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Original Research Article

Dicarboxylic acids from Caper leaves enhance antibiotic susceptibility of *Pseudomonas aeruginosa* to vancomycin

Nadhem Aissani[1]*, Pierluigi Caboni[1], Marco Saba[1], Gina De Nicola[2], Renato Iori[2] and Valentina Coroneo[3]

[1]Department of Life and Environmental Sciences, University of Cagliari, Via Ospedale, 72, 09124 Cagliari, Italy
[2]Consiglio per la Ricerca e la sperimentazione in Agricoltura, Centro di Ricerca per le Colture Industriali (CRA-CIN), Via Di Corticella 133, Bologna 40128, Italy
[3]Dipartimento di Sanità Pubblica, Medicina Clinica e Molecolare, Laboratorio di Igiene degli Alimenti, Università di Cagliari, Cagliari, Italy
**Corresponding author*

A B S T R A C T

Plant-derived antimicrobial compounds can be potentially used as novel tools to preserve food or to decrease the antibiotic resistance of bacteria. Caper (*Capparis spinosa*) offers a natural promising alternative for food safety and bioconservation. In this work, the methanolic extract of caper leaves was tested for the ability to inhibit the growth of a range of microorganisms. This extract was found to be more effective against *Pseudomonas aeruginosa* at 225 µg/ml than other organisms as proved by the broth microdilution method. Organic acids from MCL were then extracted with a mixture of water-ethanol (50/50 v/v). *P. aeruginosa* growth inhibition was recorded for malic acid, malonic acid, succinic acid, *p*-coumaric acid at 450 µg/ml while benzoic acid was active at 225 µg/ml. The antimicrobial activity of the latter compounds and oxalic acid used as chemical control in combination with antibiotics was studied using checkerboard methods and fractional inhibitory concentration (FIC) against *P. aeruginosa*. FIC_{index} values were then calculated to characterize interactions between the inhibitors. Malic acid and oxalic acid were found highly effective in increasing the susceptibility of *P. aeruginosa* to vancomycin (FIC_{index} = 0.37 and 0.50 respectively) suggesting the possible use of dicarboxylic acids from caper leaves as natural antimicrobials against *P. aeruginosa*

K e y w o r d s

Natural antimicrobials, organic acids, FIC_{index}, Checkerboard methods, *Capparis spinosa*, *Pseudomonas aeruginosa*

Introduction

Antibiotics are generally beneficial in treating susceptible microbial infections, but such benefits may be offset by their overuse. In the United States, 50% of all antibiotics produced are administrated to animals at sub-therapeutic levels to promote growth, increase feed and production (Wegner, 2003; Jindal *et al.*, 2006). This leads to the emergence of antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Peterson and Dalhoff,

2004). Thus, new strategies are needed to control these microorganisms. Combination between antimicrobials can offer a useful solution to avoid or, at least, decrease bacterial resistance. Currently, there is a growing interest in using natural antibacterial compounds such as herbal extracts or pure compounds found in medicinal plants in health care and microbial control. The majority of their antimicrobial properties are due to secondary metabolites, such as the volatile constituents, polyphenols, organic acids and dicarboxylic acids (Fattouch *et al.,* 2007, Aissani *et al.,* 2012, Feng *et al.,* 2012).

Organic acids have a long history of being used as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients (Hazan *et al.,* 2004). Specific organic acids have also been used to control microbial contamination and dissemination of foodborne pathogens in food production and processing (Ricke, 2003). To date, no studies are published on the antimicrobial effects of *Capparis spinosa* leaves extracts and its constituents against *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa is a common Gram (-) bacterium that grows not only in normal atmospheres, but also under hypoxic conditions and it is an important food borne pathogen and spoiling bacteria (Rahman and Kang, 2008). One of the most worrisome characteristics of *P. aeruginosa* is the low antibiotic susceptibility due to the multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (*mexAB, mexXY*) and the low permeability of the bacterial cellular envelopes (Poole, 2004).

