

Editor recommendation and comments

<https://doi.org/10.1099/acmi.0.000471.v2.1>

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Saeed Ahmed; National University of Medical Sciences, Biological Sciences, PAKISTAN

Date report received: 03 April 2023

Recommendation: Accept

Comments: The work presented is clear and the arguments well formed.

Author response to reviewers to Version 1

Dear Editor,

Please find enclosed the revised version of our manuscript entitled: “ *The Enterococcus secretome inhibits the growth of Mycobacterium tuberculosis complex mycobacteria- ACMI-D-22-00112*” by Achache W. and collaborators along with the answers of the authors to the reviewers ‘comments.

Reviewer 1:

Comment 1:

This short paper describes inhibition of growth of Mycobacteria with cell free supernate (CFS) of *Enterococcus* species. The methodology to demonstrate inhibition of growth of Mycobacterial strains is simple but effective. Growth of Mycobacteria is inhibited in the presence of CFS but not when sterile culture broth is used. The authors suggest that the *Enterococcus* strains are producing a bacteriocin which is inhibiting growth of Mycobacteria. However the factor that is responsible for the inhibition is not characterised at all. Some simple experiments such as testing the effect of heat treatment of the CFS and/or protease treatment of the CFS would go a long way to strengthening this manuscript.

Some further controls could be also be included.

Authors ‘answer: The authors perfectly agree with the remark and these simple experiments have been added in the revised version of the manuscript (M & M section, lines 138-140; Results section, lines 197-199; Discussion section, lines 224-225).

Comment 2:

Are these effects specific for Mycobacteria or do you see similar effects with other bacteria?

Authors ‘answer: The present study included only mycobacteria and the authors have no experimental data regarding other bacteria. Nevertheless, inhibitory effect of *Enterococcus* was tested against several species of mycobacteria including *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium canettii* with negative control consisted in *Enterococcus* medium which was treated in the same conditions. Also, previously reported studies dealing with bacteria other than mycobacteria are reported in the Discussion section (lines 227-232).

Comment 3:

Line 183 and throughout - replace the word fractions with dilutions

Authors ‘answer: Corrected accordingly.

Comment 4:

The conclusion statement needs to be modified. Rather than the work showing that Enterococci are likely to be key players in the control of Mycobacteria in the gut a more realistic conclusion might be that the results of this study suggest that further investigations are warranted to see if enterococci play any role in vivo in the control of Mycobacteria in the gut.

Authors 'answer: The reviewer is perfectly right and the conclusion statement has been corrected accordingly (lines 249- 251). Furthermore, the findings of the present study support the need for additional research to investigate the potential contribution of *Enterococcus* to the control of mycobacteria.

Comment 5:

Presentation of the results could be improved in places. Labelling of some of the figures needs attention.

Figure 2 - it is confusing to have individual figure parts labelled A and B and then parts of the figure A labelled A and B. Please use different letters here.

Authors 'answer: Corrected accordingly.

Figure 3 M and S are explained in the figure legend but no M and S is on parts a, b or c of part A

Authors 'answer: Corrected accordingly.

Figure 5 No A and B labels on the figure but A and B referred to in the legend. For the diffusion assay label wells M and S for consistency.

Authors 'answer: Corrected accordingly.

Reviewer 2:

Comment 1:

The manuscript by Achache et al describes experiments which tested the secretome of Enterococci on *Mycobacterium tuberculosis* complex mycobacteria. The methodology involved culture supernatants in agar diffusion assays and measurement of the zones of clearance. I was excited to read this manuscript as the topic is interesting and the research questions timely and important. However, I encourage the authors to develop their manuscript for clarity and robustness of data.

Authors 'answer: The authors acknowledge this enthusiastic, positive general comment.

Comment 2:

It is not clear to me that the experiments reported have been replicated as would be the standard in biology and a requirement for good statistics. Have these experiments been performed multiple independent times? Could the authors provide details? If replicates have been performed, could the data for these replicates be included

Authors 'answer: Indeed, every single experiment was triplicate, as clarified in the M & M section in line 158. The mean of the 3 measurements (triplicate) are provided in Table 2 and Table 3.

Comment 3:

Overall, I found the manuscript confusing to read. Perhaps treating the *Enterococcus* and *Mycobacterium* separately and giving them separate methods sections might help? Also see comments below on Figure one.

Authors 'answer: The authors followed this suggestion: *Mycobacterium* strains (list, culture and confirmatory identification) are now given a separate section (lines 106-120); *Enterococcus* strains (list, culture and confirmatory identification) are now given a separate section (lines 121-126), and the following sections remained unchanged.

