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METHODS AND PROTOCOLS

Effect of berberine on *Escherichia coli*, *Bacillus subtilis*, and their mixtures as determined by isothermal microcalorimetry

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Abstract The strong toxicity of pathogenic bacteria has resulted in high levels of morbidity and mortality in the general population. Developing effective antibacterial agents with high efficacy and long activity is in great demand. In this study, the microcalorimetric technique based on heat output of bacterial metabolism was applied to evaluate the effect of berberine on *Escherichia coli*, *Bacillus subtilis*, individually and in a mixture of both using a multi-channel microcalorimeter. The differences in shape of the power–time fingerprints and thermokinetic parameters of microorganism growth were compared. The results revealed that low concentration (20 μg/mL) of berberine began to inhibit the growth of *E. coli* and mixed microorganisms, while promoting the growth of *B. subtilis*; high concentration of berberine (over 100 μg/mL) inhibited *B. subtilis*. The endurance of *E. coli* to berberine was obviously lower than *B. subtilis*, and *E. coli* could decrease the endurance of *B. subtilis* to berberine. The sequence of half-inhibitory concentration (IC_{50}) of berberine was: *B. subtilis* (952.37 μg/mL)>mixed microorganisms (682.47 μg/mL)>*E. coli* (581.69 μg/mL). Berberine might be a good selection of antibacterial agent used in the future. The microcalorimetric method should be strongly suggested in screening novel antibacterial agents for fighting against pathogenic bacteria.

Keywords *E. coli* · *B. subtilis* · Mixed microorganisms · Berberine · Isothermal microcalorimetry

Introduction

Escherichia coli, a kind of Gram-negative bacteria, mainly exists in the intestinal tract of humans and animals. They are the most common pathogenic bacteria in clinic and could cause various infections and diseases including peritonitis, cholecystitis, cystitis, bloody and non-bloody diarrhea, etc., some of which are lethal, bringing serious hazards to human and animal health (Müller et al. 2001). These pathogenic *E. coli* have been the leading causes of food-borne illness (Belongia et al. 1991), responsible for hemorrhagic colitis infections that lead to the hemolytic uremic syndrome, and are also responsible for high levels of morbidity and mortality in the general population, particularly for at-risk groups, such as infants, children, and the elderly (Santos et al. 2010). On the other hand, some pathogenic *E. coli* can produce colicin, which is poisonous to the humans. Many antibiotics or other drugs have been developed for inhibiting the growth of hazardous microorganisms. But the toxicity of current drugs to humans and the clinical resistance of this bacterium to these drugs are increasing (Sendzik et al. 2009; Arias et al. 2007). Developing effective antibacterial agents with high efficacy, low toxicity, and long activity is therefore in great demand to combat this problem (Sommer et al. 2009; Haydon et al. 2008; Zlitni and Brown 2009).

Drugs that can either inhibit the growth of pathogenic microorganisms or kill them without harming host cells are considered candidates. In recent years, berberine, as a

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broad-spectrum antibacterial agent (Yang et al. 2007; Zhao et al. 2010; Braissant et al. 2010), has attracted more and more interest and attention. However, it is believed that whether berberine could be used as a novel antibacterial agent should include the evaluation of antibacterial activity of it on more microbes including harmful and beneficial bacteria rather than the above-mentioned single species. In evaluating antibacterial activity of berberine, the agar cup, micro-dilution, antimicrobial circle, and serial dilution methods are used frequently. However, these methods are easily influenced by environmental conditions and the operators; also, some qualitative and quantitative information could not be obtained simultaneously, which are crucial for objectively evaluating the antibacterial activities of the agents. Luckily, with the development of microcalorimetric techniques, the investigations of living materials have been improved during the past 30 years. A wide range of practical applications of such techniques have been envisioned (Wadsö 1995).

