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Abstract: Indoor air of dairy industry acts as a source or vehicle of microbial contamination affecting both food safety and product shelf life. This research focused on both the monitoring of the air microbial load in selected areas of a dairy factory and in evaluating the effect of air disinfection through ozonation or chemical aerosolization by hydrogen peroxide. The air microbial load was weekly monitored in the autumn/winter season after the routinely applied sanitation procedures. Air samplings, through impaction method, were carried out in 3 critical areas (cheese making, storage and packaging). Total bacteria, moulds and yeasts resulted in mean counts of 161 (± 154) MPN m-3, 228 (± 234) MPN m-3, and 137 (± 439) MPN m-3, respectively. The dairy location exhibiting the lowest contamination was the storage cell. A large variability of microbial loads characterized the packaging area. Mycobiota pattern consisted in 11 species of moulds isolated and identified through mycological and molecular techniques. The isolates observed in the indoor air mainly consisted of Cladosporium spp., Alternaria spp., and Penicillium spp.. The yeast community was mainly represented by Cryptococcus spp., Debaryomyces spp., Bulleromyces spp., and Sporobolomyces spp.. Both ozonation and hydrogen peroxide aerosolization were effective techniques in the inactivation of airborne microorganisms. After air treatment only residual fungi were identified. We verified that their occurrence was promoted by environmental recontamination.

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DeFENS

September 12th, 2018

Dear Editor,

We thank You once again for taking care of the revision of our manuscript "Effectiveness of air disinfection by ozonation or hydrogen peroxide aerosolization in dairy environments" (Ms. Ref. No.: FOODCONT-D-18-01908). We appreciate the constructive comments and suggestions of the Reviewers and modified the manuscript accordingly. Please, find attached an itemized list of responses in separately submitted files.

We want to Thank You and the Referees for your precious advice.

Sincerely,

Fabio Masotti

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Reviewer #1: Commentary

Ms. Ref. No.: FOODCONT-D-18-01908

Title: Effectiveness of air disinfection by ozonation or hydrogen peroxide aerosolization in dairy environments

To simplify reading, our answers are in a black typeface and the original points raised by the Reviewer are in black bold.

Reviewed document is a research with the aim to study the presence of culturable bacteria, moulds and yeasts in the air within some critical areas of an artisanal dairy factory. The effectiveness of two alternative air disinfection techniques (ozonation and hydrogen peroxide aerosolization) to reduce air contamination was investigated. Moreover, the identification of residual microorganisms in the indoor environment, after air disinfection, was considered. Abstract seems to be sufficient to understand the performed work.
I included remarks in the text below.
Introduction:
Introduction is correct, to focus in the microbiological air pollution control. However, it is not considered the toxic potential of ozone to manipulators. The final ozone concentration in the air is really important. The US Clean Air Act has set an ozone level of 120 mcg/m3 as an 8 hr mean concentration to protect the health of workers. Evaluation of some studies allows establishing an average environmental ozone concentration of 90 +- 10 mcg/m3 (Velio Bocci, Emma Borrelli, Valter Travagli, and Iacopo Zanardi. 2009. The Ozone Paradox: Ozone is a strong oxidant as well as a medical drug. Medicinal Research Reviews, 29(4):646-682). When ozone is included in a reseaarch work, a control of the final oxidizing chemical must be included, to assure the workers security. This is not included in the paper.

AU: We thank the Reviewer for Her/His comments. We agree with the Reviewer on the relevance of personnel exposure to ozone in view of safety and health related aspects. As reported in the manuscript, we did not measure residual concentration of ozone after treatment. The manufacturer of the generator certified that in the treated room a time span of 20 min was sufficient to guarantee its autodecomposition at levels < 0.02 ppm (the equivalent of < 0.04 mg/m^3). The manufacturer provided us only with these data after measurements carried out with a continuous ozone analyzer under the conditions adopted (room volume and time of treatment). Furthermore, under our experimental conditions the re-entry of personnel in the treated room (about 5 h after ozone generation) (Lines 174–175) abundantly exceeded the time-span of 20 min declared as safe by the manufacturer. This level was lower than the limit of 0.05 ppm (0.1 mg/m^3) cited by the Occupational Safety and Health Administration (OSHA) for ozone exposure under heavy work conditions for 8 h a day or 40 h a week.

On my opinion, this is an interesting work for a general journal. The use of chemicals, currently used in the food industry, is not one of the topics with interest for Food Control. I think authors could search for another kind of journal.

