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Synergism between hydrogen peroxide and seventeen acids against six bacterial strains

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Research paper on improvement of bactericidal efficacy of hydrogen peroxide in terms of synergy when associated with various acids.

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Abstract

Aims: The objective of this study was to evaluate the bactericidal efficacy of hydrogen peroxide administered in combination with 17 mineral and organic acids authorized for use in the food industry.

Methods and Results: The assays were performed on a 96-well microplate using a microdilution technique based on the checkerboard titration method. The six selected strains were reference strains and strains representative of contaminating bacteria in the food industry. Each synergistic hydrogen peroxide/acid combination found after 5-min contact time at 20°C in distilled water was then tested in conditions simulating four different use conditions. Thirty-two combinations were synergistic in distilled water; twenty-five of these remained synergistic with one or more of the four mineral and organic interfering substances selected. Hydrogen peroxide/formic acid combination was synergistic for all six bacterial strains in distilled water and remained synergistic with interfering substances. Six other combinations maintained their synergistic effect in the presence of an organic load but only for one or two bacterial strains.

Conclusions: Synergistic combinations of disinfectants were revealed, among them the promising hydrogen peroxide/formic acid combination.

Significance and Impact of the Study: A rapid screening method was proposed and used to reveal the synergistic potential of disinfectant and/or sanitizer combinations.

Introduction

Disinfectants used in Europe have to meet strict requirements of efficacy and safety for consumers and the environment (Anon. 1998). Hydrogen peroxide, a strong fast-acting antimicrobial agent controlling a wide spectrum of micro-organisms including bacteria, fungi, bacterial spores and viruses, is used for both its efficacy against contaminants and its capacity for complete decomposition in water and oxygen. The beneficial effects of this oxidizing product have long been recognized in ophthalmology (Krezanoski and Houlsby 1988; Kilvington 2004), in water treatment as a disinfectant and as an oxidant allowing effective control of organic load (Houtmeyers *et al.* 1977; Poffe *et al.* 1978; Ksibi 2006), in odontology for use in mouth washes (Walker 1988) or to assure the chemical decontamination of dental unit water systems (Zanetti *et al.* 2003; Szymanska 2006) and in food industry as disinfectant (Lechner 1975; Lillard and Thomson 1983), preservative (Collins 1971; Bruhl and Coote 1999) or food additive (Parish *et al.* 2003; Sapers and Jones 2006). This active compound disintegrates biofilms, thus freeing microbial cells and facilitating their destruction (Exner *et al.* 1987; Walker and Marsh 2007). Its action is affected by organic load, but not by pH. Sagripanti and Bonifacino (1996) showed that activity is little affected by changes in pH between pH 2 and 10 (in Block 2001). However, acids are frequently used in the food industry as cleaners (Loncin 1986; Sirami 1987), preservatives (Collins 1971; Bruhl and Coote 1999), microbial inhibitors (Gillet 1984; Exner *et al.* 1987; Sirami 1987; Krezanoski and Houlsby 1988; Cherrington *et al.* 1990, 1992; Parish *et al.* 2003; Raybaudi-Massilia *et al.* 2009) or veterinary

disinfectants (Böhm 1987). Acids, in particular mineral acids, are considered to be corrosive at high concentrations. Their microbicide effect is dependent on pH, or more accurately pKa. However, the molecular weight and composition of the anion portion of the acid can affect the acid's ability to diffuse through the cell wall. The fungicidal and bactericidal efficacy of acids varies depending on the strain considered (Martin and Maris 2005). Moreover, acids improve the stability and efficacy of peroxygens (Baldry and Fraser 1988). The bactericidal and fungicidal effects of the combination of hydrogen peroxide with peracetic acid or acetic acid are well established. Moreover, antimicrobial washing solutions containing combinations of hydrogen peroxide with certain other acids, such as citric acid (Ukuku *et al.* 2005), lactic acid (Lin *et al.* 2002; Venkitanarayanan *et al.* 2002; Rupasinghe *et al.* 2006), have also been found to be effective against *Escherichia coli*, *Listeria monocytogenes* or *Salmonella enterica*. We investigated the bactericidal efficacy of hydrogen peroxide as disinfectant when associated with a mineral or an organic acid. We selected 17 acids representative of a large diversity of acids' chemical structures. Before European regulation comes into force, most of them had been authorized for use as disinfectant and/or food additives and technological auxiliaries by French regulation (Anon. a 1997; Anon. a 2006). Some of them (formic, benzoic, lactic acids) are included in the European review programme (Anon. 2007) within the framework of European Biocides Directive (Anon. 1998) and are in assessment phase for use as disinfectants in food and feed domains. The efficacy of such combinations was tested towards a panel of bacterial strains including reference strains and bacteria associated with foodborne diseases or contamination in the food industry. A micromethod developed in our laboratory (Martin and Maris 1993) was used to study the synergistic effect of the association of hydrogen peroxide with each of the 17 selected acids, and to test the maintenance of the synergistic effects of selected combinations in conditions simulating practical utilization planned for disinfectants.

Materials and methods

Chemicals

Hydrogen peroxide and the 17 mineral and organic acids were provided by three suppliers. Hydrogen peroxide (50% m/v), phosphoric acid (purity 84%), nitric acid (purity 65–69%), formic acid (purity 100%), acetic acid (purity 100%) and oxalic acid (purity 75%) were provided by Prolabo (VWR, Fontenay-sous-Bois, France). Boric acid (purity 99·8%), sulphuric acid (purity 95–97%), citric acid (purity 91·4%), benzoic acid (purity 91·4%), adipic acid (purity 100%), glutaric acid (purity > 99%), propionic acid (purity 99%), succinic acid (purity 99·5%), tartaric acid (purity 99·5%) and mandelic acid (purity 99%) were provided by Merck (VWR, Fontenay-sous-Bois, France). Sulphamic acid (purity 100%) and lactic acid (purity 85%) were provided by Sigma (Saint-Quentin-Fallavier, France). Stock solutions were prepared in sterile distilled water at 1% (m/v) and 2% (m/v) for adipic and boric acids, at 2% (m/v) for benzoic acid, at 5% (m/v) for oxalic and succinic acids, at 10% (m/v) for mandelic and sulphamic acids and at 20% (m/v) for citric, glutaric, tartaric acids or sulphuric and nitric acids. Stock solutions of the other acids (formic, acetic, phosphoric, propionic and lactic acids) were prepared, when necessary, at 20%, 40% or 80% (v/v).