In any living system, various metabolic events occur within the cells involving heat-producing reactions. Isothermal microcalorimetry (IMC) could measure the heat output of all kinds of biological processes, which is proportional to the rate at which a given chemical or physical process takes place. When the microorganisms, including bacteria, fungi, etc, are added into a 20-mL disposable glass ampoule (Fig. 1a), they would utilize the limited oxygen and the nutrition of the liquid media in the ampoules for a chemical or physical process in which the heat is released. After the ampoules were sealed and introduced into an isothermal calorimeter (Fig. 1b), previously equilibrated at 37 °C, the heat output is recorded over time displaying as different thermogenic power–time curves (Fig. 1c) for various microorganisms until the heat signal returned to baseline indicating no further measurable microbial metabolic activity. The measurements are in the microwatt range under essentially isothermal conditions (Wadsö 2001), which allows small variations of the sample temperature (up to 0.1 °C). Variation in the sample temperature mostly does not affect the heat-sink temperature significantly (Braissant et al. 2010). In addition, the measurements are safe since the specimen is in a sealed ampoule at all times and simple because it is only necessary to note whether a rising, above-baseline heat signal is measured. Another advantage of IMC is that inoculation of closed, disposable, sealed ampoules only requires a biosafety level (BSL) 2 laboratory. In contrast, several promising methods require a BSL 3 laboratory (WHO 2008), therefore, increasing the cost of equipment and personnel training, which can be a problem for developing countries. More importantly, some qualitative information, such as the real-time thermogenic fingerprints (power–time curves in Fig. 1c), and many quantitative parameters, such as growth rate constant (k), the maximum heat-output power (P_{max}), the appearance time of maximum heat-output power (t_{max}), total heat effect (Q_t), inhibition ratio (I), and half-inhibitory concentration (IC_{50}), could be read or calculated through the measurements by this IMC instrument for the determination of microbial processes and evaluating the performance of antimicrobial drugs. Due to the above-mentioned advantages of IMC, it has been increasingly applied to study the effects of drugs, heavy metal and other substances on various microorganisms for screening new antimicrobial agents (Kong et al. 2009, 2011, 2012; Zhuang et al. 2011).

However, previous reports mainly focused on the evaluation of effects of other substances on a single microorganism, few studies tried to investigate the interactions of two or more microorganisms, or to continue in-depth exploration. So, the aims of the present study are: (1) to investigate the interaction relationships of *E. coli* and *Bacillus subtilis*, (2) to probe the possible mechanism of action (inhibition, promotion, or syntrophism) between the two microorganisms, and (3) to evaluate the effects of berberine on *E. coli*, *B. subtilis*, and their mixtures under a closed, static experimental environment by IMC using a TAM air multi-channel microcalorimeter for screening new antimicrobial agents with high efficacy and long activity.

Materials and methods

Instrument

The thermometric 3114/3236 thermal active monitor (TAM) air microcalorimeter (Fig. 1b) manufactured by Thermometric AB, Stockholm, Sweden, is an isothermal heat conduction calorimeter operating in the microwatt range. It has eight calorimetric channels which can keep the temperature within ±0.02 °C. All channels were mounted together to form a single heat-sink block housed in a temperature

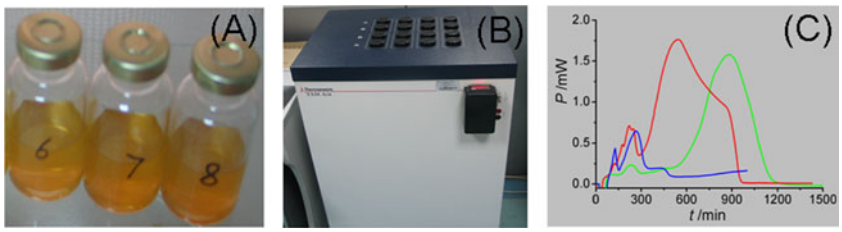

Fig. 1 **a** Twenty-milliliter disposable microcalorimetric ampoule containing medium, microorganism(s) and drugs. **b** Thermometric 3114/3236 TAM air isothermal calorimeter. **c** Thermogenic curves for the growth of different microorganisms

controlled air thermostat. Each calorimetric channel was constructed in twin configuration with one side for the sample and the other side for a static reference. The twin configuration of sample and reference within a channel allows the heat-output power from the sample to be compared directly with the heat-output power from the inert reference. The voltage difference is a quantitative expression of the overall rate of heat production in the sample. It has a limit of detection of 2 μW and baseline draft <20 μW over 24 h. A schematic representation of the instrument was reported in Li et al. (2011) and Zhang et al. (2012).