AU: We are grateful to the Reviewer for Her/His observation. Microbial food safety and Environmental control and safety are some of the issues covered by Food Control. Through the years air management received renewed interest due to the fact that the food industry has been under pressure to deliver products minimally processed, fresher in taste and appearance, more

conveniently produced and packaged, with less preservatives and prolonged shelf life. In our manuscript we pointed out both the quality of air and the effect of two alternative disinfection procedures of the air. The interest in this item is of major concern especially in those premises with faults in the hygienic design of structures. Previous researches cited in references and published in the Food Control journal (Byrne, Lyng, Dunne, & Bolton, 2008; Ocón, Garijo, Sanz, Olarte, López, Santamaría, et al. 2013; Schön, Schornsteiner, Dzieciol, Wagner, Müller, & Schmitz-Esser, 2016) focused only on the microbial quality of the air in different food sectors.

Material and methods:

All the work is performed in only one plant.

AU: Type and amounts of microorganisms in the air of a food plant can vary widely as a function of the area and on a day-to-day basis in the same environment. These variations are ascribed to different factors, namely hygienic design of structures, zone separation, presence of employees, etc.. We focused on the microbial pattern of an artisanal dairy factory. In particular, we evaluated the microbial load in 3 critical areas (cheese making, storage and packaging room).

Sanitization process is not correctly described. Ln 108-113: Which kind of disinfection process is performed? A one-way process, including a foam with chlorine is not enough to assure a correct sanitization process.

AU: We thank the Reviewer for Her/His comments. Traditionally, the sanitation process in dairy sector consists of a cleaning step (alkaline followed by acid detergent) to remove soil from surfaces and a subsequent disinfection treatment (to remove residual microorganisms). Anyway, dairy manufacturers when the soil is not particularly tenacious prefer the use of the one-step or monophase sanitation to save time for processing and water for rinsings. This technique is effective for both open surfaces (with foam technology) and closed surfaces (with CIP technology) (Marriott N. G. & Gravani R. B. Principles of food sanitation, 2006, Springer, New York). Really, an alkaline chlorinated cleaner can perform rapid cleaning of soil giving disinfection by residual chlorine. Marriott and Gravani (2006) reported that: "A chlorinated alkaline cleaning compound can clean, sanitize, and deodorize in one operation if the soil is light". The alkaline oxidizing cleaning is more effective than the alkaline cleaning alone, thanks to the good oxidant action of chlorine itself (Stanga M. *Sanitation. Cleaning and disinfection in the food industry*. Wiley-WCH, Weinheim, 2010; Stanga M. *Latte: detergenza e disinfezione dalla produzione alla caseificazione*. Chiriotti Ed., Torino, 2015). This type of sanitizer is widely adopted in dairy sector and offered in different formulations by several manufacturers (Sealed Air Diversey, Ecolab, Christeyns).

We revised the text. Please, see the paragraph 2.2 in the revised manuscript.

To compare the results using a Tukey's multiple comparisons method must be justified. Authors performed before an average comparison using ANOVA with "a posteriori" contrast of Turkey? This has been not explained.

AU: We thank the Reviewer for Her/His comment and modified the text as follows: "The statistical analyses were performed using Minitab® software (Release 18, 2017; State College, PA, USA). Comparisons of experimental data were performed with one-way ANOVA followed by Tukey's post-hoc test to determine differences between means of microbial load at a significance level of 0.05." (Revised lines 199–202).

Data of microbial load in Table 1 are justified as reported in the title (n=5). Each value is the mean of five data corresponding to the five sampling point of the packaging room.

Reviewer #2: Commentary

Ms. Ref. No.: FOODCONT-D-18-01908

Title: Effectiveness of air disinfection by ozonation or hydrogen peroxide aerosolization in dairy environments

To simplify reading, our answers are in a black typeface and the original points raised by the Reviewer are in black bold.

Reviewer #2:

General comments:

This is a reasonably sound study. The experiments performed by Masotti and co-workers have been conducted rigorously, with sample sizes being large enough to produce robust results. The methods and reagents used have been described in sufficient detail. The data presented in the manuscript do support the conclusions reached. However, although the manuscript has been written in acceptable English, it would definitely have benefitted from a final round of revision to remove grammatical errors and to clarify several sentences which leave one guessing as to their meaning.

AU: We are grateful to the Reviewer for all Her/His amendments and suggestions to improve the quality of the manuscript.

Specific comments:

*** I suggest that the last word of the manuscript title (i.e. environment) be written in plural (i.e. environments)**

AU: We modified the title as suggested.

*** "Criptococcus" should be replaced by "Cryptococcus" all through the manuscript.**

AU: We corrected the term throughout the text and in Table 2.