Capparis spinosa exhibits strong resistance to harsh environmental conditions (Sharaf *et al.,* 2000) and is found in the dry regions of west or central Asia and is widely grown in the Mediterranean basin. It has been employed as a flavouring agent in cooking, as a diuretic, hypertensive, and tonic since ancient times (Baytop 1984, Rivera *et al.,* 2003). It is used also as culinary spice as well as an anti-inflammatory agent in folk medicine, especially to treat ear ache and cough, and to expel intestinal worms (Zhou *et al.,* 2010). Recently, it was discovered that volatile secondary metabolites from caper, such as 2−thiophenecarboxaldehyde and methyl isothiocyanate were active against root knot nematodes *Meloidogyne incognita* (Caboni *et al.,* 2012).

The aims of the present work were to evaluate the antimicrobial activities of *Capparis spinosa* leaves methanolic extract (MCL) as well as the major constituents of this plant part essentially dicarboxylic acids against a set of bacteria, including *P. aeruginosa*. To assess the synergistic interaction between these agents and antibiotics those are naturally not active against this bacterium.

Materials and Methods

Chemicals and antimicrobial agents

Standards of malic acid, succinic acid, oxalic acid, benzoic acid, *p*-coumaric acid, malonic acid, methyl isothiocyanates and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich (Milano, Italy).

Cefpodoxime (Cefodox 200 mg, Scharper, Italy), lomefloxacin (Uniquin 400 mg, Madaus, Italy), cefixime (Supracef 400 mg, Lusofarmaco, Italy), cefditoren (Giason 200 mg, Zambon, Italy), ciprofloxacine (Ciproxin 500 mg, Bayer Schering Pharma,

Italy), rifaximin (Normix 200 mg, Alfa Wasserman, Italy), vancomycin, ceftazidime, polymyxine B and gentamycin were obtained from Microbiol, Italy. Methanol and water were of high-performance liquid chromatography (HPLC)-grade. Antibiotics were dissolved in distilled water to prepare working solution and tested for the calculation of their minimal inhibitory concentration (MIC).

Extraction and Chemical Characterization

Plant materials

Leaves of *C. spinosa* were collected from Cagliari (Sardinia, Italy) in February, 2014, and were dried in the absence of light at room temperature. They were then sealed in paper bags, stored at room temperature, and kept in the dark until use. Voucher specimens were deposited at the Department of Life and Environmental Sciences, University of Cagliari (Italy) for species identification. For extraction, dried leaves (100 g) were ground and extracted with methanol (1:10, w/v) in a sonicator apparatus for 15 min, filtered through Whatman no. 40 filter paper, and centrifuged for 15 min at 13000 *g*. Then, the extract was sterilized by filtration using 0.45 µm filter and used for antimicrobial activity.

Purification of glucocapparin and myrosinase

Glucocapparin was isolated from powdered freeze-dried caper (*C. spinosa*) leaves, according to a procedure developed at CRA-CIN (Bologna, Italy) (Baasanjav-Gerber *et al.* 2011). In brief, glucocapparin was purified by two sequential chromatographic steps: isolation through anion exchange chromatography, followed by gel filtration. The purity was assessed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method, yielding glucocapparin with a purity of 90% (European Economic Community, Commission Regulation, 1990).

The enzyme myrosinase was isolated from seeds of *Sinapis alba* L. as described by Pessina *et al.* with some modification (Pessina *et al.,* 1990). The specific activity of the stock solution used in the present study was 60 U/mg of soluble protein. The enzymatic activity was 35 U/mL and the solution was stored at 4°C in sterile saline solution at neutral pH until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 µmol/min of sinigrin at pH 6.5 and 37°C.

Sample preparation and derivatization

Organic acids contained in caper leaves methanolic extract were quantified. The extraction method used for dried samples was as follows: 50 ml of 50% aqueous ethanol was added to 1 g of dried leaves. The extraction mixture was then sonicated for 45 min in a water bath at 50°C. Afterwards, the mixture was filtered with 0.45 µm PTFE filter, reduced to 25 ml by rotary evaporation of ethanol (40°C) and then extracted with 30 ml (3 × 10 ml) ethyl acetate. The organic layer was collected and reduced to 10 ml by rotary evaporation (40°C) and centrifuged for 10 min. Anhydrous Na_2SO_4 was then added to remove moisture. Then, 1 ml of the organic layer was derivatized after evaporation of the solvent under nitrogen stream. For the silylation procedure a mixture of BSTFA - TMS (99:1) (200 µl) were added, vortexed in screw cap glass tubes and consecutively placed in a water bath at 80°C for 45 min. The silylated mixture was made up to 1 ml with hexane and 1 µl was analyzed by GC-MS.

