Modulatory Effects of Caffeine and Pentoxifylline on Aromatic Antibiotics: A Role for Heterocomplex Formation

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Original article

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Abstract

Antimicrobial resistance is a major healthcare threat globally. Xanthines, including caffeine and pentoxifylline, are attractive candidates for drug repurposing, given their well-established safety and pharmacological profiles. This study aimed to analyze potential interactions between xanthines and aromatic antibiotics (i.e., tetracycline and ciprofloxacin), and their impact on antibacterial activity.

UV-vis spectroscopy, statistical-thermodynamical modeling, and isothermal titration calorimetry were used to quantitatively evaluate xanthine-antibiotic interactions. The antibacterial profiles of xanthines and xanthine-antibiotic mixtures towards important human pathogens *Staphylococcus aureus*, *Enterococcus faecium, Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* were examined.

Caffeine and pentoxifylline directly interact with ciprofloxacin and tetracycline, with neighborhood association constant values of 15.8–45.6 M^{-1} and enthalpy change values up to -4 kJ M^{-1}. Caffeine showed antibacterial activity (minimum inhibitory concentration, 4–8 mg/mL) toward Gram-negative bacteria. Caffeine enhanced the antibacterial activity of the tested antibiotics in most pathogens tested. Antagonistic effects of caffeine were observed only with ciprofloxacin toward Gram-positive pathogens.

Xanthines interact with aromatic antibiotics at the molecular and *in vitro* antibacterial activity level. Given considerable exposure to caffeine and pentoxifylline, these interactions are relevant for the effectiveness of antibacterial pharmacotherapy, and may help to identify optimal treatment regimens in the era of multidrug resistance.

Keywords:

antibacterial agent; caffeine; ciprofloxacin; drug repositioning; tetracycline

1. Introduction

Increasing antimicrobial resistance (AMR) is a major healthcare threat globally. According to recent estimates in the European Union, AMR contributes to more than 670,000 infections and 33,000 deaths annually [1]. Among drug-resistant bacteria, third-generation cephalosporin-resistant *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* represent the most frequent and deadly causes of infection [1]. The burden of infections due to AMR has increased since 2007, and currently exceeds that of tuberculosis, influenza, and HIV infections combined [1]. In the United States, the extent of AMR is similar, with nearly three million antibiotic-resistant infections and 35,000 deaths reported each year [2].

Most infections with antibiotic-resistant bacteria are associated with healthcare institutions [1]. Therefore, the most vulnerable individuals hospitalized due to, e.g., chronic conditions, anticancer treatment, or organ transplant, are at the highest risk of acquiring difficult to treat infections caused by antibiotic-resistant bacteria [2]. The ongoing COVID-19 pandemic, as well as the past H1N1 influenza outbreak, show that, despite preventive antibiotic treatment [3,4], hospitalized patients are prone to developing secondary bacterial infections, which may significantly worsen their prognosis [4–6]. Indeed, bacterial co-infections were reported as a negative prognostic factor in the 2009 influenza A H1N1 pandemic, during which one in four patients suffered secondary bacterial infections [7]. This further emphasizes the urgent need to provide the healthcare system with a wide range of effective broad-spectrum antimicrobials.

The pipeline for new antimicrobials, especially those to treat multidrug-resistant bacteria, is narrow [8]. Remarkably, between the 1960s and 2000s, no novel class of antibiotics entered the market [9]. Recent advances in drug discovery, especially those focusing on natural products rather than synthetic compounds, combined with a growing body of initiatives aimed at promoting antimicrobial research, led to the discovery of promising new candidates with

diverse modes of action [10]. However, derivatives of well-established antibiotic classes prevailed in the 2018 clinical pipeline of antibacterial agents, and most candidates showed only a limited level of innovation [11]. The analysis highlighted a particular demand for new compounds with no pre-existing cross-resistance to treat infections caused by Gram-negative bacteria [11].

Apart from attempts to develop new chemical entities, which have proven limited success to date, a proposed solution is to repurpose existing drugs (including antibiotics and compounds with other indications) for treating bacterial infections. Such drug repositioning has been successfully applied for thalidomide, which was initially used for morning sickness in the 1950s and later approved for multiple myeloma treatment in 2006 [12], and thioguanine, originally used for leukemia treatment and then as a rescue immunosuppressant in inflammatory bowel disease [13]. Reviving old antibiotics has also proven effective in treating infectious diseases. Indeed, several compounds registered five or six decades ago (but abandoned due to their unfavorable safety profile or limited efficacy) were recently redeveloped and applied in clinical practice [14]. For example, colistin, which fell out of favor in the 1970s, is increasingly used as a last-line therapy in critically-ill patients [15] as it retains significant *in vitro* activity against key Gram-negative pathogens [16]. Thus, uncovering cryptic antimicrobial activities of drugs with other indications is a cost- and time-effective alternative to *de novo* antibiotic discovery and development [17].

Existing drugs could also be used as adjuvants of existing antibiotics, for example, to overcome drug resistance or to reactivate the target for the antibiotic. As evidence, amphotericin C was approved to treat visceral leishmaniasis [18] and doxycycline for chemoprophylaxis and malaria treatment [19]. More recently, the anthelmintic niclosamide was reported as a promising antibacterial agent [20]. The co-administration of β-lactam antibiotics with β-lactamase inhibitors, such as clavulanic acid, is another successful

synergistic strategy [21]. Furthermore, the antidepressant sertraline was recently evaluated in late clinical trials as an adjuvant for antifungal treatment, and the antiprotozoal pentamidine proved effective in sensitizing Gram-negative bacteria to antibiotics and overcoming colistin resistance [22].

Xanthines, including caffeine and pentoxifylline, have well-established safety and pharmacological profiles, making them attractive candidates for drug repurposing. Caffeine (a component of popular beverages, foods, dietary supplements, and drugs) is the most abundantly consumed pharmacologically-active substance worldwide. The estimated mean daily caffeine intake is 165 mg, and approximately 105 mg is related to drinking coffee [23]. The average annual consumption of caffeine-containing beverages (mostly coffee and carbonated soft drinks) is 348 L per person in North America and 200 L per person in Europe [24]. As a drug, it is used to treat apnea of prematurity by reducing bronchopulmonary dysplasia [25]. It is also used in combination with analgesics as a pain reliever [26], and in the treatment of hypersomnia [27]. Meanwhile, pentoxifylline has anti-inflammatory and rheological properties, and is used to treat vascular diseases, including intermittent claudication, venous leg ulcers [28], and heart failure [29,30].

Caffeine and pentoxifylline have also been shown to diminish the activity of a broad range of small aromatic compounds (e.g., model mutagens [31], anticancer drugs [32,33], neurotoxins [34], or foodborne carcinogens [35,36]) through their sequestration in transient non-covalent complexes and subsequent lowering of bioavailability. Although caffeine and pentoxifylline are considered safe, even at relatively high doses (up to 400 mg/day for caffeine and a typical dose of 1200 mg/day for pentoxifylline) [29,37], and their pharmacological profile is well-established, their antibacterial properties and modulatory effects on clinically-used antibiotics remain unclear.

This study aimed to investigate whether two aromatic-containing antibiotics (tetracycline and ciprofloxacin) interact non-covalently with caffeine and pentoxifylline. As such sequestration might affect biological activity of compounds captured in the complexes, we also evaluated the impact of xanthines on the *in vitro* activity of antibiotics toward a panel of seven Gram-positive and Gram-negative human pathogens.