*** Lines 121 and 122: It is not clear in what way the "2 series of samplings" in the packaging area differed. Although this has been indirectly clarified in the Results and discussion section (lines 263 to 266), it would require some explanation in subsection 2.3 as well.**

AU: We thank the Reviewer. We modified the text as follows: “The former (carried out in October-November) was the blank series relative to the subsequent ozone treatment, the second (carried out in December-February) was the blank series relative to the subsequent hydrogen peroxide aerosolization.” (Revised lines 124–126).

*** Line 129: The headquarters (i.e. city) of LCB Food Safety needs to be given.**

AU: We introduced the name of the city in the text.

*** Lines 149 and 340: The Italian "e" should be replaced with the English "and".**

AU: We corrected the text.

*** Line 155: "Roma" should be replaced by "Rome".**

AU: We replaced the name of the city.

*** Line 260: "Kang & Frank, 1990" should be replaced by "Kang & Frank, 1989".**
AU: We corrected the text.

*** Lines 296 and 480: "Chebotarevm" should be replaced by "Chebotarev".**
AU: We corrected the name.

*** Line 310: Italian Ministry of Health (2010) is missing from the list of references.**
AU: We introduced the reference.

*** Lines 318 and 319: Based on the results presented in Table 2, "Cladosporium herbarum (75%)" should be replaced with "Alternaria alternata (75%)".**
AU: We are grateful to the Reviewer for the amendment.

*** Line 374: "Infection control & Hospital epidemiology" should be replaced by "Infection Control & Hospital Epidemiology".**
AU: We corrected the text.

*** Line 376: "Greek Dairy Plant" should be replaced with "Greek dairy plant".**
AU: We corrected the text.

*** Line 395: "Factors Influencing Yeasts" should be replaced by "factors influencing yeasts".**
AU: We corrected the text.

*** Line 404: "european parliament and of the council" should be replaced with "European Parliament and of the Council".**
AU: We corrected the text.

*** Line 414: "allergens: Resolved" should be replaced by "allergens: resolved".**
AU: We corrected the text.

*** Lines 426 and 427: "Applied Dairy Microbiology" should be replaced with "Applied dairy microbiology".**
AU: We corrected the text.

*** Line 443: "…of food Microbiology" should be replaced by "…of Food Microbiology".**
AU: We corrected the text.

*** Line 448: "Advanced dairy science and technology" should be italicized.**
AU: We italicized the text.

*** Line 450: "Food control" should be replaced with "Food Control".**
AU: We corrected the text.

*** Line 462: "Trichocomaceae" should be italicized.**

AU: We corrected the text.

*** Line 466: Penicillium digitatum, P. italicum, and Geotrichum candidum should be italicized.**
AU: We italicized the text.

*** Line 478: "The Etiology of Bioaerosols in Food Environments" should be replaced with "The etiology of bioaerosols in food environments".**
AU: We corrected the text.

*** Line 486: "industry: A review" should be replaced by "industry: a review".**
AU: We corrected the text.

*** Line 487: "67, 1-20" should be replaced with "69, 157-168".**
AU: We corrected the text.

*** Title of Table 2: It is unclear what "number of CFU in 25 plates" actually means. Is it the combined number of colonies enumerated in a total of 25 Petri dishes, or the mean value of 25 plates? Please clarify why these data have not been expressed in terms of CFU/m3.**
AU: We thank the Reviewer for Her/His amendment and observation. We modified the title of Table 2 as follows: "Air microbial load (expressed as total colonies enumerated in 25 Petri dishes) before and after air disinfection treatments".
In view of the species listed in captions of Table 2, we hypothesized that results of microbial load expressed as total colonies enumerated in 25 Petri dishes could make the reader more confident with the relative contribution of each species after the disinfection treatment.

Effectiveness of air disinfection by ozonation or hydrogen peroxide aerosolization in dairy environments

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Abstract

Indoor air of dairy industry acts as a source or vehicle of microbial contamination affecting both food safety and product shelf life. This research focused on both the monitoring of the air microbial load in selected areas of a dairy factory and in evaluating the effect of air disinfection through ozonation or chemical aerosolization by hydrogen peroxide. The air microbial load was weekly monitored in the autumn/winter season after the routinely applied sanitation procedures. Air samplings, through impaction method, were carried out in 3 critical areas (cheese making, storage and packaging). Total bacteria, moulds and yeasts resulted in mean counts of 161 (± 154) MPN m^{-3}, 228 (± 234) MPN m^{-3}, and 137 (±

439) MPN m^{-3}, respectively. The dairy location exhibiting the lowest contamination was the storage cell. A large variability of microbial loads characterized the packaging area. Mycobiota pattern consisted in 11 species of moulds isolated and identified through mycological and molecular techniques. The isolates observed in the indoor air mainly consisted of *Cladosporium* spp., *Alternaria* spp., and *Penicillium* spp.. The *yeast* community was mainly represented by *Cryptococcus* spp., *Debaryomyces* spp., *Bulleromyces* spp., and *Sporobolomyces* spp.. Both ozonation and hydrogen peroxide aerosolization were effective techniques in the inactivation of airborne microorganisms. After air treatment only residual fungi were identified. We verified that their occurrence was promoted by environmental recontamination.