Bacterial strains

We selected six bacterial strains, including the reference strains *Enterococcus hirae* ATCC 10541, *Staphylococcus aureus* ATCC 9144, *Escherichia coli* ATCC 11229 and *Pseudomonas aeruginosa* CIP A22 approved by the current European and/or French standards; these four strains were used in suspension tests to determine the bactericidal efficacy of disinfectants. We also included two bacteria associated with foodborne diseases or contamination in the food industry, *L. monocytogenes* ATCC 19115 and *Salmonella typhimurium* 276 (internal numbering). This last strain, provided by the Laboratory of Bovine Pathology (Anses-Lyon, France), was isolated from bovine breeding environment and frequently used as representative of *Salmonella* sp. in our laboratory to test the bactericidal efficacy of disinfectants against this genus. Bacterial suspensions were prepared from stock cultures stored at −70°C.

Assay conditions

Combinations were tested first in sterile distilled water. Then, synergistic combinations were tested using interfering substances. Four interfering substances were chosen among those recommended by the French standard institution (A.F.NOR) in NF T 72-170 (Anon. 1988). Two hard water levels (300 mg l^{-1} and 600 mg l^{-1} $CaCO_3$) were tested to evaluate the impact of water hardness on bactericidal activity of combinations. Two organic substances were chosen, one to simulate clean conditions (0·3% bovine albumin with hard water at 300 mg l^{-1} $CaCO_3$) and the other to simulate dirty conditions (1% bovine albumin + 1% yeast extract) according to the conditions of use of products in food industry area. The concentrations indicated were the final concentrations in the assays.

Test procedure

The ND/1500 checkerboard titration method (see description of ND/1500 method below) was used also to study the efficacy of each component of the combination against each strain. The minimal bactericidal concentrations (MBC) were thus obtained under basic conditions (with distilled water) or conditions simulating the practical use (with interfering substances). The ND/1500 method was based on the checkerboard titration method which has long been used for antibiotics (Krogstad and Moellering 1980). It was developed in our laboratory to test disinfectant combinations against bacteria (Martin and Maris 1993). This method was developed in line with the recommendations of the A.F.NOR NF T 72-150 (Anon. 1987) and NF T 72-170 (Anon. 1988). The ND/1500 method was used to prevent insufficient neutralization of the products. This method involved four steps, combining the use of a 1/1500 dilution with the use of a chemical neutralizer to ensure sufficient neutralization of the products. In the first step, dilutions of each product were prepared in tubes and immediately distributed in a first standard 96-well microplate (Falcon, AES chemunex, Bruz, France). Serial twofold dilutions of each product were prepared in distilled water at the following concentrations test: 4 × MBC, 2 × MBC, MBC, 1/2 × MBC, 1/4 × MBC, 1/16 × MBC, 1/32 × MBC, 0. Successively, the eight dilutions of the two products were added (multipipet 50–200 μl, Labsystem; Cergy-Pontoise, France) in microplate 1. The acid control (concentration 0 without acid) was distributed first into wells 1 to 8 (100 μl per well) of row H, and the highest acid concentration (4 × MBC) was distributed last into wells 1 to 8 (100 μl per well) of row A. Then, the hydrogen peroxide control (concentration 0 without hydrogen peroxide) was distributed into wells A to H (100 μl per well) of column 1, and the highest hydrogen peroxide concentration (4 × MBC) was distributed into wells A to H (100 μl per well) of column 8, microplate 1. Among the 64 combinations so prepared, 49 of them were mixtures of 100 μl acid with 100 μl hydrogen peroxide, 7 of them were mixtures of 100 μl of each acid dilution with 100 μl distilled water, seven of them were mixtures of 100 μl of each hydrogen peroxide dilution with 100 μl distilled water, and the last one (well H1) was without any product (200 μl distilled water). These 14 wells filled with products alone were used to measure their MBC values during ND/1500 assay. In the second step, these 64 combinations were transferred into a microplate 2 and left in contact with bacterial suspension. So, 75 μl of the 64 combinations was immediately transferred into 64 wells of microplate 2 already filled with 60 μl of distilled water or interfering substances and 15 μl inoculum at 1×10^8 or 3×10^8 CFU ml^{-1} (in sterile peptone saline diluent). These reaction mixtures were left in contact for 5 min (±10 s) at ambient temperature (about 20°C). In the third step, after contact time, the bactericidal action of combinations of products was immediately stopped using two transfers to a neutralizing solution. Reaction mixtures (10 μl) in microplate 2 were first diluted (using multipipet 5–50 μl; Labsystem) in 90 μl of pre-prepared neutralizing solution filled into a third microplate. After stirring, 10 μl of the first neutralized mixtures from microplate 3 was transferred into 1·5 ml of the same neutralizing solution already distributed in transfer tubes (2-ml sterile transfer tubes, Dutscher; Issy-Les-Moulineaux, France). The time must not exceed 15 s for the first transfer and 30 s for the second. The neutralization solution used consisted of lecithin 3 g; histidine l g; disodium phosphate 34 g; sodium thiosulphate 5 g; polysorbate 80 15 g; and distilled water 1000 ml (sterilization at 121°C ± 1°C during 20 min). This neutralizing solution was diluted in sterile distilled water (1/10) just before assay. After stirring and a minimal 10-min exposure time, transfer tube contents were poured into Petri dishes and immediately covered with the appropriate agar. Bacterial cultures were incubated on PCA agar (Difco, Beckton-Dickinson, Le Pont de Claix, France). Agar medium was kept at 48°C in a water bath before pouring. *Ent. hirae* was incubated at 37°C for 72 h; other bacterial strains were incubated at 37°C for 48 h. We then noted for each agar plate culture growth or total bacterial destruction. MBC value of a product was the lowest product concentration for which no bacteria were detected on agar plates. Synergistic combinations were detected and their strength was calculated when no bacteria were detected on agar plates corresponding to the combinations of products at the lowest concentrations. For each MBC and each synergistic combination, pH value in assay conditions was measured.