GC-MS analysis

The chromatographic separation for the identification of silylated components was performed on an Agilent Technologies 6850 coupled with a Mass Selective Detector 5973 and a 7683B Series Injector autosampler. Injection was performed in a splitless mode. Data were elaborated using MSD ChemStation D.03.00611. The column was 5% phenylmethylpolysyloxane, 30 m x 0.25 mm x 0.25 µm. The injector was set at 290°C. The temperature program was as follows: from 70°C to 135°C with 2°C/min, hold for 10 min; from 135°C to 220°C with 4°C/min, hold for 10 min; from 220°C to 270°C with 3.5°C/min and then hold for 20 min. A post run of 10 min at 70°C was sufficient for the next injection. The flow rate of carrier gas (helium) was maintained at 1.5 ml/min. Identification of compounds was obtained by comparing the retention times with those of authentic compounds and the spectral data obtained from the NIST libraries. Each determination was carried out in triplicate.

Microbiological Assay

The antimicrobial activity of caper leaves methanolic extract was studied. The experiments were carried out using a set of microorganisms: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14024), and *Pseudomonas aeruginosa* (ATCC 9027) strains for Gram(-), *Listeria monocytogenes* (ATCC 35152) and *Staphylococcus aureus* (ATCC 25932) strains for Gram (+), *Aspergillus niger* (ATCC 16404), *Aspergillus flavus* (isolated in our laboratory) and *Penicillium* spp. as fungal strains. We also used *P. aeruginosa* strains previously isolated in our laboratory for this study. For each of the above-mentioned bacterial strains, the minimum inhibitory concentration (MIC) was determined using the broth microdilution method. Stock standard solutions of compounds deduced by GC/MS were prepared at 1% (v/v) in DMSO. Working solutions of glucocapparin were prepared by dilution in microplates at concentration values ranging from 3.5 to 900 µg/ml in 100 mmol/l phosphate buffer. For the experiment of the *in situ* release of methyl isothiocyanate, myrosinase (5 µl) was added into each well and the hydrolysis was performed at 37°C for 24 h.

In all experiments, the bacterial suspensions were added in the microwells at a concentration of 10^5 colony-forming units/ml (CFU/ml). The plates were incubated aerobically at 37°C for 24 h. Bacterial growth was revealed by the presence of turbidity and a pellet on the well bottom. The MIC value was considered the highest dilution of the extract that did not show the production of pellet. The same experiments were repeated with antibiotics at concentrations between 0.43 and 1800 µg/ml.

Inhibited microorganisms were tested using the standard disk method to determine the minimal bactericide concentration (MBC). The MBC was considered the lowest concentration of a substance determining a bactericidal effect of 99.9%. The experimental determination of MBC consists of measuring the viability percent without visible growth.

For fungal strains, inhibition tests were carried out by inoculation, with mycelia fragments of 6 mm in diameter, in Petri dishes containing potato dextrose agar (PDA). After the addition of the extract (900 µg/ml), plates were sealed with parafilm and incubated in the dark at 22°C. Control samples with the mycelia in PDA and distilled water were incubated under the

same conditions. The effectiveness of the treatments was evaluated by measuring the average diametric growth of the colonies after 4, 8 and 12 days of the inoculation. The percentage of inhibition (I) was calculated according to the formula of Zygadlo and Guzman (Zygadlo *et al.*, 1994).

$$I\ (\%) = [(C-T)/C] \times 100$$

Where C is the average diameter of fungal growth in PDA + water, and T is the average diameter of fungi cultivated in PDA with the extract. When no mycelium growth was observed, the fungal fragment was transferred to a plate containing only PDA and incubated for 48 h, to determine if the inhibition was fungistatic or fungicide. All experiments were repeated three times.