Comment 4:

Many of the diffusion assays presented show zones of clearance that breach the agar plate and I don't believe these can be used to measure inhibition zones reliably. These agar plate might inform the authors, but the formal analysis should only relate to complete zones of clearance.

Authors 'answer: The perfectly is perfectly right and in this situation indeed the authors tabulated the measurable (smaller) diameter. This point is now clarified in the M & M section, line 155.

Comment 5:

Statistics: Can the authors confirm the number of independent replicates? Can the authors discuss their statistical approach? It is not clear to me what value a one-way ANOVA brings to this story, without multiple comparisons within the ANOVA.

Authors 'answer: The experiment was performed once with three agar plates for each test. The inhibition diameters were measured for each agar plate and an average of the inhibition diameters was calculated. The analysis is based on a single variance (one way) that targets a single factor, *M. tuberculosis* strains susceptible or not.

Comment 6:

Line 87 all strains were identified ... if they authors wish to include this statement, can they also include the data or reference if this has already been published

Authors 'answer:This M & M sentence is now referenced, accordingly (new reference 24).

Comment 7:

Suggestion: the extensive list of strains might be better placed in a table than text. This might aid readability.

Authors 'answer:This list is now deleted from the text and presented in a new Table 1, accordingly.

Comment 8:

Line 126 the abbreviation DPBS is explained here but has already been cited at an earlier line without explanation. Please define abbreviation at the first use.

Authors 'answer:Corrected accordingly.

Comment 9:

Line 137 could the authors please clarify why cell free culture supernatant was pH adjusted?

Authors 'answer:Indeed, the authors did adjusted the pH to eliminate any pH-dependent biological bias in the results and highlighting the intrinsic anti- *M. tuberculosis* activity of the supernatants. This point is now clarified in the M & M section in lines 135.

Comment 10:

Line 141 could the authors please indicate why 1/5 dilution was chosen?

Authors 'answer:In fact, preliminary experiments (not reported in the manuscript) indicated this dilution was optimal to accurately observe the inhibition zones.

Comment 11:

I find the descriptions of CFU/ mL and/or MacFarland standars equivalants difficult to follow when their use is interchangeable in the text. Could the authors please use both or just CFU / mL alone.

Authors 'answer:The reviewer is perfectly right and in order to avoid any further confusion, authors deleted CFU and kept the McFarland unit data.

Comment 12:

Line 173 - 177 I find this sentence confusing. The texts describes a similar inhibitory effect of CSURQ1712 and CSURP7988 but cites p values of 0.1 and 0.2. These p values do not indicate significant differences. Could the authors please correct this. I suspect there is either a typo here, or the p value doesn't relate to the inhibition zones shown. This section required clarification.

Authors 'answer:Indeed the authors fully agree with the reviewer that in the context of these experiments, p value of 0.1 and p value of 0.2 are NOT indicative of any significance; as already written in the manuscript: there was no significant difference between these four *M. tuberculosis* strains (line 174). This section has been modified based on the comments in Figure 2 (Lines 172-179).

Comment 13:

Line 187 The protein values cited in the text should have standard deviations.

Authors 'answer:The Qubit assay consists in establishing a standard range that automatically generates a standard deviation, for each sample, three measurements are performed.

Comment 14:

Multiple mentions of standardisation of the well diffusion assay to the 1/5 dilution. The methods would benefit from detailing exactly what this standardisation is and how its performed.

Authors 'answer:see above answer.

Comment 15:

Line 218 "negativity of negative controls" Rephrasing this to "absence of zones of clearance around the negative controls" or similar scientific language is recommended.

Authors 'answer:Corrected, accordingly (Line 219).

Comment 16:

Line 219 The inhibitory effect was proportional to the inoculum... but this contradicts line X which states an effect was only observed at Y. I understood the statement in line X to indicate the effect was an on/off type effect. Please clarify these statements for accuracy.

Authors 'answer:The authors did not observe an "on/off" but indeed, they did observe a linearly dilution-dependent effect of inhibition. This point was clarified in the Discussion section, in line 220.

Comment 17:

Line 231 "at large" suggestion to remove "at large"

Authors 'answer:Deleted, accordingly.

Comment 18:

Line 257 is "validation of manuscript" correct here?

Authors 'answer:Deleted, accordingly.

Comment 19:

It is surprising that the protein concentration differs so little between the 1/8 dilution and the 1/10 dilution, however the zone of clearance effect is completely lost. Could the authors speculate or discuss this in the discussion?

Authors 'answer:This point was a little bit discussed in term of minimal inhibitory concentration, accordingly (Discussion section line 221).