Control of the neutralization and validation of the results

For each ND/1500 assay, the neutralization step was systematically controlled during checking of MBC value of hydrogen peroxide alone and acid alone as previously described (Martin and Maris 1993, 2005). So, 10 μl of products dilutions was transferred from microplate 1 to a fourth microplate pre-prepared with neutralizing solution (190 μl per well). After stirring and 10-min contact, 10 μl of neutralized dilutions of the microplate 4 was immediately transferred into transfer tubes containing 1·5 ml neutralizing solution. Then, 100 μl bacterial suspension (diluted to have 1 or 3 10^2 CFU/transfer tube) was inoculated into transfer tubes and left at least 15 min in contact with the neutralized products

(including concentrations $\geq$ MBC value). Neutralization was considered sufficient if results of numerations collected on agar plates for neutralized product concentrations were higher or equal to the half of the control numeration. To definitely validate our results, the consistency of MBC values was checked for each product with the MBC values using a reference method (Maris *et al.* 1982). If acid MBC value and hydrogen peroxide MBC value measured in ND/1500 assay were in accordance with the MBC values (±1 dilution) of reference method, just one experiment was entertained. If discordance was noticed for only one product, experiments were repeated until MBC value of each product was in conformity with reference method or until having at least one confirmation of the result (in the case of MBC value with ND/1500 $\geq$ 4 $\times$ MBC value with reference method).

Exploitation of results

Minimal bactericidal concentrations (MBC): for the microplate row or column corresponding to each product alone, bacterial growth or total microbial destruction was noted. First, dilutions without bacterial growth were considered to be the minimal bactericidal concentrations (MBC). At these concentrations, 4 log reduction in viable count was theoretically reached. MBC values were expressed in acid or hydrogen peroxide percentages (v/v or m/v).

Fractional bactericidal concentration Index (FBC): each result was expressed as a fractional bactericidal concentration index representing the degree of interaction of products when used in association. FBC values were expressed as a fraction of the MBC (FBC = MBC product in association/MBC product alone). We then calculated the sum of the FBC values (Σ FBC = MBC product A in association/MBC product A + MBC product B in association/MBC product B) for the interpretation of the results based on the following criteria:

i Σ FBC $\leq$ 0·50: synergism,
ii 0·50 < Σ FBC < 2: addition,
iii Σ FBC $\geq$ 2: antagonism.

Interference indices: to facilitate the interpretation of data on the effect of the hard water and organic substances on the efficacy of the combinations, MBC values representing optimal synergy were converted into interference indices. These interference indices corresponded to the ratio of the MBC value of each product when tested in combination in the presence of an interfering substance (hard water or organic substance) to the MBC value of the same product tested in combination in the absence of an interfering substance. We used the interference classification system previously described (Guiraud-Dauriac and Crémieux 1984) to determine the effect of each of the four interference substances on each disinfectant tested in combination, for each species. The intervals, chosen arbitrarily, were as follows:

i Class 1: no effect, interference index $\leq$ 1.
ii Class 2: weak effect, interference index between 1·1 and 5.
iii Class 3: intermediate effect, interference index between 5·1 and 10.
iv Class 4: strong effect, interference index between 10·1 and 25.
v Class 5: very strong effect, interference index > 25.

Results

Bactericidal efficacy of hydrogen peroxide in distilled water

Median MBC values (±1 dilution) obtained in basic assay conditions using the ND/1500 micromethod showed that hydrogen peroxide was bactericidal at concentrations varying from 1·56% for the more sensitive strains (*E. coli* and *Ps. aeruginosa*) to 12·5% for the less sensitive strain (*Ent. hirae*). The means and the standard errors calculated from the 17 results of MBC collected for each strain in distilled water conditions gave a more precise result for these MBC values and confirmed the results furnished by median values (Table 1). The hydrogen peroxide MBC values collected for each synergistic combination detected in basic assay conditions during each ND/1500 checkerboard titration assay are indicated in Table 2.

Bactericidal efficacy of the 17 mineral and organic acids

MBC values obtained for the 17 acids against the six strains tested were previously published by Martin and Maris (2005). For each MBC, the pH reached in assay conditions was measured and the ratio between undissociated and dissociated acid was calculated. The results obtained were analysed separately for each strain and for each acid. For each bacterium tested, between 11 and 14 acids displayed bactericidal activity. The most sensitive bacterial strain was *Ps. aeruginosa*, which was killed at acid concentrations varying between 0·006–0·009% (sulphuric, phosphoric, oxalic, nitric acids) and 0·5–1·25% (propionic, acetic, glutaric acids). The most resistant strains were *Ent. hirae* and *Staph. aureus*, for which MBC values in acid varied between 0·15–0·625% (nitric, sulphuric, oxalic acids) and 10–40% (glutaric, acetic, lactic, phosphoric acids). The MBC values of acids collected for each synergistic combination detected in basic assay conditions during each ND/1500 checkerboard titration assay are indicated in Table 2.

Table 1 Minimal bactericidal concentrations values of hydrogen peroxide in distilled water with ND/1500 method (median and mean values)

Strains	MBC*% median value (m/v or v/v) (± 1 dilution)	MBC*% mean value ± standard error (m/v or v/v)
Enterococcus hirae ATCC 10541	12·5	12·50 ± 0
Staphylococcus aureus ATCC 9144	3·12	3·58 ± 1·33
Listeria monocytogenes ATCC 19115	6·25	4·78 ± 1·61
Salmonella sp. 276	3·12	2·75 ± 1·52
Escherichia coli ATCC 11229	1·56	1·56 ± 0·68
Pseudomonas aeruginosa CIP A22	1·56	1·88 ± 1·21

*Minimum number of assays per result: 17.