Keywords: food contamination; impaction air samplers; ozonation; chemical aerosolization

1. Introduction

Good sanitation practices of surfaces and indoor air are a pre-requisite in the control of microbial spoilage in the food industry. In this sector, sources of contamination from the air are mainly concerned with microbial contaminants (Burfoot, 2005). Air in dairy factories represents a dissemination medium of bacteria, molds, yeasts and viruses, potentially leading to contamination of final products. The assessment of the air quality in a food facility is a way to control any potential mycological food safety hazards (Asefa et al., 2009). Rules on the hygiene of foodstuffs (EC No 852/2004, 2004) laid down the key role of design, siting and size of food premises to avoid or minimize airborne contamination thus resulting in acceptable hygienic performance at all stages of production. Spoilage of dairy products through airborne microorganisms has long been ascertained in dairy processing plants (Mostert & Jooste, 2002; Shale & Lues, 2007) and emphasized for its impact on the safety of dairy products and their shelf life. In literature a complex interrelation of factors and manifold sources of aerosols have been identified, namely outdoor environments, structure and plant design, processing practices, personnel, dairy equipments, air-conditioning systems, packaging materials and cleaning operations by hosing or brushing (Burfoot,

Reavell, Tuck, & Wilkinson, 2003; Holah & Lelieveld, 2011; Salustiano, Andrade, Brandão, Cordeiro-Azeredo, & Kitakawa-Lima, 2003). Composition and amount of microorganisms in aerosols varies within and among dairy plants/areas and over time (Mostert & Jooste, 2002). Anyway, results on monitoring of airborne microorganisms are difficult to compare, also due to the different types of commercial samplers (Kang and Frank, 1989; Wirtanen, Miettinen, Pahkala, Enbom, & Vanne, 2002). Settle plates are commonly adopted for a qualitative evaluation, whereas impaction samplers are preferred for quantitative results. When low bioaerosols prevail impaction sampling methods are preferred due to the high recovery rates with respect of other collection methods (Stetzenbach, Buttner, & Cruz, 2004). There are few, if any, internationally agreed limits to assess airborne microbial contamination. Kang and Frank (1989) proposed limits for airborne counts in various areas of the dairy environment. The maximum satisfactory levels suggested by Lück and Gavron (1990), range from 100 to 200 colony forming unit (CFU) m^{-3} for bacteria and from 50 to 100 CFU m^{-3} for moulds and yeasts. These threshold values were considered strict but achievable in practice (Mostert and Jooste, 2002).

Clean airflows can be generated by the use of filtration systems acting as barriers against cross contamination from the environment. Air filter systems allow also more air into the room than normal, thus establishing a positive air pressure (Mostert & Buys, 2008). As a consequence, opening the room of sensitive areas (processing lines or ripening/packaging areas) prevents the access of air, reducing microbial contamination (Byrne, Lyng, Dunne, & Bolton, 2008; Sørhaug, 2011). Different approaches have been developed to reduce viable microbial counts in the air, including chemical aerosolization, ozonation or ultraviolet (UV) irradiation. The former consists in the fine dispersion of a solution as fine mist in air. This technique reduces both the number of airborne microorganisms and on surfaces that may be difficult to reach (Bagge-Ravn, Gardshodn, Gram, & Fonnesbech Vogel, 2003; Burfoot, Hall, Brown, & Xu, 1999; Oh, Gray, Dougherty, & Kang, 2005; Park, et al. 2012). Anyway, fogging disinfection through dry mist system is an issue of food safety concern not so much dealt with on research papers (Bore & Langsrud, 2005). Hydrogen peroxide in aerosol mist form is an effective disinfectant for most microorganisms due to its oxidative action leading to the formation of hydroxyl-free radicals capable to

attack membranes, DNA and other cell components (Barbut, Menuet, Verachten, & Girou 2009). Ozonation is an alternative method for inactivating airborne microorganisms (Cullen & Norton, 2012). This cost-effective and eco-friendly treatment has been successful for inactivating airborne moulds in cheese ripening and storage facilities (Varga & Szigeti, 2016). Wirtanen et al. (2002) outlined the synergistic effect of air-handling systems coupled to ozonation as a tool to reduce microbial air counts.