3. Results

3.1 Antibiotic-xanthine interactions: spectrophotometric and statistical modelling analysis

To investigate the direct interactions between antibiotics and xanthines in aqueous solutions, we performed UV-vis spectroscopic titrations of the antibiotic solution with caffeine or pentoxifylline. All absorption spectra were analyzed at wavelengths >320 nm, for which xanthine absorption is negligible. Absorptions spectra normalized to the concentration of the absorbing ligand (antibiotic) for antibiotic-caffeine titrations are shown in **Figure 2**.

In the analyzed range of concentrations, tetracycline and ciprofloxacin are present as monomers (no dimerization or higher-order aggregation was recorded). Observed spectral changes (represented most prominently by the hypochromic shift) can therefore be attributed to a new component that emerged upon the addition of xanthine solution (i.e., the antibiotic-xanthine complex). The presence of an isosbestic point at 372 nm for tetracycline-caffeine mixtures (and at 339.5 nm for ciprofloxacin-caffeine mixtures) indicates that only two absorbing entities are present in the mixture (i.e., antibiotic monomer and antibiotic heterocomplex with xanthine). Corresponding spectral changes were observed for tetracycline-pentoxifylline and ciprofloxacin-pentoxifylline mixtures, thus demonstrating ligand hetero-aggregation.

Once theoretical spectra of the xanthine-antibiotic complex were calculated, based on the law of spectra additivity, it was possible to estimate the molar fraction of free and complexed antibiotic in each mixture during spectrophotometric titration. Examples of such two-component spectra decomposition for selected ciprofloxacin-caffeine and tetracycline-caffeine mixtures are shown in **Figure 3**.

To quantitively analyze the interactions, and calculate interaction constants, the statistical-thermodynamical model described by Zdunek et al. [38] was applied. The model assumes infinite aggregation of one type of ligand (xanthine), and limited aggregation of the heterocomplex formation of the other type of ligand (antibiotic). Experimental and theoretical concentrations of all components present in a mixture of tetracycline and caffeine are given in **Table 1**. Values of the neighborhood association constant K_{AC} for antibiotic-xanthine interactions, calculated with the model, were in the range of 10 M^{-1} (**Table 2**). The fit of the model to the experimental data for ciprofloxacin-caffeine and tetracycline-caffeine interactions is shown in **Figure 4**.

Upon the addition of caffeine, concentrations of antibiotics present in a free form markedly decreased. A ~250–300-fold excess of caffeine molecules over antibiotic molecules was needed to sequester half of the antibiotic molecules in heteroaggregates.

3.2 Thermal effects of antibiotic-xanthine interactions

To further characterize the interactions of tetracycline and ciprofloxacin with the xanthines, ITC measurements were performed (**Figure 5**). Peaks from thermograms associated with titrations of antibiotic (or buffer) with xanthine (or buffer) were integrated to estimate the heat of interaction. The net heat effect of antibiotic-xanthine was calculated as the heat effect of antibiotic-xanthine titration corrected for the heat of dilution of the antibiotic and the xanthine (recorded in control buffer titrations). To estimate the enthalpy change (ΔH) of antibiotic-

xanthine hetero-aggregation, the net heat of interaction per mole of titrant added was extrapolated for antibiotic concentration → zero (as shown in **Table 2**).

3.3 Antibacterial activity of caffeine and pentoxifylline

To evaluate the modulatory effects of the xanthines on selected antibiotics, the antibacterial activity of caffeine and pentoxifylline alone was first determined. MIC values for caffeine and pentoxifylline were evaluated for a series of Gram-positive and Gram-negative pathogens using the broth microdilution procedure (**Table 3**). Caffeine was not active against *S. aureus*, *E. faecium*, and *P. aeruginosa* up to 16 mg/mL, and demonstrated limited antibacterial activity against Gram-negative *E. coli*, *A. baumannii*, *K. pneumoniae*, and *E. cloacae* with MIC values of 4–8 mg/mL. Pentoxifylline showed no antibacterial activity at concentrations up to 16 mg/mL.

3.4 Modulation of antibiotic activity by caffeine and pentoxifylline

To investigate the possible impact of caffeine and pentoxifylline on the antibacterial activity of antibiotics, the inhibitory effects of xanthine-antibiotic mixtures on microbial growth were investigated for a broad range of concentrations using a checkerboard titration technique, and the corresponding MIC values were determined for each mixture.

Figure 6 shows an overview of the concentration-dependent effects of the xanthines on the antibacterial activity of ciprofloxacin and tetracycline, expressed as isobolograms obtained for all analyzed pathogens and antibiotic-xanthine combinations. The modulatory effect of the xanthines is presented as the change in inhibitory concentration of an antibiotic for increasing xanthine concentration. Full-size isobolograms for all pathogens and each antibiotic-xanthine combination are given in **Supplementary Figures S1–7**.

184 The profile of antibiotic activity modulation is distinct for caffeine and pentoxifylline.
185 Caffeine potentiates the antibacterial activity of both ciprofloxacin and tetracycline in all
186 Gram-negative pathogens evaluated with mostly additive effects, as determined by FICI
187 values (synergy was observed only for caffeine toward ciprofloxacin in *K. pneumoniae*).
188 However, in Gram-positive bacteria, only tetracycline antibacterial activity increased upon
189 caffeine addition, while ciprofloxacin activity was inhibited. Meanwhile, the effects of
190 pentoxifylline on the antibacterial potential of ciprofloxacin and tetracycline were less
191 pronounced than those of caffeine. Meaningful potentiation of antibiotic activity by
192 pentoxifylline was observed only for *A. baumannii*. Slight inhibitory effects of pentoxifylline
193 were reported for ciprofloxacin in *S. aureus* and *E. cloacae*, as well as for tetracycline in *P.*
194 *aeruginosa*. The MIC values for antibiotics alone and in combination with the highest sub-
195 inhibitory concentration of caffeine and pentoxifylline are listed in **Table 4**.

196 **4. Discussion**

197 We showed that the xanthines caffeine and pentoxifylline are capable of forming non-
198 covalent heterocomplexes with two aromatic antibiotics, tetracycline and ciprofloxacin. This
199 heterocomplexation leads to a substantial decrease in the free antibiotic concentration when
200 the xanthine is at ~100-fold or more excess compared to the antibiotic. We also proved that
201 caffeine and pentoxifylline influence the antibacterial properties of tetracycline and
202 ciprofloxacin; i.e., they potentiate or reduce their activity, depending on the bacterial species.

203 Being the vital component of the broadly available beverages with psychostimulatory
204 properties (e.g., coffee or tea), caffeine is consumed on a daily basis. The single doses of
205 xanthines administered as a part of everyday diet (caffeine) or in pharmacotherapy
206 (pentoxifylline) reach levels of up to 100–400 mg (for caffeine-containing beverages
207 equivalent to up to 2–7 mg/mL) [40], and peak plasma concentrations of 1–10 mg/L [41,42].

208 Yet, knowledge on the antibacterial activity of xanthines and their possible impact on
209 antibiotic therapy is limited. Previous studies showed that caffeine at concentrations of
210 2 mg/mL inhibited the growth of Enterobacteria, particularly *Serratia marcescens* and *E.*
211 *cloacae* [43]. Likewise, the calculated IC_{50} value of caffeine against *Salmonella enterica* was
212 2.6 mg/mL, demonstrating that caffeine concentration in coffee extracts is sufficient to inhibit
213 growth of this pathogen [43]. Similarly, caffeine at concentrations of ≥5 mg/mL inhibited the
214 growth of the pathogenic *E. coli* O157 strain [44]. Finally, caffeine at a concentration of
215 4 mg/mL prevented growth of a wild-type *E. coli* strain, whereas knock-out mutants
216 (particularly those lacking functions related to DNA repair) had increased caffeine sensitivity
217 with growth inhibition present at 2.5 mg/mL or lower [45]. In our study, caffeine showed
218 MIC values of 4–8 mg/mL against *E. coli*, *E. cloacae*, *K. pneumoniae*, and *A. baumannii*.
219 Meanwhile, pentoxifylline was previously shown to lack antibacterial activity against *E. coli*
220 with MIC values >1 mg/mL [46], and our study confirmed this finding, with no inhibitory
221 effects observed against a panel of Gram-positive and Gram-negative pathogens at
222 concentrations up to 16 mg/mL.