Characteristics of the combinations of products: detection of synergism

Synergistic combinations detected for Gram-negative and Gram-positive bacteria are shown in Table 2. For each synergistic combination, the MBC values of hydrogen peroxide (%) and acid (%) and pH values are indicated either when tested alone or when tested in combination. Σ FBC values calculated from the 102 ND/1500 checkerboard titration assays are listed in Table 3. The number of combinations showing synergistic effects is indicated for each acid. We found 32 combinations showing synergism after 5-min contact time at 20°C. Hydrogen peroxide combined with formic acid had synergistic effects on all six strains. Combinations with acetic acid or oxalic acid were synergistic for five and four of the six strains, respectively. Synergistic action of the remaining hydrogen peroxide/acid combinations was observed on only two of the strains for five acids and on only one strain for seven of the remaining acids. Strains affected by lactic and tartaric acids were two Gram-positive bacteria (*Ent. hirae* and *L. monocytogenes*). Those affected by nitric and succinic acids were two Gram-negative bacteria (*Salmonella* sp. and *Ps. aeruginosa*). Concerning citric acid, one Gram-negative bacterium and one Gram-positive bacterium (*Ent. hirae* and *Salmonella* sp.) were affected. No synergism was found on bacteria after 5-min contact time for glutaric and boric acids, and no antagonistic effect was detected within the range of acid concentrations tested in this study. In conclusion, among the 32 combinations with synergistic effect, 14 of them had a Σ FBC value equal to 0·50, corresponding to a combination of each product at a concentration four times less than MBC value measured for the product alone. The strongest synergistic effects (Σ FBC = 0·28 or 0·12) were found for combinations of hydrogen peroxide with formic acid or for certain product combination/bacterial strain pairs. Thus, the concentration of hydrogen peroxide needed to detect bactericidal effects on *Ent. hirae* decreased from 12·5% to 0·78% upon combination with 0·312% formic acid, to 1·56% with 2·5% phosphoric acid and to 3·12% with 5% tartaric or citric acid, 2·5% acetic or lactic acid, 0·625% sulphamic acid or with 0·156% oxalic acid. The concentration of hydrogen peroxide needed to kill the sensitive Gram-negative bacterium *Ps. aeruginosa* decreased from 1·56% to 0·195% - 0·039% when combined with 0·0015% formic acid, 0·002% nitric acid, 0·0195% succinic acid, 0·062% propionic acid, 0·156% acetic acid, 0·25% adipic acid or 0·025% benzoic acid. *Staph. aureus* was also killed after 5 min of treatment with 0·39% hydrogen peroxide combined with 0·156% formic acid. For *E. coli*, synergisms were found when 0·19%, 0·31% and 0·39% hydrogen peroxide were respectively combined with 0·078% formic acid, 0·625% acetic acid and 0·078% oxalic acid. Results expressed in percentage of product concentration (%) at the point of optimal synergistic effect are summarized in Table 2.

Maintenance of synergism between hydrogen peroxide and acids in the presence of interfering substances

We determined whether the synergistic effects detected under basic assay conditions were maintained in conditions that simulate those of the intended practical use of the disinfectants. We thus tested the 32 synergistic combinations selected (Σ FBC $\leq$ 0·50) in the presence of the four interfering substances described above, using 128 assays. The concentration of each component of the hydrogen peroxide/acid combination and pH values at the optimal point of synergy in distilled water, and at the optimal point of synergy detected in the presence of interfering substances, are indicated for combinations in which synergism was still detected (Table 2). Twenty-six of the combinations tested demonstrated synergistic effects in the presence of at least one interfering substance (mineral or organic), 17 synergistic combinations showed in the presence of the two hard water solutions (300 and 600 mg l^{-1} $CaCO_3$) and 12 with at least one of the two organic interfering substances. Synergism was maintained in the presence of all the four interfering substances for only seven product combination/bacterial strain pairs among the 32 synergistic couples selected in distilled water: four pairs using formic acid combination, one with acetic acid, one with propionic acid and one with succinic acid.

Table 2 Values of acid and hydrogen peroxide concentrations (%/%) and pH measured in combinations staying synergistic with interfering substances