In this study, the presence of culturable bacteria, moulds and yeasts in the air within some critical areas of an artisanal dairy factory was evaluated. The effectiveness of two alternative air disinfection techniques (ozonation and hydrogen peroxide aerosolization) to reduce air contamination was investigated. Focus was addressed also to the identification of residual microorganisms in the indoor environment after air disinfection and to the interpretation of their occurrence.

2 Materials and methods

2.1 Dairy factory

The investigation was carried out in a dairy factory in Northern Italy (Po river valley) in the timespan October-February of 2017/2018. The amount of milk daily processed ranged from 1,200 to 1,400 L and about 65 % of it was transformed into soft fresh cheeses (Crescenza and "primosale" i.e., a short-ripening time variant), Ricotta and semi-hard cheeses (Caciotta type). The former varieties were ripened up to 3 and 1 d, respectively in the storage/ripening cell of the factory, whereas the ripening of Caciotta cheese was carried out in another dairy factory. The remaining amount of milk was used in the manufacture of ice creams, yogurts and vanilla/chocolate puddings. The factory was firstly visually inspected to reveal potential sources of microbial contamination. Factors taken into consideration included: plant design (separation of areas), ventilation, adoption of positive air pressure, state of floor-drains, number of employees, packaging materials.

105 *2.2 Sanitation of structures and open plants*

106

107 In the cheese making room, one-step (monophase) sanitation of structures and open plants was
108 daily performed with foam technology after each production day. Pre-washing with cold water using low-
109 pressure (< 200 kPa) was followed by the application of chlorinated alkaline detergent (dissolved in water
110 to 3.5 %) at room temperature to avoid corrosion phenomena. This type of cleaner can perform rapid
111 cleaning of soil giving disinfection by residual chlorine. The solution was sprayed as foam at low pressure
112 on the equipment, floor and walls up to a height of 2 m. After 15–20 min the foam on surfaces was rinsed
113 off with water. On a monthly basis, an acidic detergent was used as descaler. Occasionally, tenacious soil
114 was removed by brush or cloth deeped in mild detergents. Storage cell and packaging room were cleaned
115 and disinfected on weekly basis with foam technology.

116

117 *2.3 Sampling plan*

118

119 The sampling plan consisted in monitoring air contamination both after the routinely applied
120 sanitation procedure (blank samples) and after air disinfection with ozone or hydrogen peroxide
121 aerosolization (test samples). Three critical areas, i.e., cheese making, storage, and packaging rooms,
122 were selected. In cheese making room and storage cell, the air sampling was done on weekly basis for 5
123 consecutive weeks. In the packaging area, 2 series of samplings (each of 5 weeks) were undertaken
124 between October 2017 and February 2018 (Table 1). The former (carried out in October-November) was
125 the blank series relative to the subsequent ozone treatment, the second (carried out in December-
126 February) was the blank series relative to the subsequent hydrogen peroxide aerosolization. Air ozonation
127 was performed only in packaging room, whereas chemical aerosolization was applied both in cheese
128 making and in packaging rooms. No disinfection treatment of air was carried out in the storage cell.

129

130 *2.4 Sampling and microbiological analysis of air*

Microbiological analysis of air was performed by impaction method using a portable air sampler by LCB Food Safety (AirTest®, Boz, France). The active air-sampler collected a volume of air per min of 100 L. Contact plates with a suitable agar medium were clipped in place and covered with a lid with a precision pattern of holes (n = 256). Selected volume of air was 100 L. The sucked air striked on 90 mm diameter Petri plates. At the end of the suction step, the air sampler lid was removed, and the agar plate recovered and incubated at an appropriate temperature. Total bacteria count (TBC) was carried out on Plate Count Agar (PCA, International PBI, Darmstadt, Germany) followed by incubation at 30 °C for 72 h. Moulds and yeasts in the air were collected on Yeast Extract Glucose Chloramphenicol Agar (YGC, Sacco System, Como, Italy) and subsequent incubation in the dark at 25 °C for 5 d. A total of 350 air samples were analyzed.

The mean value of CFU in the 5 sampling points of each room was converted to the most probable number (MPN) per m^3 of air by using the Feller's law (Feller & Higgins, 1968), according to the manufacturer's instructions. Air samples were collected by positioning the air sampler at 1.5 m height. In each area, 5 air sampling points (1 to 5 in Figure 1) were selected.