Materials

E. coli (CCTCC AB91112) was provided by China Center for Type Culture Collection, Wuhan University, Wuhan, People's Republic of China. *B. subtilis* (CMCC(B) 63501) was provided by National Center for Medical Culture Collections, Beijing, People's Republic of China. *E. coli*, *B. subtilis* and their mixed microorganisms were all inoculated in Luria–Bertani (LB) culture medium, which was prepared by dissolving 10 g peptone, 5 g yeast extract, and 5 g NaCl in 1,000 mL deionized water (pH 7.0–7.2) and sterilized by autoclaving at 0.1 MPa and 121 °C for 30 min.

Analytical-grade berberine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA) for preparing the solution of berberine. All other chemicals used were of analytical grade and available locally.

Microcalorimetric measurement

All microcalorimetric experiments were performed using the eight-channel isothermal calorimeter (Fig. 1b) with ampoule method in batch mode. The calorimeter was brought to equilibrium temperature over night in advance. Five-milliliter LB culture medium was introduced into the 20-mL sterilized ampoule (Fig. 1a) containing berberine compound at different concentrations, and the suspensions of *E. coli*, *B. subtilis* and their mixed microorganisms at the cell density of 1×10^6 colony forming units (CFU)/mL were inoculated into each ampoule. Then, the ampoules were sealed, shaken-up slightly, and put into the microcalorimeter. The power–time signals were recorded at an interval of 1 min and were collected continuously using the dedicated software package until the recorder returned to the baseline. The temperature of the calorimeter was controlled at 37 °C. All apparatus were cleaned and sterilized by autoclaving before using.

Results

Normal power–time curves of *E. coli*, *B. subtilis*, and their mixed microorganisms

When the suspensions of single microorganism and their mixtures are introduced into the ampoules, the heat-output signals are recorded to form the normal power–time curves (Fig. 2) of *E. coli*, *B. subtilis*, and the mixed microorganisms of *E. coli* and *B. subtilis* (1:1, *v*/*v*) in the absence of berberine. These three curves in Fig. 2 could all be divided into five phases, that is, a lag phase, the first exponential growth phase, a transition phase, the second exponential growth phase and a decline phase (Kong et al. 2010, 2011). There are two peaks, peaks I and II in each curve, that represent the two maximum heat-output powers in the first and second exponential growth phase. When comparing the three curves, it could be found that the power–time curve of mixed microorganisms in Fig. 2c is very similar with that of *E. coli* in Fig. 2a, and significantly different from that of *B. subtilis* in Fig. 2b, including the peak form, peak heights, and appearance times of the first and second peaks. The possible reason might be that *E. coli* have killed *B. subtilis* or inhibited the growth of *B. subtilis* so the growth character of *B. subtilis* could not be presented. The detailed mechanism of action would be discussed in the next study. All these indicated that *E. coli* (the typical harmful or pathogenic bacteria) would kill *B. subtilis* (the beneficial or probiotic bacteria) or inhibit their growth when they co-existed, which would be more harmful to the human or other living system. So, it is urgent and necessary to develop effective antibacterial agents to combat this problem (Coates et al. 2002; Xie et al. 1988). The ideal antibacterial agents should have strong antibacterial effects on *E. coli* and/or have poor antibacterial effects on *B. subtilis* or promote the growth of *B. subtilis*.

Power–time curves of *E. coli*, *B. subtilis*, and their mixed microorganisms in the presence of berberine

When the water solution of berberine was added into the internal system of *E. coli*, *B. subtilis*, and their mixed microorganisms' growth in the glass ampoule, the metabolism of the bacteria would be influenced. These influences could be intuitively shown from the heights, appearance times of the two peaks in the power–time curves of *E. coli*, *B. subtilis*, and their mixed microorganisms (Fig. 3) in the presence of berberine. But, these curves were similar and the five phases of the curves still existed.