Comment 20:

Figure 1I commend the authors for including a schematic figure describing their methodology and I would strongly encourage the authors to improve this figure. I have the following suggestions:

1.remove all shadows from boxes and pictures

Authors 'answer:Done accordingly.

2. revise colour scheme from a red/green to a colour-blind friendly pallet

Authors 'answer:Done accordingly.

3. slightly reduce the size of the agar plates at the bottom of the figure

Authors 'answer:Done accordingly.

4. use the exact same agar plate diagram and size for each plate - change the colour to indicate media and insert circles to indicate well.

Authors 'answer:Done accordingly.

5. Make all test tubes the same size and if you must have them at an angle, use the exact same angle for each test tube.

Authors 'answer:Done accordingly.

6. Make arrows uniform in size and colour

Authors 'answer:Done accordingly.

7. Make text uniform - don't present text in a coloured box or circle and remove coloured boxes.

Authors 'answer:Done accordingly.

8. The figure required further explanation in the legend - what are 1, 2 and 3 in the schematic referring to?

Authors 'answer:Done accordingly.

Figure 2

1. Perhaps the deviation bars or data points could be slightly different colours or thicknesses to aid visualisation? Currently, it is difficult to see individual data points that sit on the error bars. Perhaps grey error bars would help?

Authors 'answer:Done accordingly.

2. Remove the shadow from behind the agar plate picture. Also, the blue box outline is unnecessary and could be removed.

Authors 'answer:Done accordingly.

3. Have the ANOVA tests been performed to test significance of individual strain combinations? Could the authors explain the logic of performing an ANOVA on this group of strains (S10, S14, S15, S16) without making individual comparisons. This can easily be done in Graphpad and would add value to these data. For example, I suspect S15 treated individually might be significantly different from S14 (if only slightly and probably not meaningfully) in the CSURQ1712 graph.

Authors 'answer:Done accordingly.

4. Each graph should be its own panel and therefore have an identifying letter. E.g. Figure 2 B-F.

Authors 'answer:Done accordingly.

5. Line 426 the first description of this strain being obtained from human stool should not be in the figure legend. Please remove this detail from the legend and document this in the methods.

Authors 'answer:Done accordingly.

Figure 3

1. It is not advisable to have figure 3, panel a sub-panel a,... please relabel these sub panels (i, ii... etc) or make each plate it's own panel.

Authors 'answer:Done accordingly.

2. Panel A (a) here an asymmetric diffusion pattern is shown. Ideally, this assay should be performed with either an agar plate large enough or a well small enough to allow a symmetric and entire diffusion pattern occur. Could the authors please explain why this hasn't been possible? I believe the 1/8 dilution shown in (c) is sufficient to measure the zone of clearance, but the authors have indicated they used the 1/5 which also doesn't show a full diffusion pattern.

Authors 'answer:The perfectly is perfectly right, in this condition, asymmetric diffusion patterns may be due to the concentration of the supernatant and how it is deposited and diffused onto the agar plate.

3. B I believe this graph would benefit from larger data points and perhaps a grey error bar.

Authors 'answer:Done accordingly.

4. I have concerns about how the data for dilution 1/2 and 1/5 were recorded in light of the images presented in this figure. It appears only the 1/5 dilution has error bars, but I would expect error in these types of data. Please clarify.

Authors 'answer:The graph has been modified so that the error bars are visible.

5. C The legend indicates a 1/10 dilution that is not present on the graph. It is difficult to see which bars correspond to the patterns shown in the legend. Perhaps increasing the bar size would help. Are there again no errors bars from these data? If not, please explain.

Authors 'answer:The values for the 1/10 dilution are nul and were removed from the graph.

6. Please remove the blue boxes surrounding the figure

Authors 'answer:Done accordingly.

Figure 4

Remove all shadows from boxes

Authors 'answer:Done accordingly.

The panel labels (A, B...) are not aligned and make the figure difficult to interpret. These panel labels could also benefit from being bigger. I would again strongly advise the red/green colour scheme and encourage a colour-blind friendly scheme. The legend for the red and green colours is poorly placed and closer to the strain names than the colour label.

Authors 'answer:Done accordingly.

Figure 5

This figure is missing panel labels, while the wells seem to be labelled C and D.

I have concerns about how this diffusion was measured given that the agar plate is too small to measure the full zone of clearance.

Authors' answer: Done accordingly. See above answer.

Table 1

Each cell showing a measurement in the table should be the same size and appear uniformly in this table.

Authors' answer: Done accordingly.

As the authors did answer all the reviewers' remarks and queries, did perform additional experiments as required, and revised the manuscript accordingly, they hope that this revised version will be accepted for publication.

