Technical report

Test protocol for assessing the effectiveness of the reduction of micro-organisms intentionally inoculated in plates using the Jonix Mate air ionisation system.

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Introduction

This protocol has been implemented to verify reduction of micro-organisms intentionally inoculated in culture media and exposed for predetermined periods of time to the effect of ionized air. A control test has been effected for each trial using the same inoculated media not subject to treatment.

Inoculated strains

The following micro-organisms were tested:

- *Staphylococcus aureus* (Gram + , asporogenous, aerobe - facultative anaerobe)
- *Listeria monocytogenes* (Gram + , asporogenous, aerobe - facultative anaerobe)
- *Aspergillus niger* (fungus - Mould)
- *Kluyveromyces marxianus* (perfect form of *Candida pseudotropicalis*)
- Various micro-organisms in association (Enterobacteriaceae – Total bacterial count)

Materials and methods

Various micro-organisms were directly inoculated in Plate Count agar (Oxoid, Italy) and Malt Agar (yeasts and moulds) (Oxoid, Italy), treated for various times 0 (control), 30, 60, 90, 120 min and 12 and 24 h with the ioniser. After treatment the plates were incubated at 25 °C for 2-5 days. After that the colonies that survived treatment were counted. The results were expressed in % of inactivation/decay.100 – (Initial number/Final number %)

Preparation of the microbial suspension and inoculation

Staphylococcus aureus

Inoculation consisted of 2 strains of *Staphylococcus aureus* from international collections (DSMZ 4910) and Collection of the Department of Agri-Food, Environmental and Animal Husbandry Sciences of the University of Udine (DIAL). Individual suspensions were prepared with one loop of *St. aureus* added to sterile peptone water (0.8% NaCl). The spectrophotometer evaluated optical density equal to 0.1 at 600 nm. In order to evaluate the load of each suspension, the same were

diluted in sterile peptone water. After that 0.1 ml of each dilution was inoculated in Plate Count Agar (Oxoid, Italy). The plates were incubated at 37 °C for 48 hours and the grown colonies were counted. Each suspension contained on average approximately 10^7 CFU/ml. Decimal dilutions were carried out and 0.1 ml of dilution 10^{-3} CFU/ml was spread in Plate Count Agar. The open plates were placed in the chamber with ioniser turned on 24 hours before. At the above time frames the plates were recovered and placed to incubate at 37 °C for 48 hours. 5 replicas (samples) were analysed at each interval.

Listeria monocytogenes

Inoculation consisted of 2 strains of *Listeria monocytogenes*: L. monocytogenes , L. monocytogenes from International Collections and plants and stored in the Collection of the Department of Foodstuff Sciences of the Faculty of Agriculture of the University of Udine (DIAL). Individual suspensions were prepared with one loop of *L. monocytogenes* added to sterile peptone water (0.8% NaCl). The spectrophotometer evaluated optical density equal to 0.1 at 600 nm. In order to evaluate the load of each suspension, the same were diluted in sterile peptone water. After that 0.1 ml of each dilution was inoculated in Plate Count Agar (Oxoid, Italy). The plates were incubated at 37 °C for 48 hours and the grown colonies were counted. Each suspension contained on average approximately 10^7 CFU/ml. Five ml of each suspension were mixed and diluted in sterile peptone water and 0.1 ml of dilution 10^{-3} CFU/ml was spread in Plate Count Agar. The open plates were placed in the chamber with ioniser turned on 24 hours before. At the above time frames the plates were recovered and placed to incubate at 37 °C for 48 hours. 5 replicas (samples) were analysed at each interval.

Kluyveromyces marxianus

Kluyveromyces marxianus was seeded in malt agar (Oxoid, Italy), incubated at 25 °C for 3 days, then one colony was diluted in sterile peptone water (NaCl 0.1%, Peptone 0.8%, Water 1000 ml). The spectrophotometer evaluated optical density equal to 0.1 at 600 nm. In order to evaluate the load of the suspension, the same were diluted in sterile peptone water. After that 0.1 ml of each dilution was inoculated in plates containing Malt Agar (Oxoid, Italy). The plates were incubated at 25 °C for 3-5 days and the grown colonies were counted. The suspension contained on average approximately 10^7 CFU/ml. Decimal dilutions were carried out and 0.1 ml of dilution 10^{-4} CFU/ml was spread in Malt Agar. The open plates were placed in the chamber with ioniser turned on 24

hours before. At the above time frames the plates were recovered and placed to incubate at 25 °C for 3-5 days. 5 replicas (samples) were analysed at each interval.