223 Prior studies showed that extracts of roasted coffee exhibited antibacterial activity against
224 human pathogens like *S. aureus* or *Streptococcus mutans*; however, the intrinsic antibacterial
225 activity of caffeine was weak [47]. Yet, the addition of alpha-dicarbonyl compounds to
226 caffeine synergistically increased its antibacterial properties [47]. Indeed, Kang et al.
227 described the synergistic effects of caffeine on the aminoglycoside antibiotics, kanamycin and
228 neomycin, in *E. coli* [45]. The authors concluded that aminoglycosides generated damage to
229 bacterial DNA bases, and the synergistic action of caffeine was attributed to slowing, and
230 ultimately blocking, DNA replication. Unexpectedly, the opposite effects were observed with
231 the fluoroquinolone, ciprofloxacin: caffeine suppressed its antimicrobial effects toward *E. coli*
232 and *Bacillus anthracis*. No possible explanation of such caffeine action was given [45]. As an

aromatic molecule, caffeine may directly interact with ciprofloxacin, thus decreasing its antibacterial activity, which could explain its contradictory effects on ciprofloxacin compared to kanamycin and neomycin. A similar pattern of caffeine action was described for two model nitrogen mustard mutagens: caffeine prevented cytotoxicity of an aromatic, heterocyclic quinacrine mustard, whereas no modulatory effect of caffeine was shown for the aliphatic mechlorethamine [48].

In our study, using UV-Vis spectroscopy combined with statistical-thermodynamical modeling and ITC, we provided evidence for non-covalent complex formation between xanthines (caffeine and pentoxifylline) and two aromatic antibiotics, ciprofloxacin and tetracycline. The neighborhood association constants (K_{AC}) and enthalpy changes (ΔH) were lower than those determined for xanthines and model mutagens, heterocyclic foodborne carcinogens, or anticancer drugs (K_{AC} in 10 M^{-1} range; ΔH values 20–30 kJ M^{-1}) [31,36,49]. This may be due to the restricted availability of aromatic rings within the antibiotic molecules when compared with classic aromatic ligands, which are characterized by dominating conjugated planar aromatic and/or heterocyclic structures. Nevertheless, the interception of antibiotic molecules was effective for a xanthine:antibiotic molar ratio of $\geq$100.

The concentrations of xanthines used in our checkerboard experiments were typically at least 1,000-fold higher than the concentrations of antibiotics. Under such conditions, heterocomplexation at the molecular level should be relevant, with at least 75% of the antibiotic molecules sequestered, according to xanthine-antibiotic association constants (calculated based on UV-Vis spectroscopy measurements). Such sequestration could result in the reduction of antibiotic activity. However, our *in vitro* analysis of antibacterial activity showed the opposite: for most studied pathogens, caffeine enhanced rather than diminished effects of both tetracycline and ciprofloxacin. Inhibitory effects of xanthines were reported for

ciprofloxacin only, and were restricted to Gram-positive pathogens (*S. aureus* and *E. faecium*).

The observed reduction of ciprofloxacin activity in the presence of xanthines is in line with reports by Kang et al., who showed antagonistic activity of caffeine toward ciprofloxacin in *E. coli* [45], and by Masadeh et al., who described the inhibitory effects of pentoxifylline toward ciprofloxacin in a panel of Gram-positive and Gram-negative pathogens, including *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* [50]. Our findings indicate that mixed stacking aggregate formation between xanthines and antibiotics only reduce antibiotic activity if the mechanism of action of the affected antibiotic depends on DNA binding (as is the case for ciprofloxacin but not tetracycline). This is in agreement with the inhibitory effects of xanthines against model mutagens, anticancer drugs, or food-derived carcinogens described previously, as all of these compounds exert their biological action at least in part through non-covalent (intercalation) or covalent (adduct formation) DNA binding [31,32,36].

In contrast to Gram-positive bacteria, caffeine potentiated the antibacterial activity of both tetracycline and ciprofloxacin in Gram-negative pathogens. It seems plausible that the attenuation of ciprofloxacin by caffeine observed in Gram-positive bacteria, even if present in Gram-negative pathogens, is surpassed by another mechanism of caffeine action, which is specific for Gram-negative bacteria. It could be speculated that heterocomplexation of an antibiotic with xanthine increases antibiotic solubility and/or membrane permeability (as xanthines penetrate into membranes easily) [51], which could lead to increased antibacterial response. However, heterocomplexation of aromatic antibiotics was reported for both caffeine and pentoxifylline, and the strength of interaction was comparable for both xanthines, whereas prominent potentiation of antibacterial activity of ciprofloxacin and tetracycline was only observed for caffeine. Therefore, the potentiating effect of caffeine on antibiotic action is most likely dependent on the antibacterial activity of caffeine itself rather than its interplay

with antibiotics, as the observed modulatory effects were, at best, additive. Indeed, the potentiation of tetracycline and ciprofloxacin activity was only observed in *P. aeruginosa* caffeine concentrations of >1 mg/mL, although the bacteria were resistant to caffeine alone at concentrations up to 16 mg/mL. In contrast, pentoxifylline lacked antibacterial activity at concentrations up to 16 mg/mL, which likely explains its lack of its influence on antibiotic activity for most studied organisms.

Overall, the activity of caffeine, both alone and in combination with ciprofloxacin and tetracycline, towards Gram-negative pathogens appears promising. The elucidation of its mechanism of action is appealing, especially considering there is a shortage of effective pharmacological treatment options particularly for Gram-negative-associated infections [52]. In addition, further evaluation of the possible modulatory effects of xanthines on quinolones is warranted, as quinolones represent one of the most intensively explored class of antibiotics, and several new fourth-generation fluoroquinolones have recently entered the market or are in late phase drug development [53].

In conclusion, xanthines such as caffeine and pentoxifylline interact with aromatic antibiotics at the molecular and *in vitro* antibacterial activity level. Given the considerable exposure to caffeine and pentoxifylline, these interactions are relevant for the effectiveness of antibacterial pharmacotherapy. According to our findings, caffeine potentiates the antibacterial activity of selected antibiotics in Gram-negative pathogens. Therefore, as a compound with a well-established safety profile, caffeine may be worth repurposing for optimal treatment regimens in the era of multidrug resistance.

Materials and Methods

Materials

All chemicals, including xanthines CAF (1,2,3-trimethylxanthine) and PTX (3,7-dimethyl-1-(5-oxohexyl)xanthine), and antibiotics tetracycline hydrochloride and ciprofloxacin hydrochloride, were purchased from Sigma-Aldrich (St. Louis, USA). Structures of the above mentioned compounds are shown in **Figure 1**. A 0.1 M sodium phosphate buffer (pH 6.8), containing Na_2HPO_4 and NaH_2PO_4 (purchased from Avantor Performance Materials, Gliwice, Poland) was used in UV-Vis spectroscopy and isothermal titration calorimetry (ITC) measurements. The buffer was filtered through a 0.2 μm pore Millex Millipore filter and degassed before experiments. CAF and PTX stock solutions were prepared by dissolving their weight amounts in a sodium phosphate buffer (pH 6.8) or deionized water at concentrations of approximately 10^{-1} M, and stored at 4 °C. Antibiotic stock solutions were prepared by dissolving their weight amounts in a sodium phosphate buffer (pH 6.8) or deionized water immediately before the experiments. The concentrations of antibiotic solutions were assessed by UV-Vis spectroscopy using determined molar absorption coefficients (ε_λ), ε_{358} = 14 900 M^{-1} cm^{-1} and ε_{322}= 12 380 M^{-1} cm^{-1} for tetracycline and ciprofloxacin, respectively.