Synergistic test

To evaluate the synergistic effect of the isolated antimicrobial substances with antibiotics, checkerboard method was used against *P. aeruginosa* as previously described (Rand *et al.*, 1993, Palaniappan and Holley, 2010). This method is often combined with calculation of a fractional inhibitory concentration FIC_{index} to test the antimicrobial potencies of drugs in medical laboratories (Pei *et al.*, 2009). Synergistic interactions involving the natural antimicrobial substance A (organic acid) and substance B (antibiotic) were tested. The concentrations of the agents used were started from the double of their MIC value and were evaluated by calculating the FIC index for each combination using the following formula: FIC of substance A = MIC of substance A in combination / MIC of substance A alone; FIC of substance B = MIC of substance B in combination / MIC of substance B alone; FIC index = FIC of substance A + FIC of substance B (Mandalari *et al.*, 2010). Synergy was defined as a $FIC_{index} \leq 0.5$. When the FIC_{index} is between 0.5 and 4.0 indicates no interaction between substances while a FIC index > 4.0 indicates an antagonism (Hemaiswarya *et al.*, 2008). For synergistic activity confirmation, a suspension of 10^5 CFU/ml of the bacterium was prepared in which the concentration of the antimicrobials corresponded to the observed synergistic activity were added then, 100 µl was plated 3 times in agar TBX (Microbiol-Cagliari, Italy). A control with no antimicrobial was included. The bacterium was incubated overnight at 37°C, and the number of survived bacteria was counted.

Statistical Analysis

All tests of the assessment of bacterial growth were run in triplicate and averaged. Means, standard errors, standard deviations and degrees of significance using Student's test were calculated from replicates within the experiments, and analyses were done using Microsoft Excel 2010. If natural compound-antibiotic combination appeared synergistic ($FIC_{index} \leq 0.5$), this observation was confirmed only after it was demonstrated that the combination also caused a significant decrease ($P < 0.05$) in the bacterial growth.

Results and Discussion

Antimicrobial activity of the extract

Using the microdilution method, MCL showed an antimicrobial activity only against both the reference *P. aeruginosa* (ATCC 9027) and isolated strains with a MIC at 225 µg/ml (Table 1). Methyl isothiocyanate the major compound found in caper stems and leaves methanolic extracts (Caboni *et al.*, 2012), did not show any antimicrobial activity. This isothiocyanate is naturally released, when the plant is

stressed, by the contact of myrosinase and glucocapparin. Similarly, both glucocapparin alone and methyl isothiocyanate produced *in situ* by the addition of myrosinase to the precursor glucocapparin, showed no activity. This leads us to hypothesize that organic acids found in MCL were responsible for activity. Subsequently, levels of organic acids were determined by GC-MS after extraction with aqueous ethanol and reported in Table 2.

Antimicrobial activity of organic acids deduced by GC-MS analysis

Malic acid, succinic acid, malonic acid and *p*-coumaric acid as components of MCL showed an activity against this *P. aeruginosa* with MIC at 450 µg/ml while benzoic acid showed an activity of 225 µg/ml (Table 2). Same level of activity (450 µg/ml) was found when we used oxalic acid as an external control confirming the antimicrobial activity of organic acids and their use as food additives and preservatives (Ricke, 2003). The antibacterial mechanisms of organic acids are not fully understood, they are probably capable of exhibiting bacteriostatic and bactericidal properties based on the physiological status of the organism and the physicochemical characteristics of the external environment, but in all cases pH is considered a primary determinant of effectiveness because it affects the concentration of undissociated acid formed.

Undissociated acid forms can penetrate the bacterial membrane and generate ions and protons in the neutral pH of cytoplasm (Cherrington *et al.,* 1990, Cherrington *et al.,* 1991). Export of excess protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy (Ricke, 2003). Organic acids can be toxic for microorganisms by membrane uncoupling capabilities. These compounds interfere with cytoplasmic membrane structure and proteins so that the electron transport is uncoupled and subsequent ATP production is reduced, dissipating pH values and electrical gradients across cell membranes (Axe and Bailey, 1995). This hypothesis was tested by adding dicarboxylic acids to the *P. aeruginosa* culture decreasing the pH value from 7.4 to 4.0.