***Coliforms* and *Escherichia coli* (environmental/faecal contamination)**

The inoculation consisted of 2 strains of *E. coli* isolated from flours and stored in the Collection of the Department of Foodstuff Sciences of the Faculty of Agriculture of the University of Udine (DIAL), one strain of *Pantoea (Enterobacter) agglomerans* of environmental origin. Individual suspensions were prepared with one loop of each micro-organism added to sterile peptone water (0.8% NaCl).

The spectrophotometer evaluated optical density equal to 0.1 at 600 nm. In order to evaluate the load of each suspension, the same were diluted in sterile peptone water. After that 0.1 ml of each dilution was inoculated in Plate Count Agar (Oxoid, Italy). The plates were incubated at 37 °C for 48 hours and the grown colonies were counted. Each suspension contained on average approximately 10^7 CFU/ml. Five ml of each suspension were mixed and diluted in sterile peptone water and 0.1 ml of dilution 10^{-3} CFU/ml was spread in Plate Count Agar. The open plates were placed in the chamber with ioniser turned on 24 hours before. At the above time frames the plates were recovered and placed to incubate. 5 replicas (samples) were analysed at each interval.

Aspergillus niger

Spores, produced from colonies of *Aspergillus niger*, seeded in Malt agar (Oxoid, Italy) incubated at 25 °C for 5 days, were diluted in peptone water (NaCl 0.1%, Peptone 0.8%, Water 1000 ml). After homogenisation the spores, following dilution, were counted in Malt agar plates (Oxoid, Italy) and 0.1 ml of dilution 10^{-3} were spread in Malt Agar plates. The open plates were placed in the chamber with ioniser turned on 24 hours before. At the above time frames the plates were recovered and placed to incubate at 25 °C for 3-5 days. 5 replicas (samples) were analysed at each interval.

Ozone concentration was also measured during treatment in order to evaluate the healthiness of the method.

Ioniser model used

A Jonix Mate ioniser model by the company Jonix srl was used. Viale Spagna 31-33, 31020 Tribano (PD). The ioniser, placed in a food grade cold store room of approximately 60 m3 (6x3.6x 2.70 m) kept at average temperature of 15 °C., had been switched on 2 days prior to the start of the

experimentation in order to eliminate interference due to air contamination in the cold store room. The open plates were positioned on metal shelves and subject to treatment leaving the ioniser on throughout the

tests.

Results

The outcome of the tests are outlined below. The figures are the approximate average result of five plates. The data are expressed in CFU/ml in 90mm plate.

Table 1: Change in the concentration of *Staphylococcus. aureus*

Time min/h	Average CFU/ml	st. dev.	% decrease
0	58400000	10822199.41	
30	31320000	7121235.84	37.1
60	34100000	3090307.42	31.5
90	21640000	3710525.56	56.5
120	4600000	854400.37	90.8
12 h	40000	89442.71	99.9
24 h	0	0	100.0

Key: st. dev.: standard deviation

Table 2: Change in the concentration of *Listeria monocytogenes*

Time min/h	Average CFU/ml	st. dev.	% decrease
0	65280000	1752712.18	
30	65080000	7150314.67	0.3
60	51360000	25677772.49	21.3
90	3520000	1605303.71	94.6
120	2006000	1080823.76	96.9
12 h	980000	752994.02	98.5
24 h	960000	409878.03	98.5

Key: st. dev.: standard deviation

Table 3: Change in the concentration of *Kluyveromyces marxianus*

Time min/h	Average CFU/ml	st. dev.	% decrease
0	2184480	505268.19	
30	2292400	622791.13	4.9
60	2336000	782642.95	6.9
90	2266000	979938.77	3.7
120	2158000	864823.68	1.2
12 h	0	0	100.0
24 h	0	0	100.0

Key: st. dev.: standard deviation

Table 4: Change in the total bacterial count

Time min/h	Average CFU/ml	st. dev.	% decrease
0	70240000	7029082.44	
30	63420000	3692153.84	9.7
60	82480000	16818799.01	17.4
90	54800000	10471867.07	21.9
120	57960000	16288277.99	17.5
12 h	0	0	100.0
24 h	0	0	100.0

Key: st. dev.: standard deviation

Table 5: Change in the concentration of *Aspergillus niger*

Time min/h	Average CFU/ml	st. dev.	% decrease
0	1300000	200000	
30	740000	54772.25	43.1
60	1000000	519615.24	23.1
90	1000000	353553.39	23.1
120	1260000	421900.46	30.8
12 h	360000	207364.41	72.3
24 h	180000	130384.04	82.7