In Fig. 3a and c, it could be found that the heights of the highest peaks lowered while increasing the concentration of berberine in the range of 0–1,200 μg/mL, while in Fig. 3b, the heights rose while increasing the concentration of

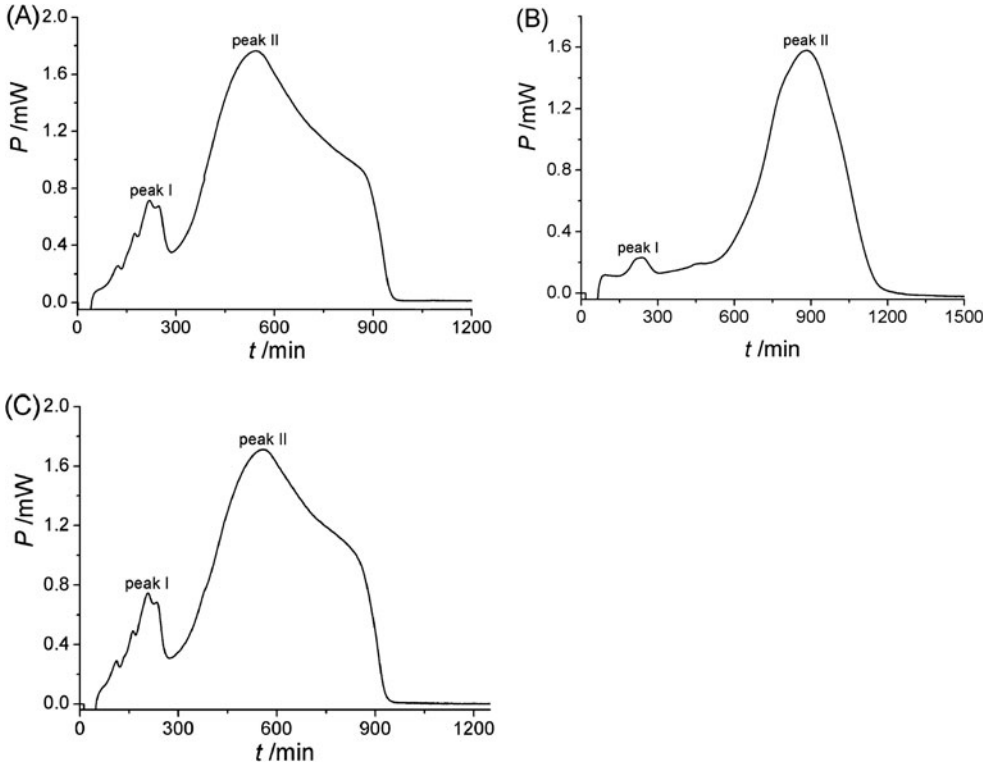

Fig. 2 The normal thermogenic HFP–time curves of **a** *E. coli*, **b** *B. subtilis*, and **c** mixed microorganisms at 37 °C

berberine in the range of 0–100 μg/mL, and then lowered in the range of 100–1,200 μg/mL. All these indicated that in the concentration range of 0–1,200 μg/mL, berberine inhibited the growth of *E. coli* and the mixed microorganisms all the time, but at a low concentration, it promoted the growth of *B. subtilis* and then inhibited the growth at high concentration within the increased concentration range.

Important thermokinetic parameters from the power–time curves

The influences of berberine on the growth of *E. coli*, *B. subtilis*, and their mixed microorganisms could be mainly and quantitatively reflected from the changes of some important thermokinetic parameters in Table 1 from the