Sincerely yours,

Prof. Michel DRANCOUYRT, MD, PhD

Corresponding author.

VERSION 1

Editor recommendation and comments

<https://doi.org/10.1099/acmi.0.000471.v1.5>

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Saeed Ahmed; National University of Medical Sciences, Biological Sciences, PAKISTAN

Date report received: 21 December 2022

Recommendation: Major Revision

Comments: The work presented is clear and the arguments well formed. The reviewers have highlighted major concerns with the work presented. Please ensure that you address their comments.

Reviewer 2 recommendation and comments

<https://doi.org/10.1099/acmi.0.000471.v1.3>

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Anonymous.

Date report received: 19 December 2022

Recommendation: Major Revision

Comments: The manuscript by Achache et al describes experiments which tested the secretome of Enterococci on Mycobacterium tuberculosis complex mycobacteria. The methodology involved culture supernatants in agar diffusion assays and measurement of the zones of clearance. I was excited to read this manuscript as the topic is interesting and the research questions timely and important. However, I encourage the authors to develop their manuscript for clarity and robustness of data. Major Concerns: It is not clear to me that the experiments reported have been replicated as would be the standard in biology and a requirement for good statistics. Have these experiments been performed multiple independent times? Could the authors provide details? If replicates have been performed, could the data for these replicates be included. Overall, I found the manuscript confusing to read. Perhaps treating the Enterococcus and Mycobacterium separately and giving them separate methods sections might help? Also see comments below on Figure one. Many of the diffusion assays presented show zones of clearance that breach the agar plate and I don't believe these can be used to measure inhibition zones reliably. These agar plate might inform the authors, but the formal analysis should only relate to complete zones of clearance. Statistics: Can the authors confirm the number of independent replicates? Can the authors discuss their statistical approach? It is not clear to me what value a one-way ANOVA brings to this story, without multiple comparisons within the ANOVA. Further comments: Line 87 all strains were identified ... if they authors wish

to include this statement, can they also include the data or reference if this has already been published. Suggestion: the extensive list of strains might be better placed in a table than text. This might aid readability. Line 126 the abbreviation DPBS is explained here but has already been cited at an earlier line without explanation. Please define abbreviation at the first use. Line 137 could the authors please clarify why cell free culture supernatant was pH adjusted? Line 141 could the authors please indicate why 1/5 dilution was chosen? I find the descriptions of CFU/ mL and/or MacFarland standards equivalents difficult to follow when their use is interchangeable in the text. Could the authors please use both or just CFU / mL alone. Line 173 - 177 I find this sentence confusing. The text describes a similar inhibitory effect of CSURQ1712 and CSURP7988 but cites p values of 0.1 and 0.2. These p values do not indicate significant differences. Could the authors please correct this. I suspect there is either a typo here, or the p value doesn't relate to the inhibition zones shown. This section required clarification. Line 187 The protein values cited in the text should have standard deviations. Multiple mentions of standardisation of the well diffusion assay to the 1/5 dilution. The methods would benefit from detailing exactly what this standardisation is and how it's performed. Line 218 "negativity of negative controls" Rephrasing this to "absence of zones of clearance around the negative controls" or similar scientific language is recommended. Line 219 The inhibitory effect was proportional to the inoculum... but this contradicts line X which states an effect was only observed at Y. I understood the statement in line X to indicate the effect was an on/off type effect. Please clarify these statements for accuracy. Line 231 "at large" suggestion to remove "at large" Line 257 is "validation of manuscript" correct here? It is surprising that the protein concentration differs so little between the 1/8 dilution and the 1/10 dilution, however the zone of clearance effect is completely lost. Could the authors speculate or discuss this in the discussion? Figure 1 I commend the authors for including a schematic figure describing their methodology and I would strongly encourage the authors to improve this figure. I have the following suggestions: 1. remove all shadows from boxes and pictures 2. revise colour scheme from a red/green to a colour-blind friendly palette 3. slightly reduce the size of the agar plates at the bottom of the figure 4. use the exact same agar plate diagram and size for each plate - change the colour to indicate media and insert circles to indicate well. 5. Make all test tubes the same size and if you must have them at an angle, use the exact same angle for each test tube. 6. Make arrows uniform in size and colour 7. Make text uniform - don't present text in a coloured box or circle and remove coloured boxes. 8. The figure required further explanation in the legend - what are 1, 2 and 3 in the schematic referring to? Figure 2 1. Perhaps the deviation bars or data points could be slightly different colours or thicknesses to aid visualisation? Currently, it is difficult to see individual data points that sit on the error bars. Perhaps grey error bars would help? 2. Remove the shadow from behind the agar plate picture. Also, the blue box outline is unnecessary and could be removed. 3. Have the ANOVA tests been performed to test significance of individual strain combinations? Could the authors explain the logic of performing an ANOVA on this group of strains (S10, S14, S15, S16) without making individual comparisons. This can easily be done in Graphpad and would add value to these data. For example, I suspect S15 treated individually might be significantly different from S14 (if only slightly and probably not meaningfully) in the CSURQ1712 graph. 4. Each graph should be its own panel and therefore have an identifying letter. E.g. Figure 2 B-F. 5. Line 426 the first description of this strain being obtained from human stool should not be in the figure legend. Please remove this detail from the legend and document this in the methods. Figure 3 1. It is not advisable to have figure 3, panel a sub-panel a,... please relabel these sub panels (i, ii... etc) or make each plate its own panel. 2. Panel A (a) here an asymmetric diffusion pattern is shown. Ideally, this assay should be performed with either an agar plate large enough or a well small enough to allow a symmetric and entire diffusion pattern occur. Could the authors please explain why this hasn't been possible? I believe the 1/8 dilution shown in (c) is sufficient to measure the zone of clearance, but the authors have indicated they used the 1/5 which also doesn't show a full diffusion pattern. 3. B I believe this graph would benefit from larger data points and perhaps a grey error bar. 4. I have concerns about how the data for dilution 1/2 and 1/5 were recorded in light of the images presented in this figure. It appears only the 1/5 dilution has error bars, but I would expect error in these types of data. Please clarify. 5. C The legend indicates a 1/10 dilution that is not present on the graph. It is difficult to see which bars correspond to the patterns shown in the legend. Perhaps increasing the bar size would help. Are there again no error bars from these data? If not, please explain. 6. Please remove the blue boxes surrounding the figure. Figure 4 Remove all shadows from boxes The panel labels (A, B...) are not aligned and make the figure difficult to interpret. These panel labels could also benefit from being bigger. I would again strongly advise the red/green colour scheme and encourage a colour-blind friendly scheme. The legend for the red and green colours is poorly placed and closer to the strain names than the colour label. Figure 5 This figure is missing panel labels, while the wells seem to be labelled C and D. I have concerns about how this diffusion was measured given that the agar plate is too small to measure the full zone of clearance. Table 1 Each cell showing a measurement in the table should be the same size and appear uniformly in this table.