UV-Vis spectroscopy measurements

The 2 mL aliquots containing the antibiotic were placed in a quartz cuvette (1 cm light path) and titrated with 5-150 μL of CAF or PTX solution. The absorption spectra of each mixture were measured using a Beckman DU 650 or a Jena Analytic Specord 50 Plus spectrophotometer (equipped with a water bath or a Peltier thermostat, respectively) at 0.5 nm intervals, and stored in a digital form. All measurements were done in a 0.1 M sodium phosphate buffer (pH 6.8) at 25 °C (±0.1 °C). Absorption spectra are given in the form of molar absorption coefficient (ε_λ, M^{-1} cm^{-1}).

Quantitative analysis of antibiotic-xanthine interactions

To reflect changes only in a structure of antibiotics, all the UV-Vis spectra were analyzed in the range of wavelengths above 320 nm, for which light absorption of xanthines is negligible. The theoretical spectrum of antibiotic-xanthine complex was calculated by extrapolation of molar extinction coefficient (for each wavelength) to $C_{TA}/C_{TC} \rightarrow 0$ (where C_{TA} and C_{TC} are the total concentrations of the antibiotic and the xanthine, respectively). The spectra of mixtures containing the antibiotic and the xanthine were decomposed into a weighted sum of components by non-linear regression analysis. This allowed estimation of the concentration of free antibiotic and antibiotic complexed with xanthine for all mixtures analyzed spectroscopically.

Calculations with statistical-thermodynamical model

Mixed association constant values ($\boldsymbol{K_{AC}}$) for antibiotic-CAF and antibiotic-PTX complexation along with the concentrations of all mixture components were determined with statistical thermodynamics of mixed aggregation based on the Zdunek et al. model [38]. The model describes interactions in two-component ligand-xanthine mixtures, where one component, *C* (xanthine) is capable of both homo- and heteroaggregation, and the other, *A* (in this analysis the antibiotic), is only capable of heteroaggregation with xanthine. To determine neighborhood $\boldsymbol{K_{AC}}$ (hetero-neighborhood) and $\boldsymbol{K_{CC}}$ (homo-neighborhood) equilibrium constants with the model, a weight function for each oligomer needs to be calculated. $\boldsymbol{K_{CC}}$ equilibrium constant values were determined using constant values of CAF homoaggregation reported previously [54]. The equations used to calculate concentrations of each component in every form in the antibiotic-xanthine mixture are listed below:

$$C_{TA} = C_A[\frac{1 - C_C(K_{CC} - K_{AC})}{1 - C_C(K_{CC} + K_{AC}^2 C_A)}]^2 \quad (1)$$

$$C_{TC} = C_A[\frac{1 + K_{AC} C_A}{1 - C_C(K_{CC} + K_{AC}^2 C_A)}]^2 \quad (2)$$

$$C_{AC} = 2K_{AC}C_A C_C (1 + K_{AC}C_A)\frac{1 - C_C(K_{CC} - K_{AC})}{[1 - C_C(K_{CC} + K_{AC}^2 C_A)]^2} \quad (3)$$

$$C_{CC} = K_{CC}[\frac{C_C(1 + K_{AC}C_A)}{1 - C_C(K_{CC} + K_{AC}^2 C_A)}]^2 \quad (4)$$

where C_{TA} and C_{TC} are total concentrations of antibiotic and xanthine, respectively, C_A and C_C are concentrations of free antibiotic and xanthine molecules, respectively, C_{AC} is the concentration of antibiotic-xanthine heteroneighborhoods, while C_{CC} is the concentration of xanthine homoneighborhoods. The calculations were performed using SigmaPlot 11 (Systat Software, Inc.), Microsoft Office Excel (Microsoft), and Mathcad Prime 6 (Parametric Technology Corporation) software.

Isothermal titration calorimetry (ITC)

All ITC experiments were done in deionized water at 25 °C using an AutoITC isothermal titration calorimeter (MicroCal, Malvern Panalytical Inc., MA, USA) with 1.4491 mL of sample and reference cells. The cell containing deionized water was used as the reference. All solutions were degassed before titrations. The experiment consisted of injecting 10.02 µL (20 injections, 2 µL for the first injection only) of buffer solution of the appropriate antibiotic (1 mM) into the reaction cell initially containing xanthine (15 mM). The titrant was injected in 5 minute intervals to ensure that the titration peak returns to the baseline prior to the next injection. Each injection lasted 20 s. Background titrations were run using identical titrant with the pure buffer solution placed in the sample cell. To account for the heat of dilution, the result of a background titration was subtracted from each experimental titration. To achieve a homogeneous mixing in the cell, the stirrer speed was established at 300 rpm. To remove the effect of titrant diffusion across the syringe tip during the equilibration process, an initial 2 µL injection was removed from each data set before analysis. The data, specifically the heat normalized per mole of injectant, were processed with Origin 7 software from MicroCal.

374 *Antibacterial assays*

375 Cation-adjusted Mueller-Hinton broth (CA-MHB) for antimicrobial susceptibility testing by
376 broth microdilution method was purchased from Beckton Dickinson (BD Difco™ BBL™).
377 Following bacterial strains were used in the study: Gram-positive *Staphylococcus aureus*
378 ATCC 25923 and *Enterococcus faecalis* ATCC 19433; Gram-negative *Pseudomonas*
379 *aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC
380 19606, *Klebsiella pneumoniae* ATCC 700603, and *Enterobacter cloacae* ATCC 700323.

381 Antimicrobial potential of tested agents was determined by broth microdilutions method
382 according to CLSI guidelines [55]. Minimal Inhibitory Concentration (MIC) of antibiotics and
383 xanthines was defined as their lowest concentration at which no visible bacterial growth was
384 observed after 24 hour stationary incubation at 37 °C. The following gradients of compounds
385 concentration, obtained by serial 2-fold dilutions of medium, were applied: from 128 to
386 0.015625 µg/mL for antibiotics, and from 16 to 1 mg/mL for xanthines. From thus prepared
387 solutions, 100 µL aliquots were transferred into 96-well plates. Next, wells were inoculated
388 with 10 µL aliquots of bacterial suspension containing approximately 1×10^7 CFU/mL.
389 obtained from liquid cultures in CA-MHB medium (6 hours, 37 °C, 150 rpm) diluted in fresh
390 medium. Checkerboard titration method was used to evaluate interactions of antibiotics and
391 xanthines by applying two-dimensional combination of their concentration gradients. Results
392 were analysed with two following methods: calculation of Fractional Inhibitory Concentration
393 Index (FICI) for each tested combination (according to Odds) [39], and isobologram analysis
394 [42]. FICI values were used to characterize following types of interaction: i) synergistic for
395 FICI $\leq$ 0.5, ii) additive for FICI between 0.5 and 2.0, iii) antagonistic for FICI $\geq$ 4.0 [56]. All
396 microbiological experiments were done at least as biological triplicates.