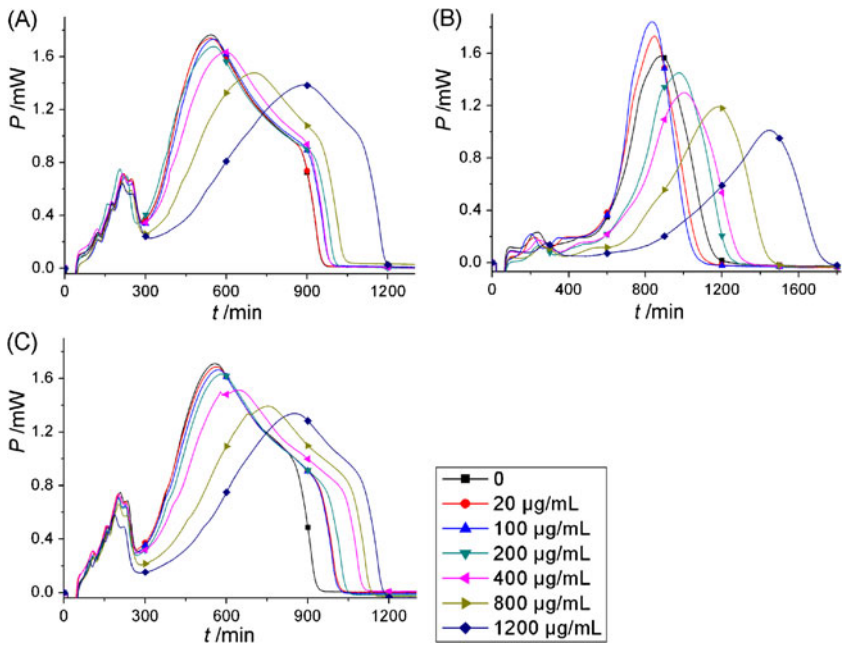

Fig. 3 The power–time curves of **a** *E. coli*, **b** *B. subtilis*, and **c** the mixed microorganisms in the presence of different concentrations of berberine

Table 1 Thermokinetic parameters obtained from the power–time curves of the growth of *E. coli*, *B. subtilis*, and the mixed microorganism in the presence of berberine

Microorganism	c^a (μg/mL)	k (min^{-1})	t_G (min)	t_{max} (min)	P_{max} (mW)	Q_t (J)	I (%)	IC_{50} (μg/mL)
E. coli	0	0.00785	88.3	542.0	1.7649	53.65	0	581.69
	20	0.00702	98.7	541.3	1.7393	50.61	10.57	
	100	0.00647	107.1	551.7	1.7338	49.04	17.58	
	200	0.00571	121.4	553.3	1.6762	47.82	27.26	
	400	0.00404	171.5	597.0	1.6348	45.83	48.54	
	800	0.00205	338.0	706.0	1.4778	39.79	73.89	
	1200	0.00144	481.3	892.3	1.3878	32.88	81.66	
B. subtilis	0	0.00624	111.1	881.3	1.5793	37.34	0	952.37
	20	0.00645	107.4	845.6	1.7313	44.38	−3.37	
	100	0.00686	101.0	835.0	1.8408	47.32	−9.94	
	200	0.00532	130.3	975.3	1.4517	33.33	14.74	
	400	0.00475	145.9	1,006.3	1.3003	29.61	23.88	
	800	0.00362	191.4	1,179.3	1.1921	24.64	41.99	
	1,200	0.00211	328.4	1,445.3	1.0123	20.09	66.19	
Mixed microorganism	0	0.00716	96.8	556.7	1.7120	48.92	0	682.47
	20	0.00632	109.7	559.3	1.6877	46.11	11.73	
	100	0.00543	127.6	572.6	1.6663	42.51	24.16	
	200	0.00481	144.1	584.3	1.6346	39.67	32.82	
	400	0.00369	187.8	643.0	1.5144	32.19	48.46	
	800	0.00257	269.6	751.0	1.3934	28.02	64.11	
	1,200	0.00197	351.8	852.0	1.3392	22.23	72.49	

power–time curves. The growth rate constant (k) of the second exponential growth phase of the second peak could be calculated according to the equation:

$$\ln P_t = \ln P_0 + kt \quad (1)$$

where P_0 and P_t are the heat-output powers at time 0 and t (min), respectively. The generation time (t_G) of these microorganisms could be calculated from the formula:

$$t_G = (\ln 2)/k \quad (2)$$

It could be found in Fig. 4 that k for *E. coli* and the mixed microorganisms decreased and t_G increased indicating that the growth rates of *E. coli* and the mixed microorganisms were slowed and lengthened; while increasing the concentration of berberine in the concentration range of 0–1,200 μg/mL, their growth was significantly inhibited. While k and t_G for *B. subtilis* increased and decreased showing that berberine had stimulation effects in certain low concentration ranges on *B. subtilis*.

P_{max} and t_{max} of the highest peak, and Q_t of the total metabolic progress of these microorganisms could be also obtained from the power–time curves, which were all listed in Table 1. Q_t could be obtained by integrating the peak areas under the power–time curves.