2.4.1 Moulds isolation and identification

Mould colonies grown in YGC agar from pre and post-treatment samplings were subjected to macro- and microscopic examination to identify their genus (Dragoni, Cantoni, Papa, & Vallone, 1997). *Penicillium* colonies were isolated and purified on potato dextrose agar, then cultured on Czapek Yeast extract Agar (CYA, Merck, Darmstadt, Germany), Malt Extract Agar (MEA, Scharlau, Barcelona, Spain) and Glycerol Nitrate Agar (G25N, Merck) at 5, 25 and 37 °C for 7 days and were identified according to Pitt and Hocking (1997).

2.4.2 Yeasts isolation and identification

Yeast colonies grown in the post-treatment sampling plates were isolated, purified by streaking on Yeast Malt agar (YM, Scharlau) and identified by rDNA sequencing. Total DNA was extracted from 1 mL of the overnight cultures by the Microlysis kit (Aurogene s.r.l., Rome, Italy) following the manufacturer's instructions. Yeasts were identified by PCR amplification of D1/D2 region of the 26S rRNA gene amplification by using primers NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3 and NL4 (5'- GGTCCGTGTTTCAAGAC-GG-3') according to Kurtzman and Robnett (1998). Amplification products were sequenced by Macrogen Europe (Amsterdam, the Netherlands) and raw sequence data were carefully reviewed by CHROMAS software (Griffith University, Queensland, Australia). Sequence homologies were then analyzed through MycoBank database (http://www.mycobank.org/BioloMICSSequences.aspx?expandparm=f&file=ALL) and species names were assigned whenever the degree of homology with the closest known relative species was higher than 97%.

2.5 Ozonation

In the packaging area, ozonation was realized through a corona discharge ozone generator (Model Coccinella, C.G.C. Coccinella Ecosan snc, Bergamo, Italy) using atmospheric air as source of oxygen. The apparatus produced ozonated air piped in the treated rooms at a constant flow rate of 40 L min^{-1} and it generated 1.5 g ozone per hour. Above conditions were suggested by the manufacturer of the ozone generator. The air disinfection was carried out overnight for 3 h (11 to 12 p.m. and 1 to 3 a.m.) from Friday to Sunday. The premise was closed during treatment and no personnel was present. After each experimental run, no scrubbers were adopted to reduce the residual ozone concentration. The manufacturer of the equipment prescribed a time span of 20 min to guarantee healthy and safe re-entry of the employees in the room. Under these conditions the manufacturer reported that the ozone level in the room was reduced to a level judged safe for the employees (< 0.02 ppm). The personnel access to the room for air sampling took place only at the beginning of the subsequent working day (about 5 h after ozone generation).

183

184 *2.6 Chemical aerosolization*

185

186 Hydrogen peroxide aerosolization was realized in cheese making and packaging rooms by a
187 mobile unit (Mini Air Solution, ZR Diagnostici srl, Cremona, Italy) forcing the disinfectant solution
188 through a dedicated nozzle producing particles in the range 5–15 µm. The device was positioned in the
189 middle of the room. The chemical (Mida San 316, Christeyns, Gent, Belgium) was a solution containing
190 90 % distilled water and 10 % hydrogen peroxide stabilized with a chelating agent (data from
191 manufacturer). The apparatus produced 1 mL m^{-3}, working with air velocity of 100 m s^{-1} at the nozzle for
192 effective delivery of the chemical. As a function of the room volume, the aerosolization took place in 16
193 and 20 min in cheese making and packaging rooms, respectively. The mist dispenser produced particles
194 of aerosolized hydrogen peroxide freely circulating having access to all surfaces. After the air treatment,
195 an exposure time of 20 min was guaranteed to allow aerosol decomposition.

196

197 *2.7 Statistical evaluation*

198

199 The statistical analyses were performed using Minitab® software (Release 18, 2017; State
200 College, PA, USA). Comparisons of experimental data were performed with one-way ANOVA followed
201 by Tukey's post-hoc test to determine differences between means of microbial load at a significance level
202 of 0.05.