397

398

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Author contribution statement

Conceptualization, AW, GG, JP; data curation, AW; investigation, AW, MKM, GG, AB, AF, DW; data analysis and interpretation, AW, GG, JP; methodology, AW, MKM, DW, JP; project administration, AW; supervision, AW, JP; visualization, AW; funding acquisition, AW; writing – original draft, AW; writing – review & editing, AW, MKM, GG, AB, AF, DW, JP. All authors have read and agreed to the published version of the manuscript.

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559 **Table 1.** Concentrations of all components present in tetracycline-caffeine mixtures during spectrophotometric titration (experimental and

560 calculated with Zdunek et al. model [38]).

Sample #	C_{TC}, mM	C_{TA}, µM	C_C, mM	C_{CC}, mM	C_{AC}, µM	C'_A, µM	C_A, µM	X'_{BA}, µM	X_{BA}, µM	K_{AC}, M^{-1}
0	0.00	43.18	0.00	0.000	0.00	43.18	43.18	0.00	0.00	-
1	0.30	43.07	0.29	0.001	1.14	41.58	41.94	1.50	1.13	60.50
2	0.59	42.97	0.58	0.004	2.24	40.25	40.76	2.72	2.21	56.75
3	1.18	42.76	1.14	0.015	4.29	38.35	38.58	4.41	4.18	48.26
4	1.76	42.55	1.68	0.033	6.18	38.00	36.59	4.55	5.96	33.81
5	2.33	42.34	2.21	0.058	7.93	35.26	34.78	7.08	7.56	42.24
6	2.90	42.14	2.71	0.089	9.55	34.29	33.13	7.85	9.01	38.70
7	4.02	41.74	3.68	0.167	12.45	31.33	30.22	10.41	11.52	40.10
8	5.12	41.34	4.59	0.265	14.95	28.91	27.74	12.43	13.60	40.41
9	7.78	40.38	6.64	0.583	19.92	24.72	22.92	15.66	17.46	38.71
10	10.32	39.47	8.42	0.982	23.54	19.44	19.44	20.03	20.03	45.57
11	15.06	37.76	11.41	1.942	28.24	12.59	14.80	25.17	22.96	55.84

561 C_{TC}, total caffeine concentration; C_{TA}, total tetracycline concentration; C_C, caffeine monomer concentration; C_{CC}, caffeine homoaggregate neighborhood concentration; C_{AC},
562 tetracycline-caffeine heteroaggregates neighborhood concentration; C'_A, tetracycline monomer concentration (determined spectrophotometrically); C_A, tetracycline monomer

concentration; X'_{BA}, tetracycline in heteroaggregates with caffeine concentration (determined spectrophotometrically); X_{BA}, tetracycline in heteroaggregates with caffeine concentration; K_{AC}, tetracycline-caffeine neighborhood association constant. Mean K_{AC} ± standard error = 45.6 M^{-1} ± 2.5 M^{-1}.

565 **Table 2.** Determined thermodynamical parameters of antibiotic-xanthine complex formation

Complex	K_{AC} **(SE), M^{-1}**	ΔH **(SE), kJ × mol^{-1}**
Tetracycline-caffeine	45.6 (2.5)	-3.17 (0.14)
Tetracycline-pentoxifylline	15.8 (0.6)	-4.00 (0.06)
Ciprofloxacin-caffeine	24.7 (0.9)	-1.44 (0.07)
Ciprofloxacin-pentoxifylline	18.4 (1.0)	-2.01 (0.06)

566 K_{AC}, neighborhood association constant; SE, standard error; ΔH, enthalpy change

567

568

569 **Table 3.** Antibacterial activity of xanthines: caffeine and pentoxifylline against selected

570 Gram-positive and Gram-negative pathogens

Pathogen	MIC (mg/mL)	
	Caffeine	**Pentoxifylline**
Gram-positive		
Staphylococcus aureus ATCC 25923	> 16	> 16
Enterococcus faecium ATCC 19433	> 16	> 16
Gram-negative		
Pseudomonas aeruginosa ATCC 27853	> 16	> 16
Escherichia coli ATCC 25922	4	> 16
Acinetobacter baumannii ATCC 19606	4	> 16
Klebsiella pneumoniae ATCC 700603	8	> 16
Enterobacter cloacae ATCC 700323	8	> 16

571 MIC, minimal inhibitory concentration

572

573 **Table 4.** The influence of xanthines on antimicrobial activity of selected antibiotics

	MIC_A	$MIC_{A+caffeine}$	$MIC_{A+pentoxifylline}$
		[µg/mL]	
***Staphylococcus aureus* ATCC 25923**			
Ciprofloxacin	0.5-1	8	1
Tetracycline	1	0.5	1
***Enterococcus faecium* ATCC 19433**			
Ciprofloxacin	2	8	2
Tetracycline	2	0.25	1
***Pseudomonas aeruginosa* ATCC 27853**			
Ciprofloxacin	0.5	0.25	0.25
Tetracycline	64	32	128
***Escherichia coli* ATCC 25922**			
Ciprofloxacin	0.0156	0.078	0.0156
Tetracycline	2	1	2
***Acinetobacter baumannii* ATCC 19606**			
Ciprofloxacin	2	0.5	0.25
Tetracycline	4	1	1
***Klebsiella pneumoniae* ATCC 700603**			
Ciprofloxacin	0.5	0.125	0.5
Tetracycline	32	8	32
***Enterobacter cloacae* ATCC 700323**			
Ciprofloxacin	0.03125	0.03125	0.0625
Tetracycline	4	2	4

574 MIC, minimal inhibitory concentration; A, antibiotic tested alone; A+caffeine, antibiotic tested with caffeine at
575 the highest sub-inhibitory concentration (MIC specified in **Table 3**); A+pentoxifylline, antibiotic tested with
576 pentoxifylline at the highest sub-inhibitory concentration tested (MIC specified in **Table 3**).
577

Figure captions

Figure 1. Chemical structures of studied compounds. Top, xanthines: caffeine and pentoxifylline; bottom, antibiotics: ciprofloxacin and tetracycline.

Figure 2. Spectrophotometric titrations of antibiotics with caffeine. Panel a, absorption spectra (in the form of molar extinction coefficient ε_M) of ciprofloxacin (initial concentration, 60.3 μM) titrated with caffeine (concentration range, 0.3-40.6 mM); panel b, absorption spectra (in the form of molar extinction coefficient ε_M) of tetracycline (initial concentration, 43.2 μM) titrated with caffeine (concentration range, 0.3-21.5 mM); Spectra of an antibiotic in its free form are marked in bold. Theoretical spectra of an antibiotic-caffeine complex are marked as dashed lines.

Figure 3. Examples of two-component decomposition of antibiotic-caffeine spectra. Panel a, decomposition of spectrum for ciprofloxacin (55.1 μM) and caffeine (10.6 mM), molar fraction of free ciprofloxacin = 0.64; panel b, decomposition of spectrum for tetracycline (40.4 μM) and caffeine (7.78 mM), molar fraction of free tetracycline = 0.61; Solid lines represent experimental spectra, dotted lines – sum of calculated decomposed spectra, dashed lines – calculated spectra of free antibiotics, dashed-dotted lines – calculated spectra of antibiotic complexed with caffeine. Top panels show residuals between experimental and sum of calculated decomposed spectra.