Key: st. dev.: standard deviation

Comment on results

As may be inferred from the results of the above mentioned tests, the microbial reduction obtained with the Jonix Mate air ionisation system on surfaces simulating organic material inoculated with various microbial strains is high and allows most inoculated strains to be completely inhibited within 12 - 24 hours. In fact in many cases the reduction obtained is higher than 95%. The microbial reduction effect of ionised air is similar both on Gram positives and Gram negatives as well as on yeasts and moulds, although the effectiveness often appears to depend on the species considered, specifically:

1) *Staphylococcus aureus* (Table 1). This micro-organism is typical of human and animal mucosa. It is a potential pathogen, since the species includes enterotoxin-producing strains (A, B, C, D, E, F). Enterotoxin A seems the most widespread one at food level. In fact it has been the culprit in a number of poisoning cases since it is produced -- like the other enterotoxins -- in food stored in thermal abuse. Foodstuff may be naturally contaminated by *Staphylococcus aureus* or come into contact with it due to human manipulation and contaminated environments and equipment. In the event of development the toxin persists

since it is thermostable hence it is not eliminated by pasteurisation and/or cooking. Using the ionising treatment assures complete inhibition of said micro-organism. In fact, continuous inactivation over time is observed (Table 1). In fact after 2 hours reduction is 90% and 99.9-100% at 12 and 24 hours.

2) *Listeria monocytogenes* (Table 2). This is an environmental origin psychrotrophic micro-organism. It contaminates any environment and consequently any food either as ingredient or finished product (Cocolin et al., 2005). Its presence in foodstuff (ready to eat) is regulated by microbiology criteria set out in Reg. EC 2073/05 and 1414/08, since it is a pathogen and highly virulent. Every year, in fact, a number of listeriosis cases are reported (0.4/100,000 inhabitants) following consumption of food products. Consequently, the food industry uses a number of technologies to eradicate or prevent its presence in food, as well as to prevent its growth. The Jonix system assures reduction already after 90 min of 94% of *L. monocytogenes* present, and at 12-24 hours reduction is greater than 98%. To achieve total eradication (100%) prolonged treatment is required (36 hours).
3) *Kluyveromyces marxianus* (Table 3). *K. marxianus* is an innocuous yeast, considered as the perfect form of *Candida pseudotropicalis*; which, however, is a recognised pathogen, although less virulent than *Candida albicans*, a typical pathogen of human and animal mucosa. It is widespread in nature, particularly in milk and fermented milk (i.e. Kefir). It is used in the production of bio-ethanol. This yeast is particularly resistant to short ionising treatment (< 120 min). However, its presence may be eradicated from organic substrates after 12/24 hours of treatment, as effectively reported in table 3.
4) Total bacterial count (Table 4). For an assessment of the effect of the ionising treatment on the total bacterial count various micro-organisms typical of faecal (*Escherichia coli*) and environmental (*Pantoea agglomerans*) contamination were used. Also in this case treatment shorter than 120 minutes do not lead to significant reductions, while treatment of 12 and 24 hours lead to complete eradication of the 2 species considered.
5) *Aspergillus niger* (Table 5). Mould (fungus) of environmental origin and typical of vegetables, even if it has often been isolated on the surface of cured meats. It features mycotoxigenic strains. In fact black aspergilli may produce Ochratoxin A and B; Ochratoxin A is undesirable in foodstuff as it has been classified by IARC (International Agency for Research of Cancer) in group "2B" as a possible carcinogen. A number of studies, in fact, have proved its teratogenic, neurotoxic, genotoxic, immunotoxic and nephrotic properties (IARC, 1993; JEFCA, 2001). As a matter of fact in Italy the limit of 1

µg/Kg has been suggested for its presence in meat and meat-based products (Circular of the Ministry of Health no. 10-09/06/1999). Ionisation is conducive to variable reduction of said micro-organism, linked to the treatment time. In fact 12 or 24 hours are required to reduce 72 and 82 % of the *A. niger* load inoculated in organic media. To achieve complete inactivation of inoculated spores a 36-48 hour treatment is required.

Conclusion

The Jonix Mate system based on air ionisation makes it possible to inactivate microbial strains intentionally inoculated in organic culture media. The reduction percentage is strictly linked to the microbial species considered and the treatment time.

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