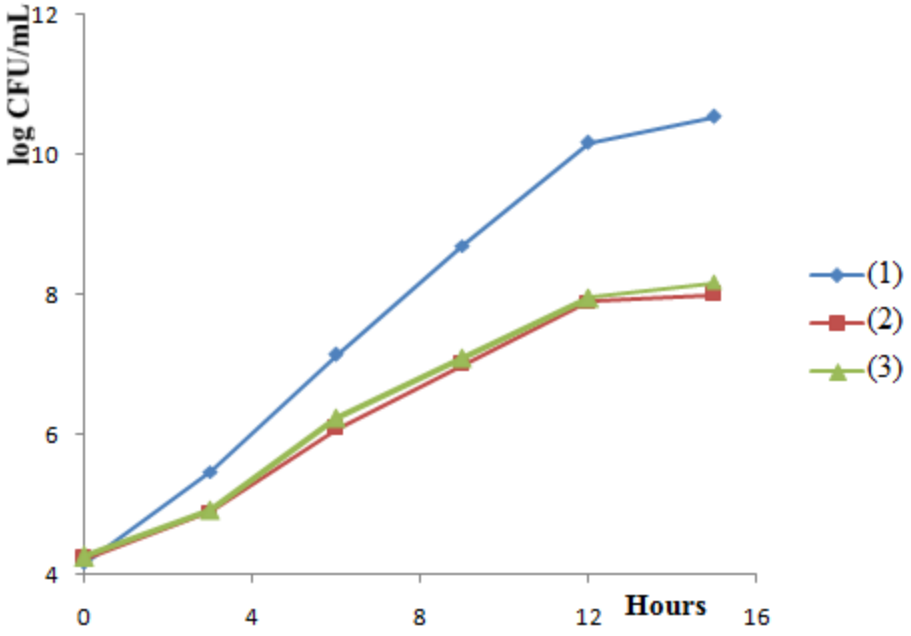
The effects of such pH decrease have been associated to the dissipation of the proton-motive force and inability to maintain internal pH followed by denaturation of acid-sensitive proteins and DNA (Ricke, 2003). Less direct antibacterial activities of organic acids have been reported and include interference with nutrient transport, cytoplasmic membrane damage resulting in leakage, disruption of outer membrane permeability and influencing macromolecular synthesis (Alakomi *et al.,* 2000).

Bacteria can develop resistance to dicarboxylic acids by adapting to acid stress and induction of acid tolerance response (Foster and Spector, 1995). No inhibition zone was detected when the disk diffusion method was used. This result let us conclude that MCL is only bacteriostatic not bactericidal against *P. aeruginosa*. Bacteria that grow around the disk can be also those that may metabolize organic acids present in MCL.

Antimicrobial activity of tested antibiotics

The resistant patterns of *P. aeruginosa* to the tested antibiotics are shown in Table 3. Only vancomycin, cefditoren and lomefloxacin showed a legible activity against this bacterium (MICs = 225, 1800 and 3.51 µg/ml, respectively).

Figure.1 Growth curves of *P. aeruginosa* at 37°C with and without treatment



(1) Control, (2) 56.25 µg/ml of malic acid + 56.25 µg/ml of vancomycin, (3) 56.25 µg/mL of oxalic acid + 112.5 µg/mL of vancomycin

Table.1 Activity of caper leaves methanol extract against bacterial and fungal strains using the microdilution method

Microorganism	**MIC (µg/ml)**
Gram (–)	
P. aeruginosa (ATCC 9027)	225
E. coli (ATCC 25922)	>900
S. typhi (ATCC 14024)	>900
Gram (+)	
L. monocytogenes (ATCC 35152)	>900
S. aureus (ATCC 25932)	>900
Fungal strains	
A. niger (ATCC 16404)	NA
A. flavus	NA
Penicillium spp	NA

NA means Not active.

Table.2 Silylated organic acid levels (mg/kg) determined by GC/MS analysis after derivatization with BSTFA and MIC values against *P. aeruginosa*

Compound	*Rt*(min)	KI	MW	concentration (mg/kg)	MIC (µg/ml)
Malonic acid	14.32	1051	248	2.8 ± 0.7	450
Benzoic acid	16.35	1169	194	148 ± 52	225
Succinic acid	20.60	1170	262	2.9 ± 0.9	450
Malic acid	31.09	1390	350	3.4 ± 1.6	450
p-coumaric acid	50.99	1674	308	4.7 ± 1.9	450

Silylated compounds are listed in order of elution from a DB5 (30 m, 0.25 mm, 0.25 µm) capillary column.