Acid	Combination	$Acid/H_2O_2$		$Acid/H_2O_2$		$Acid/H_2O_2$		$Acid/H_2O_2$		$Acid/H_2O_2$		MBC of acid* alone		MBC of H_2O_2†alone	
	Assay conditions	Distilled water		300 mg l^{-1} $CaCO_3$		600 mg l^{-1} $CaCO_3$		Bovine albumin (0·3%)		Bovine albumin + yeast extract (1%)		Distilled water		Distilled water	
In combination with H_2O_2	Strain	%/%	pH	%/%	pH	%/%	pH	%/%	pH	%/%	pH	(%)	pH	(%)	pH
Formic	*Enterococcus hirae*	0·312/0·78	2·65	0·156/0·39	2·76	0·078/0·39	2·90	0·312/1·56	2·64	0·625/3·12	3·13	5	2·02	12·5	4·72
	Staphylococcus aureus	0·156/0·39	2·82	0·078/0·39	2·90	0·156/0·39	2·72	0·156/0·78	3·19	0·312/1·56	3·33	1·25	2·37	3·12	6·14
	Listeria monocytogenes	0·039/0·39	3·15	0·078/0·78	2·90	0·078/0·78	2·91	0·078/1·56	3·47	0·625/0·78	3·22	0·156	2·86	6·25	5·59
	Salmonella sp.	0·039/0·78	3·13	0·078/0·19	2·93	0·039/0·39	3·03	0·078/0·78	3·44	_		0·156	2·86	3·12	6·14
	Escherichia coli	0·078/0·19	3·02	0·078/0·3	2·92	_‡		_		_		0·312	2·70	1·56	6·58
	Pseudomonas aeruginosa	0·0015/0·195	4·50	0·0031/0·39	3·25	0·0125/0·39	3·28	0·025/0·39	3·64	0·025/0·39	4·26	0·025	3·17	1·56	6·58
						0·0062/0·7	3·43								
Acetic	*Enterococcus hirae*	2·5/3·12	2·53	2·5/3·12	2·35	2·5/6·25	2·23	_		_		10	2·42	12·5	4·72
	Staphylococcus aureus	1·25/1·56	2·61	1·25/1·56	2·52	1·25/1·56	2·53	_		_		5	2·60	6·25	5·29
	Listeria monocytogenes	1·25/1·56	2·61	_		1·25/0·78	2·56	_		_		5	2·60	6·25	5·59
	Escherichia coli	0·625/0·31	2·78	_		0·625/0·048	2·77	1·25/0·78	3·04	_		2·5	2·78	1·56	6·58
	Pseudomonas aeruginosa	0·156/0·39	3·29	0·039/0·39	3·44	0·078/0·39	3·37	0·312/0·39	3·63	0·625/0·39	3·93	0·625	3·10	1·56	6·58
Oxalic	*Enterococcus hirae*	0·156/3·12	1·96	0·312/1·5	1·74	_		_		_		0·625	1·70	12·5	4·72
	Staphylococcus aureus	0·078/1·56	2·26	0·078/0·39	2·26	0·078/0·78	2·06	_		_		0·312	1·92	6·25	5·59
	Listeria monocytogenes	0·039/0·78	2·56	_		_		_		_		0·312	1·92	3·12	6·14
	Escherichia coli	0·078/0·39	2·29	0·039/0·19	2·57	0·078/0·3	2·07	_		_		0·312	1·92	1·56	6·58
						0·039/0·78	2·32								
Nitric	*Salmonella* sp.	0·019/0·39	2·08	_		0·039/0·78	1·78	_		_		0·078	2·20	1·56	6·58
	Pseudomonas aeruginosa	0·0024/0·39	3·05	0·0048/0·09	2·71	0·0048/0·78	2·70	_		_		0·009	3·10	1·56	6·58
Lactic	*Enterococcus hirae*	2·5/3·12	1·98	0·312/1·56	1·93	0·625/0·78	2·34	—‡		——		10	1·98	12·5	4·72
	Listeria monocytogenes	0·078/1·56	2·84	0·625/0·39	2·31	1·25/0·39	2·13	—		—		1·25	2·47	6·25	5·59
Succinic	*Salmonella* sp.	1·25/0·195	2·67	0·625/0·39	2·86	—		—		—		5	2·39	3·12	6·14
	Pseudomonas aeruginosa	0·019/0·39	4·50	—		0·039/0·195	3·51	0·312/0·19	3·77	0·078/0·78	5·03	0·078	3·40	1·56	6·58
Tartaric	*Listeria monocytogenes*	0·625/1·56	2·25	0·625/0·78	2·12	5/0·19	1·66	2·5/1·56	1·89	—		5	2·04	6·25	5·59
				2·5/0·39	1·85										
	Enterococcus hirae	5/3·12	1·61	—		—		—		—		≥20	≤1·29	12·5	4·72
Citric	*Salmonella* sp.	0·625/1·56	2·15	—		—		—		—		5	2·06	6·25	5·59
	Enterococcus hirae	5/3·12	1·61	5/1·56	1·62	ND		5/3·12	1·67	—		≥20	≤1·46	12·5	4·72
Propionic	*Pseudomonas aeruginosa*	0·0625/0·39	3·54	0·0625/0·195	3·43	0·25/0·78	3·08	0·125/0·39	4·32	0·50/0·19	4·24	0·50	2·72	1·56	6·58
Phosphoric	*Enterococcus hirae*	2·5/1·56	1·11	2·5/0·39	1·14	5/0·39	0·87	—		—		10	1·08	12·5	4·72
Mandelic	*Salmonella* sp.	0·156/0·39	3·27	0·078/0·39	3·37	0·078/0·78	3·36	0·156/1·56	3·38	—		0·625	2·62	3·12	6·14

(continued)

Table 2. *(Continued)*

Acid	Combination	$Acid/H_2O_2$		$Acid/H_2O_2$		$Acid/H_2O_2$		$Acid/H_2O_2$		MBC of acid* alone		MBC of H_2O_2†alone
	Assay conditions	Distilled water		300 mg l^{-1} $CaCO_3$		600 mg l^{-1} $CaCO_3$		Bovine albumin (0·3%)		Bovine albumin + yeast extract (1%)		Distilled water		Distilled water
In combination with H_2O_2	Strain	%/%	pH	%/%	pH	%/%	pH	%/%	pH	%/%	pH	(%)	pH	(%)	pH
Sulphamic	*Enterococcus hirae*	0·625/3·12	1·97	—		—		—		—		2·50	1·30	12·5	4·72
Sulphuric	*Salmonella* sp.	0·039/0·78	3·07	—		—		—		—		0·156	2·05	3·12	5·59
Benzoic	*Pseudomonas aeruginosa*	0·025/0·039	3·32	—		—		—		—		0·10§	ND	1·56	6·58
Adipic	*Pseudomonas aeruginosa*	0·25/0·39	ND	—		—		—		—		≥1	ND	1·56	6·58

ND, not determined.
*MBC of acid: value of MBC (%) measured for acid alone during ND/1500 assay in distilled water conditions.
†MBC of H_2O_2: value of MBC (%) measured for H_2O_2 alone during ND/1500 assay in distilled water conditions.
‡Combination found not synergistic in the assay(s) condition(s) (0·50 < ΣFBC <2).
§‰.

Interference indices: the effect of mineral or organic substances on MBC values of acid and hydrogen peroxide in a synergistic combination

Interference indices calculated for hydrogen peroxide were mostly class 1 (interference index values of 0·25, 0·50 to 1) in assays using hard water and were mostly class 2 (interference index values of 2 or 4) for assays showing synergism in the presence of organic interfering substances. Interference indices calculated for acids were generally similar to those calculated for hydrogen peroxide (class 1 and 2). However, a class 3 interference indices (interference index value of 8 for example) were obtained for *L. monocytogenes* with lactic and tartaric acids in hard water conditions, and for two bacteria, class 4 interfering indices were calculated (with a value of 16, classed as a 'strong' effect). The class 4 interfering index values were found for *L. monocytogenes* (treated with a formic acid–containing combination in the presence of 1% bovine albumin and 1% yeast extract and a lactic acid combination in the presence of 600 mg l^{-1} $CaCO_3$) and for *Ps. aeruginosa* (treated with formic and succinic acid combinations in the presence of the two organic interfering substances) (Table 4).