203

204 **3. Results and discussion**

205

206 *3.1 Hygienic design of the dairy factory*

207

208 Environmental conditions of a dairy factory are of utmost importance for the quality and safety of
209 final products. A preliminary activity of this research consisted in collecting information and evaluating
210 the hygiene status of structure, equipment and utensils through direct observation and dialog with the
211 personnel. The dairy factory was located in a rural area, surrounded by crop lands and linked to main
212 roads. The perimeter area was not paved or asphalted. This aspect could be critic in the creation and
213 transfer of bioaerosols inside the factory. The area outside the building was well kept and free of solid
214 waste, but the proximity to farmyards as well as the presence of decaying vegetation were factors
215 putatively promoting contamination of indoor environment. The surface of the building was in good
216 conditions, free of hollows or cracks and with a good level of care. Floors were smooth and not slippery.
217 Access routes and employees circulation were free of hurdles. Overall, the factory was maintained in
218 good conditions of hygiene, cleaning and tidiness. A ventilation system was adopted only in the storage
219 cell to guarantee a constant airflow rate injection, keeping the temperature at 4 °C and relative humidity >
220 90%. Air filters were properly maintained according to manufacturer instructions. Positive air pressure
221 systems as a tool to control air intake were not adopted inside the plant.
222
223 *3.2 Air microbial load in different areas of the dairy factory*
224
225 Microbial air contamination is an issue of concern to manufacturers of dairy products also occurring
226 in well designed, constructed and maintained factories (Early, 2017). Bacteria and moulds are common
227 and ubiquitous airborne contaminants in the dairy environment (Johnson, 2001). Yeasts are ubiquitous
228 microorganisms that form part of the microbiota of most natural ecosystems including man-made habitats
229 such as foods systems (Deak, 2006). In our survey, the microbial quality of the air in cheese making,
230 storage and packaging areas, was monitored through 200 analyses (blank samples). We adopted for air
231 sampling, the impaction technique which is more effective and dependable than the use of sedimentation
232 techniques in dairy plants (ISO/FDIS 8086, 2004). The mean counts (± standard deviation) for TBC,
233 moulds and yeasts were 161 (± 154) MPN m^{-3}, 228 (± 234) MPN m^{-3}, and 137 (± 439) MPN m^{-3},

234 respectively. The boxplot data set, detailing the airborne microbial counts within the 3 areas, was
235 characterized by wide interval levels of microorganisms (Figure 2). Several factors were judged as
236 sources or contributors to the spreading of microorganisms, including: processing conditions, employees
237 and product traffic, equipment surfaces, the hygienic design of the structures, agricultural practices in the
238 outdoor environment, and weather conditions. Intra-variability of data among the 5 sampling sites
239 (positions 1 to 5 in Figure 1) was low ($p < 0.05$) within each type of both microorganism and area (results
240 not shown). We observed that the presence of yeasts was meanly lower in comparison to that of other
241 microorganisms, likely since yeast cells are larger and denser (Ocón et al., 2013). Our results are in line
242 with levels identified as good quality air in dairy processing plants by other authors (Kang & Frank, 1989;
243 Ren & Frank, 1992). Taking into considerations the European Community Board indications (European
244 Collaborative Action, 1993), our mean levels of air microbial load fitted the ranges of contamination
245 classified as "medium" (100–500 MPN m^{-3}) or "very low" (0–50 MPN m^{-3}).

246 Counts relative to the cheese making room characterized for distribution intervals in the range 0–842
247 MPN m^{-3}, 80–458 MPN m^{-3} and 0–245 MPN m^{-3} for TBC, moulds and yeasts, respectively (Figure 2). Air
248 samplings were carried out after cleaning of equipment (paragraph 2.3), in "in operational" conditions. In
249 this environment, we hypothesized that factors accounting for most of the variation of microbial aerosol
250 consisted of: *i)* open access door and the subsequent fluctuation of temperature and humidity, *ii)* the
251 employees traffic, *iii)* the closeness to the external access (Figure 1), *iv)* the absence of a double door
252 system between outside and the processing environment and *v)* the adoption of the passageway as a
253 changing room.

254 The dairy location exhibiting the lowest count levels was the storage cell. To maintain stable
255 microclimate conditions, this environment was always kept closed. Both the short residence time of
256 products (1–2 days) and the air supply/handling system likely played a significant role in controlling the
257 risk. Levels of TBC, moulds and yeasts were in the range 0–352 MPN m^{-3}, 20–186 MPN m^{-3} and 0–398
258 MPN m^{-3}, respectively. Data distributions characterized for reduced interquartile ranges and the
259 occurrence of outlier data for TBC and yeasts during the 2nd week (Figure 2).

The culturable microbial community in the packaging room, consisting of 2 sampling series (paragraph 2.3), varied extensively over the period considered (0–492 MPN m^{-3}, 10–1253 MPN m^{-3} and 0–2323 MPN m^{-3} for TBC, moulds and yeasts, respectively) (Figure 2). In particular, some levels recorded for moulds and yeasts suggested a high degree of air contamination. Acceptable counts of fungi in a food processing area should be < 50 CFU m^{-3} (Lück & Gavron, 1990) or in the range 70–430 CFU m^{-3} (Kang & Frank, 1989). By comparing the mean counts of the 2 sampling series, the heavier loads occurred in the former (from 27th October to 24th November) ($p < 0.01$). In particular, spike levels of moulds and yeasts were recorded during the 2nd and 5th week, respectively. The reason for this so high occasional contamination was unclear, but it was likely related to the fact in the first series "in operational" samplings were carried out in the morning in the presence of employees. Differently, the second series of samplings (from 1st December to 2nd February) was executed "at rest", early in the morning. Overall data demonstrate that the strong antimicrobial effect of chlorine-based sanitizers is not enough alone to guarantee the respect of specific hygienic requirements over time.