Relationships between dosage and heat effects for these microorganisms

The total heat effect Q_t in the growth progress of *E. coli*, *B. subtilis*, and the mixed microorganisms was an important parameter for comparing the heat output of these

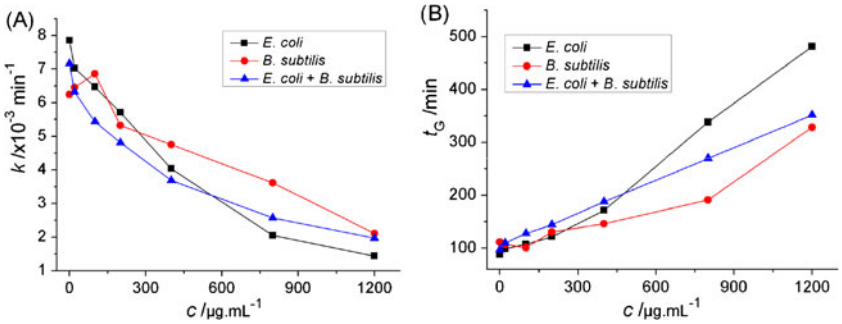

Fig. 4 The relationships of **a** k–c and **b** t_G–c for *E. coli*, *B. subtilis*, and the mixed microorganisms

microorganisms and the effects of berberine. The relationships between Q_t and c for *E. coli*, *B. subtilis*, and the mixed microorganisms were shown in Fig. 5. For the control (not adding berberine solution), Q_t for *E. coli* was larger than that for *B. subtilis*, which indicated that *E. coli* of the same cell density of 1×10^6 CFU/mL with *B. subtilis* produced more heat than *B. subtilis* although the total life periods of *E. coli* were shorter than that of *B. subtilis*. But when *E. coli* and *B. subtilis* were mixed, the values of Q_t for these mixed microorganisms were smaller than that of *E. coli* and larger than that of *B. subtilis*, which might be due to the interaction of *E. coli* and *B. subtilis*. In this progress of interaction, *E. coli* produced a large amount of antibacterial substances, which inhibited or killed *B. subtilis* following with a lot of consumption of heats. Because of the inhibition of *E. coli* to *B. subtilis*, *E. coli* predominated in the mixed microorganisms; the values of Q_t for the mixed microorganisms were still larger than that of *B. subtilis*.

When berberine was added, it could be found that, according to the data in Table 1 and curves in Fig. 5, berberine had a positive effect on *B. subtilis* when the concentration is less than 100 μg/mL, reflecting from the increased Q_t; when the dosage exceeded 100 μg/mL, this positive effect was weakened. When the dosage was increased continuously, berberine had negative effect on *B. subtilis* to inhibit the growth. While within the concentration range of 0–1,200 μg/mL, the values of Q_t for *E. coli* and the mixed microorganisms decreased all the time showing that berberine had strong inhibitory effects on *E. coli* and the mixed microorganisms of *E. coli* and *B. subtilis*, which further indicated the strong antibacterial effects of berberine especially on harmful pathogenic bacteria, such as *E. coli*.

Inhibition ratio I of berberine to *E. coli*, *B. subtilis*, and the mixed microorganisms

In all the experiments, we chose the same concentration of berberine for *E. coli*, *B. subtilis*, and the mixed microorganisms for more objective comparability. However, comparing the experiments with the single microorganism to that of mixed microorganisms, it could be seen that the inhibition ratio (I) and the effect of restrain were different although the concentration of berberine was the same. I (%) can be calculated on the basis of the growth rate constant k as Kong et al. (2010):

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (3)$$

where k_0 and k_c (in per minute) are the growth rate constants of *E. coli*, *B. subtilis*, and the mixed microorganisms in the absence of berberine (the control condition) and that inhibited by berberine at a certain concentration of c. I can show the inhibitory extent of some concentration of berberine on the microorganisms' growth, which can be directly reflected from *I–c* curve in Fig. 6. When the inhibition ratio I is 50 %, the corresponding concentration of berberine is called half-inhibitory concentration (IC_{50}, in micrograms per milliliter). IC_{50} can be regarded as the inhibition concentration of causing a 50 % decrease of growth rate constant of *E. coli*, *B. subtilis*, and the mixed microorganisms, which can quantitatively represent the effect of berberine. The values of I and IC_{50} were also depicted in Table 1.