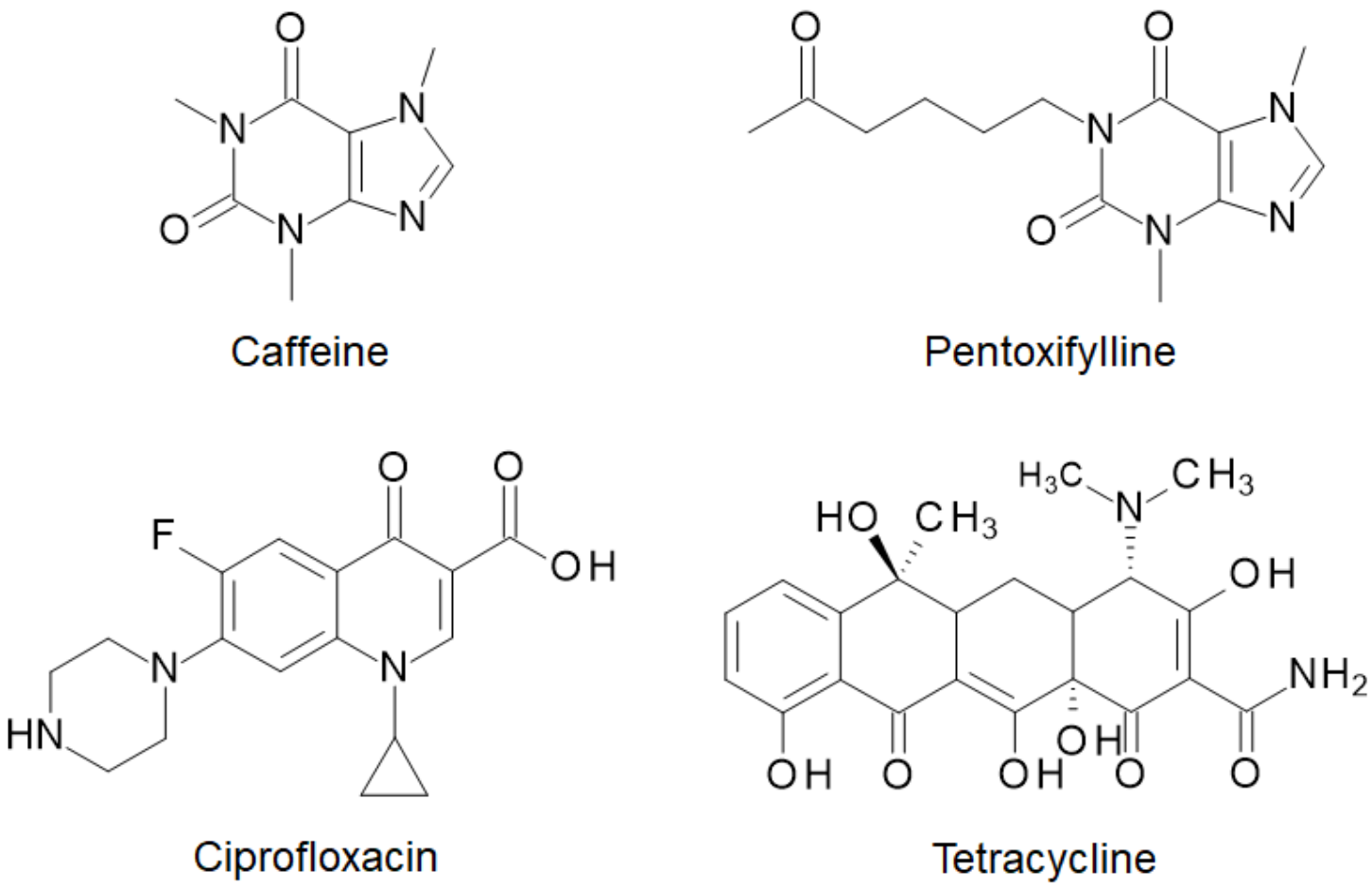
Figure 4. Comparison of experimental and theoretical concentrations in antibiotic-xanthine mixtures analyzed spectrophotometrically. Panel a, ciprofloxacin-caffeine interactions; panel b, tetracycline-caffeine interactions. Points represent concentrations of antibiotic in a free form (circles) and in complex with caffeine (triangles), calculated with two-component spectra decomposition. Lines represent concentrations of an antibiotic in a free form (solid line) and in complex with caffeine (dashed line), calculated using statistical-thermodynamical model of mixed aggregation [38] (with K_{AC} values 24.71 M^{-1} ± 0.89 M^{-1} (SE) for ciprofloxacin-caffeine interaction and 45.6 M^{-1} ± 2.5 M^{-1} (SE) for tetracycline-caffeine interaction).

Figure 5. Thermal effects of antibiotic-caffeine complex formation – analysis with isothermal titration calorimetry. Panels a-b, thermograms for analysis of ciprofloxacin-caffeine (panel a) and tetracycline-caffeine (panel b) interactions; solid line, titration of caffeine with antibiotic; dotted line, titration of caffeine with buffer; dashed line, titration of buffer with antibiotic; panels c-d, thermal effects of ciprofloxacin-caffeine (panel c) and tetracycline-caffeine (panel d) interactions; circles, titration of caffeine with buffer; squares, titration of caffeine

606 with antibiotic; triangles, titration of buffer with antibiotic. The net heat of antibiotic-caffeine interaction,
607 calculated as the difference between heat of antibiotic-caffeine titration and control (buffer) titrations, is marked
608 with crosses.

609 **Figure 6.** Dose-dependent modulation of antibiotics (ciprofloxacin and tetracycline) inhibitory potential by
610 xanthines (caffeine and pentoxifylline) towards selected bacterial pathogens. Graphs represent isobolograms for
611 each antibiotic-xanthine pair tested in concentration gradient of both compounds. Seven investigated pathogens
612 are given as separate rows. FIC, Fractional Inhibitory Concentration Index, calculated for each tested antibiotic-
613 xanthine combination according to Odds [39].

Figures



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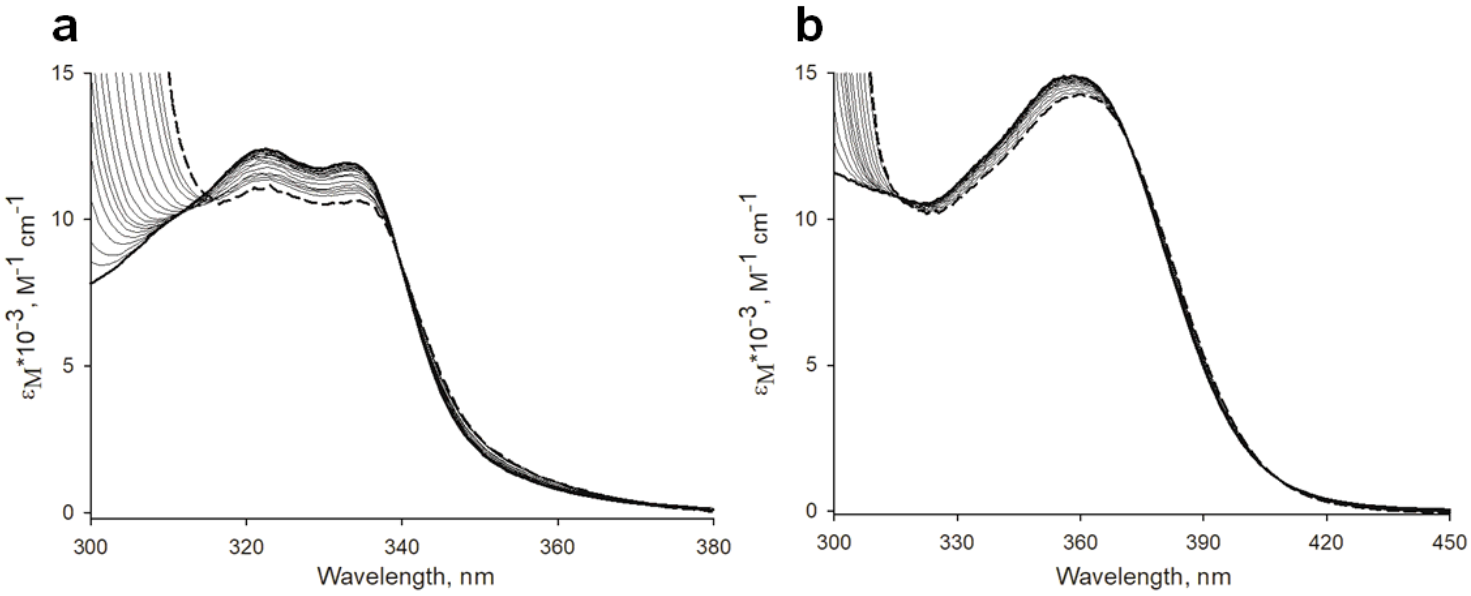