Please rate the manuscript for methodological rigour

Poor

Please rate the quality of the presentation and structure of the manuscript

Poor

To what extent are the conclusions supported by the data?

Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

No

Is there a potential financial or other conflict of interest between yourself and the author(s)?

No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Yes

Reviewer 1 recommendation and comments

<https://doi.org/10.1099/acmi.0.000471.v1.4>

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Anonymous.

Date report received: 03 October 2022

Recommendation: Minor Amendment

Comments: This short paper describes inhibition of growth of Mycobacteria with cell free supernate (CFS) of Enterococcus species. The methodology to demonstrate inhibition of growth of Mycobacterial strains is simple but effective. Growth of Mycobacteria is inhibited in the presence of CFS but not when sterile culture broth is used. The authors suggest that the Enterococcus strains are producing a bacteriocin which is inhibiting growth of Mycobacteria. However the factor that is responsible for the inhibition is not characterised at all. Some simple experiments such as testing the effect of heat treatment of the CFS and/or protease treatment of the CFS would go a long way to strengthening this manuscript. Some further controls could also be included. Are these effects specific for Mycobacteria or do you see similar effects with other bacteria? Presentation of the results could be improved in places. Labelling of some of the figures needs attention. Figure 2 - it is confusing to have individual figure parts labelled A and B and then parts of the figure A labelled A and B. Please use different letters here. Figure 3 M and S are explained in the figure legend but no M and S is on parts a, b or c of part A Figure 5 No A and B labels on the figure but A and B referred to in the legend. For the diffusion assay label wells M and S for consistency. Line 183 and throughout - replace the word fractions with dilutions The conclusion statement needs to be modified. Rather than the work showing that Enterococci are likely to be key players in the control of Mycobacteria in the gut a more realistic conclusion might be that the results of this study suggest that further investigations are warranted to see if enterococci play any role in vivo in the control of Mycobacteria in the gut.

Please rate the manuscript for methodological rigour

Very good

Please rate the quality of the presentation and structure of the manuscript

Good

To what extent are the conclusions supported by the data?

Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

No

Is there a potential financial or other conflict of interest between yourself and the author(s)?

No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Yes

SciScore report

<https://doi.org/10.1099/acmi.0.000471.v1.1>

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iThenticate report

<https://doi.org/10.1099/acmi.0.000471.v1.2>

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