From Table 1 and Fig. 6, it could be found that the inhibition ratio I for berberine on *E. coli* (with berberine 200–1,200 μg/mL), *B. subtilis* (with berberine 200–1,200 μg/mL), and the mixed microorganisms (with berberine 200–1,200 μg/mL) increased with increasing concentration of berberine. The value of IC_{50} for berberine on *E. coli* was lower than that for *B. subtilis*, which showed that the sensitivity of *E. coli* to berberine was higher than that of *B. subtilis*. This also meant that 581.69 μg/mL of berberine solution could cause a 50 % decrease of growth rate constant of *E. coli*, while for *B. subtilis*, the concentration of berberine would be up to 952.37 μg/mL. When *B. subtilis* were added into the cell solution of *E. coli*, the sensitivity of the mixed microorganisms to berberine was lower than that of *E. coli* but higher than that of the mixed microorganisms; 682.47 μg/mL of berberine would cause a 50 % decrease of growth rate constant of the mixed microorganisms, indicating that *B. subtilis* would lower the sensitivity of *E. coli* to berberine, which might be due to the interaction between *E. coli* and *B. subtilis*. The accurate mechanism of action would be explored in future research.

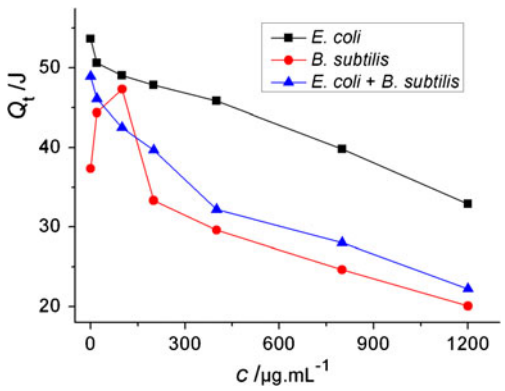

Fig. 5 Relationships between Q_t and c for *E. coli*, *B. subtilis*, and the mixed microorganisms

Endurance of *E. coli*, *B. subtilis*, and the mixed microorganisms

Based on the above-mentioned results, we preliminarily explored the endurance of *E. coli* and *B. subtilis*, and them to berberine. The data in Table 1 showed that the values of P_{max} of the mixed microorganisms were lower than those of *E. coli*, but higher than those of *B. subtilis* in the absence of berberine or in the presence of berberine at the same

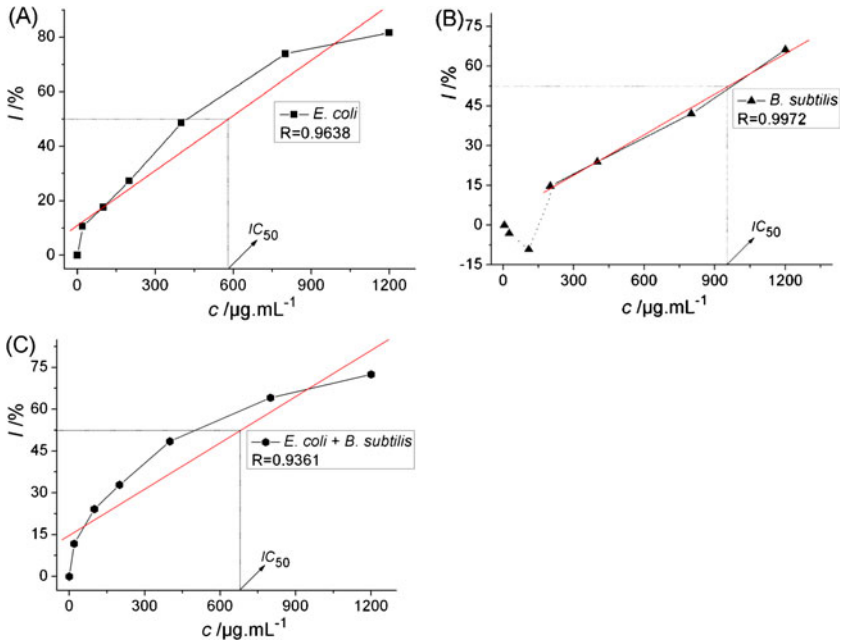

Fig. 6 The *I*–*c* relationships for *E. coli*, *B. subtilis*, and the mixed microorganisms in the presence of berberine

concentration. It might be due to the consumption of heat-output power in the interaction between *E. coli* and *B. subtilis*, or the negative effect of *B. subtilis* on *E. coli*, which further indicated that *B. subtilis* had low endurance to *E. coli*.