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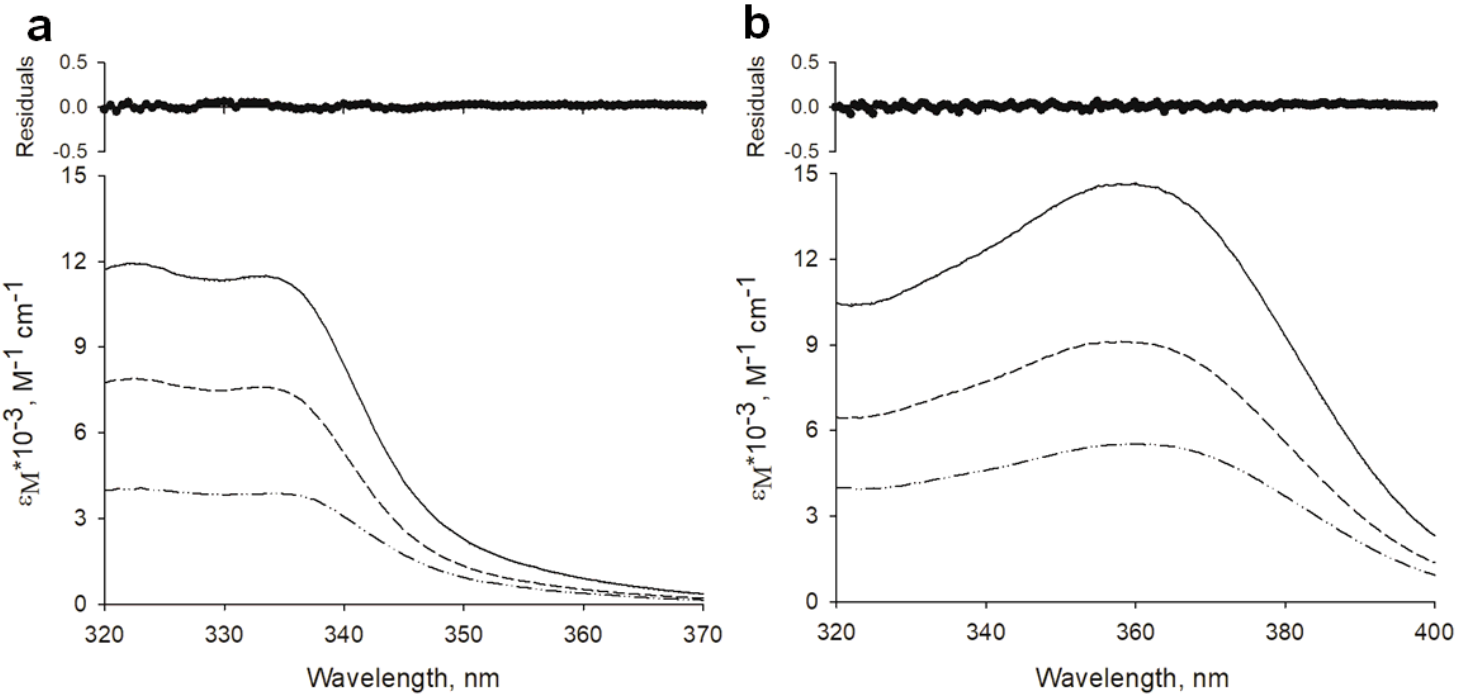

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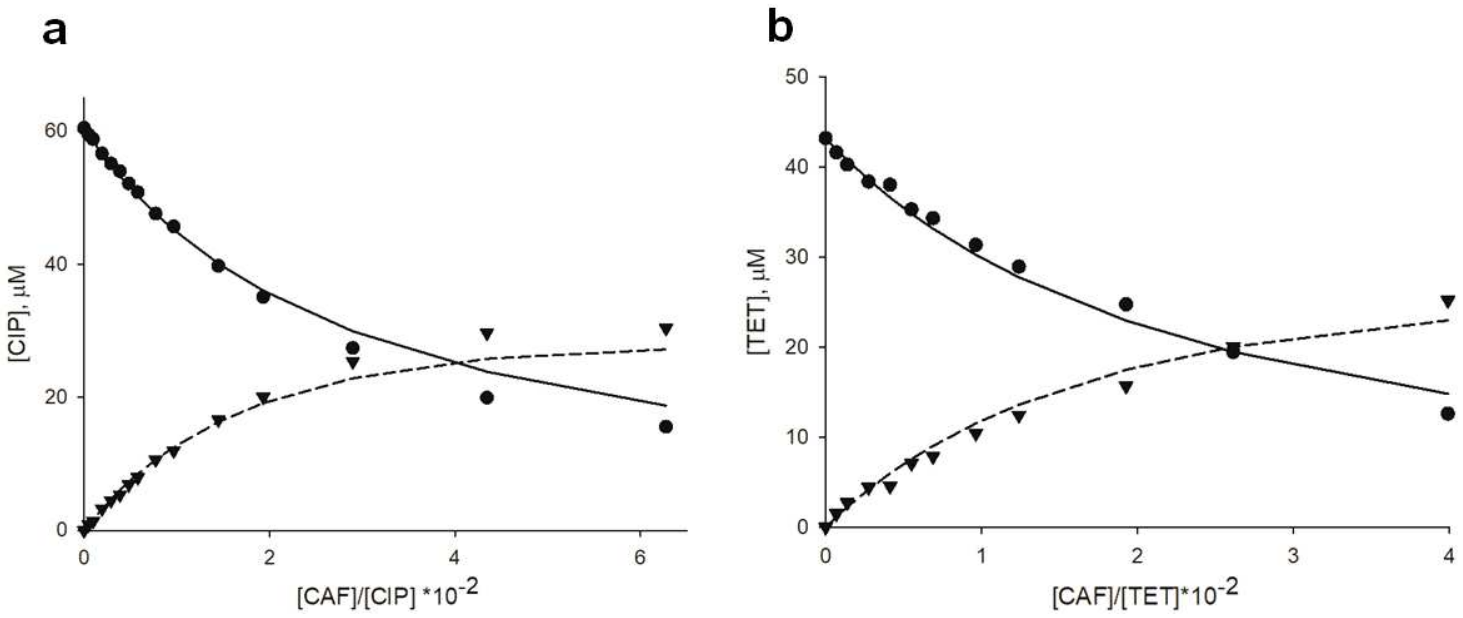