Discussion

Hydrogen peroxide demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, fungi and bacteria spores. Generally, greater effects were seen in Gram-negative than in Gram-positive bacteria (Block 2001). Our data cannot be easily compared with results cited in papers because of the variable contact times, assay temperatures and directions for use claimed. Our results confirmed the bactericidal effect of hydrogen peroxide between 1·56% (±0·68) – 2·75% (±1·52) for Gram-negative bacteria and 3·58% (±1·3) – 12·5% (0) for Gram-positive bacteria. Concentrations between 1% and 5% hydrogen peroxide are generally indicated in food washing treatments to reduce microbial populations on alfalfa sprouts, cantaloupe, melons or asparagus for a few minutes of contact time (Parish *et al.* 2003). For example, a previous study investigating the efficacy of 2·5% and 5% hydrogen peroxide washing treatments by submerging melons during 5 min found that these treatments caused a 3 $\log_{10}$ CFU cm^{-2} reduction in *Salmonella* spp. on all melon surfaces (Ukuku 2004). The same conclusions were reported by Abadias *et al.* (2011) and Alexandre *et al.* (2012), and these last authors reported that the highest total mesophile reductions (2·26 ± 0·38 and 1·59 ± 0·41 log units) were reached after washing of strawberries with respectively 5% and 1% chemical solutions of hydrogen peroxide.

Table 3 Recapitulative table of the synergistic combinations detected in distilled water. The synergy is expressed as the sum of the fractional bactericidal concentrations (Σ FBC)

Nature and characteristics of the acid in combination with hydrogen peroxide			Σ FBC						Number of synergistic combinations per acid
Acid	MW (g mol^{-1})	$pK_{1;2;3}$	*E. hirae*	*S. aureus*	*L. monocytogenes*	*S.* sp.	*E. coli*	*P. aeruginosa*	
Formic	46·02	3·74	0·12	0·37	0·31	0·50	0·37	0·37	6
Acetic	60·05	4·74	0·50	0·50	0·50	*	0·50	0·50	5
Oxalic	90·04	1·27; 4·27	0·50	0·50	0·37	*	0·50	*	4
Nitric	63·02	*[a]	*	*	*	0·50	*	0·50	2
Lactic	90·08	3·83	0·50	*	0·31	*	*	*	2
Succinic	118·09	4·16; 5·61	*	*	*	0·31	*	0·37	2
Tartaric	150·09	2·95; 4·25	$\leq$0·50	*	0·37	*	*	*	2
Citric	192·12	3·14; 4·77; 6·39	$\leq$0·50	*	*	0·37	*	*	2
Propionic	74·08	4·87	*	*	*	*	*	0·31	1
Sulphuric	98·08	†; 1·92	*	*	*	0·50	*	*	1
Benzoic	122·1	4·2	*	*	*	*	*	0·28	1
Sulphamic	97	1	0·50	*	*	*	*	*	1
Phosphoric	98	2·15; 7·21; 12·67	0·37	*	*	*	*	*	1
Mandelic	152·14	3·40	*	*	*	0·37	*	*	1
Adipic	146·14	4·42; 5·42	*	*	*	*	*	$\leq$0·50	1
Glutaric	132·11	4·34	*	*	*	*	*	*	0
Boric	61·83	9·24	*	*	*	*	*	*	0

MW, molecular weight; $pK_{1;2;3}$, pK values found coming from http://sakura.cpe.fr/pka.html

† = strong acid.

[a]Combination not synergistic (0·50 <ΣFBC <2).

Concerning the acids, the MBC values measured for the 17 acids against the six strains selected for this study were determined and discussed in a previous paper (Martin and Maris 2005). Variable bacteriostatic and bactericidal effects of organic acids such as acetic, lactic, citric, malic and tartaric acids have previously been demonstrated in culture media and food systems for concentrations varying between 0·5 and 5% and contact times varying between 2 and 30 min on bacteria such as *L. monocytogenes, Salmonella* spp. or *E. coli* 0157:H7 (Whright *et al.* 2000; Wu *et al.* 2000). More recently, Martinez-Tellez *et al.* (2009), investigating the efficacy of sanitizers such as chlorine, hydrogen peroxide and lactic acid against *Salm. typhimurium* inoculated in fresh green asparagus and green onions, found that the most effective sanitizer reducing growth close to 3 $\log_{10}$ CFU g^{-1} during short exposure times (40, 60, 90 s) was the 2% lactic acid solution. Acid concentrations used in commercial products to reduce microbial numbers on meat, vegetables or fruits, applied using longer contact times, are similar to or lower than the minimal bactericidal concentrations obtained in this study.

Very few protocols have been proposed to evaluate the synergy between disinfectant products. Thus, fifteen years ago, we developed and validated a micromethod similar to the checkerboard method used in antibiotherapy to study the efficiency of combinations of antiseptic and disinfectant molecules (Martin and Maris 1993). This method was established to reproduce criteria of the standards currently in force (inoculum concentration, temperature and contact time, nature of the interfering substances, neutralization). These criteria coming from the French AFNOR standards described above are similar to those of the current European standards EN 1040, EN 1276 (Anon. b 1997, 2006) used to test the bactericidal efficacy of antiseptics and disinfectants. We were deliberately interested in keeping combinations for which a synergy was detected towards both molecules. That is why a synergistic effect was concluded when the $\sum$ FBC was $\leq$ 0·50 and not $\leq$ 0·75 as in Alasri *et al.* (1993) study. These authors used the checkerboard titration method and defined the degree of synergy between hydrogen peroxide and acetic acid when used in combination as previously described by Berenbaum (1978). They used criteria defined by a fractional sporicidal concentration index (FSC) $\leq$0·75 and found nine synergistic combinations (see the example on *Bacillus subtilis* ATCC 6633). We also found combinations with a FBC index equal to 0·75, but we considered them as additive combinations and did not mention them. So, at the concentra-

Table 4 Interference indices calculated for acid and hydrogen peroxide for each combination remaining synergistic in the presence of interfering substances