Berberine at 20 μg/mL began to inhibit the growth of *E. coli*, 581.69 μg/mL of berberine would cause a 50 % decrease of growth rate constant of *E. coli*. While berberine at 20 μg/mL promotes the growth of *B. subtilis* when the concentration exceeded 100 μg/mL, berberine began to inhibit the growth of *B. subtilis* and 952.37 μg/mL of berberine would cause a 50 % decrease of growth rate constant of *B. subtilis*. The endurance of *E. coli* to berberine was obviously lower than *B. subtilis*. Also, berberine at 20 μg/mL began to inhibit the growth of the mixed microorganisms of *E. coli* and *B. subtilis*, 682.47 μg/mL of berberine would cause a 50 % decrease of growth rate constant of the mixed microorganisms, which showed that the endurance of the mixed microorganisms to berberine was obviously lower than *B. subtilis*, but higher than *E. coli*. This also indicated that *E. coli* could decrease the endurance of *B. subtilis* to berberine. If both microorganisms were put under the same situation in the laboratory in the presence of berberine, for instance, at the same concentration, the degree of endurance of *E. coli* was lower than that of *B. subtilis*. All these results, to some extent, elucidated that berberine might be used as a potential antibacterial drug for inhibiting the growth of some harmful bacteria such as *E. coli*, also the mixed bacteria of *E. coli* with others.

Discussion

The strong toxicity of pathogenic bacteria, such as pathogenic *E. coli*, has brought serious threats to the humans' health, which may result in high levels of morbidity and mortality in the general population. Developing effective antibacterial drugs with high efficacy and long activity is an urgent task.

In this study, by evaluating the effects of berberine on *E. coli*, *B. subtilis*, and their mixed microorganisms using IMC, we have found that: (1) the growth fingerprints of *E. coli*, *B. subtilis*, and the mixed microorganisms could be real-time recorded by IMC using a microcalorimeter; and from these fingerprint curves, some qualitative and quantitative information could be obtained for evaluating the interaction between *E. coli* and *B. subtilis* and the effects of berberine; (2) when *E. coli* and *B. subtilis* were put together, the growth character of *B. subtilis* would not be presented, which might due to the inhibitory effects of *E. coli* on *B. subtilis*; (3) low concentration of berberine began to inhibit the growth of *E. coli* and the mixed microorganisms while promoting the growth of *B. subtilis*; berberine at high enough concentration inhibited the growth of *B. subtilis*; (4) the endurance of *E. coli* to berberine was obviously lower than *B. subtilis*, and *E. coli* could decrease the endurance of *B. subtilis* to berberine; (5) berberine might be a good selection of antibacterial drug for inhibiting the growth of some harmful bacteria such as *E. coli*, also the mixed bacteria of *E. coli* with others and (6) microcalorimetric investigations of drugs on microorganisms were possible and promising. IMC is unique in its ability to easily provide rapid detection and real-time, quantitative monitoring of a wide variety of microbiologic phenomena, which was not observable by other techniques (Chardin et al. 2002; Batovska et al. 2007; Leng et al. 2007). There was ample opportunity for IMC to be transformed into a clinical tool having capabilities otherwise unavailable for evaluating the antibacterial effects

of more and more drugs on pathogenic bacteria (Coates et al. 2002).

Nevertheless, the mechanism of interaction between *E. coli* on *B. subtilis* and also the mechanism of action of berberine on *E. coli*, *B. subtilis*, and their mixed microorganisms need to be explored in future research. Still, new methods and approaches are needed for the development of antibacterial tests and studies for screening effective antibacterial drugs with high efficacy and long activity.

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