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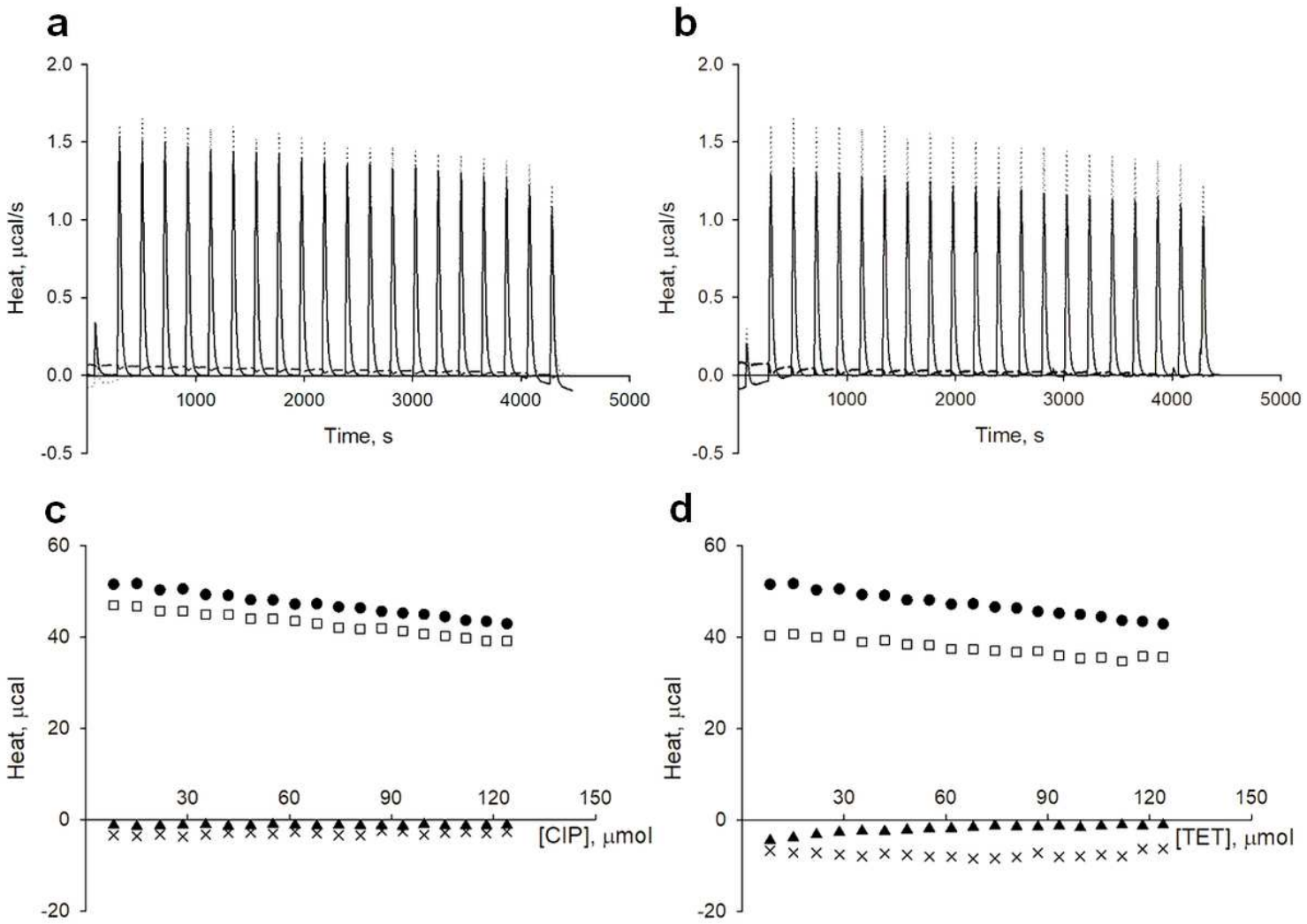

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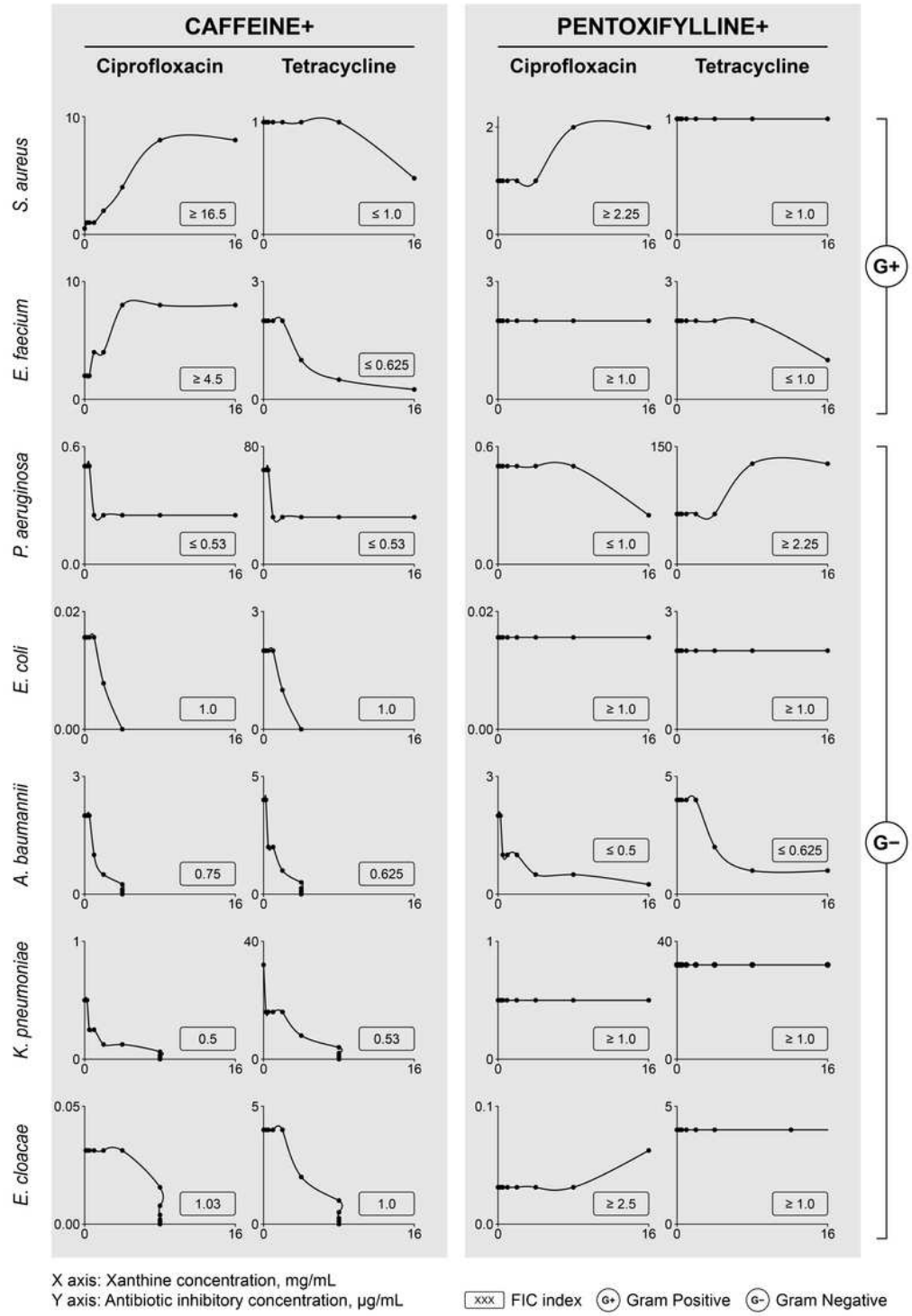

Figure 6

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