		Acid				H_2O_2			
Strain	Acid	300 mg l^{-1} $CaCO_3$*	600 mg l^{-1} $CaCO_3$†	OM 1‡	OM 2§	300 mg l^{-1} $CaCO_3$	600 mg l^{-1} $CaCO_3$	OM 1	OM 2
Enterococcus hirae	Formic	0·50	0·25	1	2	0·50	0·50	2	4
	Acetic	1	1	–	–	1	2	–	–
	Oxalic	2	–	–	–	0·50	–	–	–
	Lactic	0·12	0·25	–	–	0·50	0·25	–	–
	Phosphoric	1	2	–	–	0·25	0·25	–	–
	Citric	1	–	1	–	0·50	–	1	–
Staphylococcus aureus	Formic	0·50	1	1	2	1	1	2	4
	Acetic	1	1	–	–	1	1	–	–
	Oxalic	1	1	–	–	0·25	0·50	–	–
Listeria monocytogenes	Formic	2	2	2	16	2	2	4	2
	Acetic	–	1	–	–	–	0·50	–	–
	Lactic	8	16	–	–	0·25	0·25	–	–
	Tartaric	4	8	4	NC**	0·25	0·12	1	NC
Salmonella sp.	Formic	2	1	2	–	0·25	0·50	1	–
	Nitric	–¶	2	–	–	–	2	–	–
	Succinic	0·50	–	–	–	2	–	–	–
	Mandelic	0·50	0·50	1	–	1	2	4	–
Escherichia coli	Formic	1	–	–	–	2	–	–	–
	Acetic	–	1	2	–	–	0·12	2	–
	Oxalic	0·50	1	–	–	0·50	1	–	–
Pseudomonas aeruginosa	Formic	2	4	16	16	2	4	2	2
	Acetic	0·25	0·50	2	4	1	1	1	1
	Nitric	2	2	–	–	0·25	2	–	–
	Propionic	1	4	2	2	0·50	2	1	0·50
	Succinic	–	2	16	16	–	0·50	0·50	2

*300 mg l^{-1}: Hard water at 300 mg l^{-1} $CaCO_3$.
†600 mg l^{-1}: Hard water at 600 mg l^{-1} $CaCO_3$.
‡OM 1: 0·3% bovine albumin (in hard water at 300 mg l^{-1} CaCO3).
§OM 2: 1% bovine albumin + 1% yeast extract.
¶$0.50 < \Sigma FBC < 2$ and combination not synergistic.
**NC, interference indices not calculated (the MBC value was not reached).

tion ranges tested for each acid and for hydrogen peroxide, we identified several additive combinations, but did not detect any antagonistic effect.

Our findings suggest the significant benefit of using such combinations on pathogenic bacteria such as *L. monocytogenes* and S*almonella* sp., but their effects on the resistant bacteria *Ent. hirae* seem to be of particular interest (Tables 2 and 3). The term 'synergism' applies to the bactericidal effects observed for combinations of hydrogen peroxide and acids at concentrations at least four times less than their initial MBC values. The MBC values of the acids are related to their nature, their molecular weight, their dissociation capacity and the type of microbe that they kill or inhibit (Martin and Maris 2005). Schurman (2001) found a strong antimicrobial effect of 0·1% or 0·3% malic acid on *E. coli* in apple cider when used in combination with 0·012% or 0·017% hydrogen peroxide, but after 24-h incubation at 25°C, and found that a combination of 0·3% tartaric acid with 0·017% hydrogen peroxide reduced *E. coli* O157:H7 levels in purple grape juice, even at 4°C. A beneficial effect of a mixture of 2% hydrogen peroxide with 1·5% lactic acid was previously demonstrated on *S. enterica, L. monocytogenes* and *E. coli* O157:H7 after 5 min of treatment at 22°C on freshly cut lettuce leaves (Lin *et al.* 2002). Combinations of 1·5% hydrogen peroxide and 1·5% lactic acid were also found to be effective on *Salmonella* sp. and *L. monocytogenes* after 15 min of contact at 40°C on apples, oranges and tomatoes by Venkitanarayanan *et al.* (2002) and on apples by Rupasinghe *et al.* (2006). Using our micromethod, we also found a synergistic effect of 0·078% lactic acid associated with 1·56% hydrogen peroxide and 0·625% tartaric acid associated with 1·56% hydrogen peroxide on *L. monocytogenes* after just 5-min

contact time at 20°C, but we did not detect a synergistic effect of such combinations on *Salmonella* sp. and *E. coli*. However, the median MBC value reached for hydrogen peroxide on *Salmonella* sp. was 3·12%. So, we also detected an additive effect ($0{\cdot}50 < \Sigma$ FBC < 2) of lactic acid with hydrogen peroxide under basic assay conditions; our results confirmed that the combination of these products at half of their MBC values (e.g. 1·56% hydrogen peroxide combined with 0·15% lactic acid) was effective in reducing the bacterial level in a suspension of *Salmonella* sp. (data not shown). We revealed too the efficacy of three other combinations in reducing *L. monocytogenes* contamination: 0·39% hydrogen peroxide with 0·039% formic acid, 1·56% hydrogen peroxide with 1·25% acetic acid and 0·78% hydrogen peroxide with 0·039% oxalic acid (Table 2). Our results were not consistent for *Salmonella* sp. and *E. coli* with the previous findings by Schurman (2001) cited above. This previous study also demonstrated the effects of combinations of 0·1% to 0·3% citric acid with 0·012% or 0·017% H_2O_2 on *Salmonella* sp. in orange juice, after at least 1 h. We found a synergistic effect for combinations with higher product concentrations in distilled water: 1·56% H_2O_2 and 0·625% citric acid (Table 2). However, *Salmonella* sp. also showed a high level of sensitivity to five other combinations: 0·78% H_2O_2/0·039% formic acid, 0·195% H_2O_2/1·25% succinic acid, 0·39% H_2O_2/0·15% mandelic acid, 0·39% H_2O_2/0·019% nitric acid and 0·78% H_2O_2/0·039% sulphuric acid (Table 2).