Table.3 MIC values of ten different antibiotics against *P. aeruginosa*

Antibiotic	MIC (µg/ml)
Ceftazidime	<0.43
Polymyxine B	<0.43
Gentamycin	<0.43
Lomefloxacin	3.51
Vancomycin	225
Cefditoren	1800
Cefixime	>1800
Rifaximin	>1800
Ciprofloxacin	>1800
Cefpodoxime	>1800

Table.4 Synergistic activity of investigated organic acids and antibiotics

Combination	FIC_{index}
Malic acid+vancomycin	0.37
Oxalic acid+vancomycin	0.5
p-coumaric acid+vancomycin	0.62
Benzoic acid+vancomycin	0.75
Succinic acid+vancomycin	1.12
Malonic acid+vancomycin	NT
Malic acid+cefditoren	NA
Oxalic acid+cefditoren	NT
p-coumaric acid+cefditoren	NT
Benzoic acid+cefditoren	NA
Succinic acid+cefditoren	NA
Malonic acid+cefditoren	NA

NT means Not tested; NA means Not active.

Synergistic test

The checkerboard method, used to evaluate synergism against *P. aeruginosa*, showed a synergistic activity between malic acid and vancomycin at 56.25 µg/ml each (FIC index = 0.37) and oxalic acid and vancomycin at 112.5 and 56.25 µg/mL, respectively (FIC index = 0.5) while no interaction was observed between organic acids and cefditoren against *P. aeruginosa* (Table 4). Furthermore, after growth experiments of this bacterium were performed in the same conditions reported above (concentrations of dicarboxylic acids and vancomycin), we observed a significant decrease in the number of viable bacteria ($P < 0.05$) (Figure 1).

Aburjai *et al.* found that the methanol extract of almost 19 Jordanian plants decrease the resistance of *P. aeruginosa* to nalidixic acid, chloramphenicol, gentamicin and cephalosporin (Abujari *et al.,* 2001).

In the present work we found that dicarboxylic acids, such as malic acid and succinic acid from caper leaves acted synergistically with vancomycin. The latter is an antibiotic that inhibits the cell wall synthesis, prevents incorporation of N-Acetyl muramic acid and N-Acethyl glucosamine of Gram (+) bacteria. The synergistic activity between dicarboxylic acids and this antibiotic against *P. aeruginosa* Gram (-) bacteria may be attributable to their combined reaction with the cell membrane porins as a possible primary target site by facilitation of the penetration of the antibiotic (Sikkema *et al.,* 1994). Another mode of synergistic action can be by blocking the inhibitory effects of protective enzymes or interfering with metabolic targets of the antibiotic (Hemaiswarya *et al.,* 2008). The addition of dicarboxylic acids can also alter vancomycin speciation in a manner that enhance vancomycin uptake by *P. aeruginosa*. This last hypothesis was confirmed by Zhang using tetracycline in the presence of dicarboxylic acids against *E. coli* (Zhang *et al.,* 2014). The antagonistic interaction between dicarboxylic acids and cefditoren could be explained by the competition for target sites or inhibition of cell uptake. Benzoic acid showed the same MIC as well as the methanolic extract with 250 µg/ml but no synergistic activity was noted when tested with antibiotics. This can be due to the concentration of this compound in caper leaves (142 ± 52 mg/kg).

Conclusively, it is challenging to develop new drugs and additives to face bacterial resistance and to ameliorate food conservation. The present work showed the antimicrobial activity of organic acids from caper and the synergistic activity of dicarboxylic acids with vancomycin, an antibiotic used usually against Gram (+) bacteria. Further studies are needed to confirm this synergistic activity and develop it.

At the present we focus on the isolation of the fraction of caper leaves methanolic extract, responsible of this activity, and asses this latter *in vivo* using real food substrates such as meat and fish samples.

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