Previous findings for the bactericidal activity of acids with organic interfering substances have been inconsistent (Jacquet and Reynaud 1994). Some studies have shown a decreased level of bactericidal activity of acids in organic matter (Gelinas and Goulet 1983). Other studies have shown the effect of organic substance on *Salmonella* sp., for example, to be dependent on the nature of this organic matter and on the type of acid tested (Cherrington *et al.* 1992). More consistent findings, however, have established a loss of bactericidal activity of hydrogen peroxide in the presence of organic matter, an effect observed for all oxidizing disinfectants. We confirmed that the synergistic effect of some combinations after 5-min contact time was mostly maintained in the presence of mineral ions and sometimes in the presence of organic substances. The maintenance of these synergistic effects seemed to be related to the species tested and to the choice of the associated acid. The MBC values of the components of such combinations at the optimum point of synergism may also be affected by the nature of the interfering substances. The acid components (such as lactic or tartaric acids) were the more sensitive products to the presence of hard water conditions when applied to *L. monocytogenes*, but the interference indices never exceeded 8 or 16 (Table 4). The synergistic effect of seven of the hydrogen peroxide/acid combinations studied was still detected in hard water and organic substances. Of these, the combination showing the strongest effect and displaying synergism for the six bacteria tested consisted of hydrogen peroxide and the smallest acid molecule, formic acid. As mineral and organic interfering substances have a weak interference (interference indices not more than 4) on both components of this interesting combination when applied on the resistant strains *Ent. hirae* and *Staph. aureus*, this combination keeps all its interest with mineral and organic interfering substances not only on these two strains, but also on *L. monocytogenes and Ps. aeruginosa.*

For each strain, the pH values measured in distilled water for the various synergistic associations between hydrogen peroxide and acid remained within a narrow range. So, the lower mean of pH values was calculated for *Ent. hirae* ($1{\cdot}93 \pm 0{\cdot}50$), and the higher mean was found for *Ps. aeruginosa* ($3{\cdot}70 \pm 0{\cdot}64$). Similar means of pH values (varying between $2{\cdot}56 \pm 0{\cdot}28$ and $2{\cdot}72 \pm 0{\cdot}51$) were calculated for the four other strains. However per strain, the association between hydrogen peroxide and formic acid presented most often the higher pH values at the optimal point of synergy comparing to other combinations. Moreover, the acid and the strain considered, the pH values measured at the optimal point of synergy were superior to pH values when acids, used alone, were bactericidal. This increase in pH varied from 0·27 to 1·33 between strains when formic acid was in association with hydrogen peroxide. A similar evolution, more or less pronounced, of pH conditions in distilled water was noticed between the optimal point of synergy and the CMB of each other acid. When all the synergistic associations found per bacteria in distilled water were compared, the pH values of synergistic associations raised from 0 to 0·67 for Gram-positive bacteria and from 0 to 1·33 for Gram-negative bacteria. With 300–600 mg l^{-1} $CaCO_3$, the associations were effective at slightly lower pH values than in distilled water related to the protective effect on bacterial cells of calcium ions. On the other hand, organic interfering substances (in particular 1% bovine albumin and 1% yeast extract mixture) interfered with products and bactericidal efficacy was maintained with higher concentrations of each product and less acidic pH conditions. The antimicrobial action of an organic acid, when used alone, depends on several factors including reduction in pH, the ratio of undissociated to dissociated forms of the acid, chain length, cell physiology and metabolism (Abkas and Olmez 2007). Hydrogen peroxide is a strong oxidant, which generally displays higher levels of efficacy in acid conditions (Block 2001).

We detected a high degree of bactericidal activity for 32 combinations of the two molecules. With formic acid, this enhanced bactericidal activity was quickly detected (as soon as 5-min contact time) for all the panel of strains tested and was maintained in various use conditions. This increased activity may be due to numerous factors as the effect of the pH of the environment, the penetration of the undissociated form of the acid into the cell cytoplasm combined with the presence of the small hydrogen peroxide molecule and/or that of its peroxy acid form. An explanation of mechanisms of activity of such molecules in association is difficult to develop, but factors such as the small size of the molecules combined with their capacity to form a third-generation active substance such as performic acid (Merka and Horacek 1979; Gehr *et al.* 2009) would be arguments. Combination of small molecules may be quicker at killing bacteria. A diminution of acid and hydrogen peroxide concentrations without any loss of bactericidal activity would be an interesting improvement to decrease the corrosion of food surfaces by products and alteration in fresh produce during sanitizing operations.

In conclusion, our study is the first one that screens the bactericidal efficacy after 5-min contact time of hydrogen peroxide when associated with 17 different mineral and organic acids against reference bacterial strains representative of contaminants of the environment and food industry. We also determined whether the synergistic effects of these combinations were maintained in the presence of interfering substances. We demonstrated that the synergistic effect of hydrogen peroxide/formic acid combination was maintained for the six selected strains with most of the interfering substances. Synergism was also found both in distilled water and in the two hard water conditions for eight other hydrogen peroxide/acid combinations. However, the synergism observed for these combinations did not tend to be always maintained in assays with a low organic load (300 mg l^{-1} $CaCO_3$ and 0·3% bovine albumin) or in assays with a high organic load (1% yeast extract and 1% bovine albumin), with the exception of the effects of acetic, succinic and propionic acid combinations on *Ps. aeruginosa*. The few combinations showing synergism on other strains than *Ps. aeruginosa* in assays mixing hard water and bovine albumin as tartaric acid/hydrogen peroxide on *L. monocytogenes,* citric acid/hydrogen peroxide on *Ent. hirae* and mandelic acid/hydrogen peroxide on *Salmonella* sp. confirmed the potential interest in using such combinations. Our method appears to be a useful tool to allow the rapid screening of the efficacy of complex formulations against planktonic cells. However, the maintenance of such synergies against sessile cells on surfaces has to be confirmed with typical food process surfaces or food surfaces. First, this work on bacteria will be completed by a similar investigation on other food and environmental contaminants such as yeast, fungi and viruses during longer contact times. However, the study of maintenance of these synergisms using laboratory tests such as surface tests simulating practical conditions of application would be the further step to complete our knowledge about the efficacy of such associations.

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