3.3 Airborne mycobiota in the dairy environment

Fungal isolates in the air of the dairy factory (Figure 3) belonged to Ascomycota phylum and were classified in 7 genus and 11 species. The isolates observed in the indoor air mainly consisted of *Cladosporium herbarum* (59.8 %), followed by *Alternaria alternata* (20.7 %) and *Penicillium* spp. (13.7 %). *Aspergillus* spp. (3.0 %), *Mycelia Sterilia* (1.9 %), *Mucor racemosus* (0.7 %) and *Trichoderma viride* (0.2 %) were less frequent and their presence was not always revealed during the 5 sampling weeks (results not shown). A phenotypic approach (Pitt, Samson, & Frisvad, 2000) allowed to identify *P. expansum*, *P. nalgiovense* and *P. lanosocoeruleum*. Among *Aspergillus* genus we characterized the species *A. niger*, *A. flavus* and *A. candidus*. The relative distribution of moulds genera almost overlapped in cheese making and packaging rooms (Figure 4). Differently, in the storage cell *Penicillium* was the most abundant genus, likely as a consequence of the thermo-hygrometric conditions in this area which

286 favored its growth (Plaza, Usall, Teixidó, & Vinas, 2003). Moulds revealed in our survey are reported in
287 literature as responsible for dairy products spoilage, being *Penicillium* and *Aspergillus* the most
288 frequently reported genera (Garnier, Valence, & Mounier, 2017). Beletsiotis, Ghikas and Kalantzi (2011)
289 in a Greek dairy plant found as dominant genus both *Cladosporium* spp. and *Penicillium* spp.. Recently,
290 other authors (El-Fadaly, El-Kadi, Hamad, & Habib, 2015; Garnier et al., 2017) revealed also *Alternaria*
291 in different cheeses and dairy environments. D'amico (2014) described *Cladosporium herbarum* as a
292 common contaminant of cold rooms, ceiling in ripening rooms and air conditioning ducts. It is generally
293 accepted that the fungal dynamics of the indoor locations in a dairy factory is affected among the others,
294 by the outdoor environment (Beletsiotis et al., 2011). Fukutomi and Taniguchi (2015) reported that the
295 primary source of the genus *Cladosporium* is the outdoor environment.
296
297 *3.4 Antimicrobial effect of air ozonation*
298 Gaseous ozonation is reported to be an effective method for inactivating airborne microorganisms
299 (Cullen & Norton, 2012). This technique has been tested since several years to improve the hygiene of
300 cheese making (Serra, Abrunhosa, Kozakiewicz, Venâncio, & Lima, 2003; Shiler, Eliseeva, &
301 Chebotarev, 1978). We observed that in the packaging area the periodic (3 h/d) generation of ozone
302 overnight for 3 d per week allowed to improve the quality of air (Table 2). No bacteria isolates were
303 revealed in the plates (n = 25) during the 5-week trial, whereas the growth of moulds and yeasts was
304 observed only in some plates (n = 3 and 5, respectively) accounting for only 3 and 7 CFU, respectively.
305 Residual moulds consisted of *Alternaria alternata* and *Cladosporium herbarum*. Following PCR
306 fingerprinting to profile the yeast community after air ozonation we detected 4 species of yeasts, namely:
307 *Cryptococcus albidus*, *Debaryomyces hansenii*, *Bulleromyces albus*, and *Sporobolomyces roseus* (Table
308 2). Above genera exert a spoilage action in dairy environment (Montel et al., 2014). Also Bokulich and
309 Mills (2013) and Schön et al. (2016) reported the abundance of *Debaryomyces* in most surfaces of dairy
310 factory and floor drains. Under the conditions adopted in our trial and hypothesizing a uniform
311 distribution of the ozonated air, we calculated a predicted ozone concentration of 5 ppm at the end of each

312 treatment. Investigations carried out on the periodic ozonation of processing areas in dairy factories with
313 similar or slightly lower ozone concentrations prevented mould growth, without adversely affecting
314 chemical and sensory properties of the cheese (Pascual, Llorca, & Canut, 2007; Shiler et al., 1978). The
315 antimicrobial effect of ozonation supported the opinion of the Italian Ministry of Health endorsing the use
316 of gaseous ozone for disinfecting empty cheese ripening and storage facilities (2010).
317
318 *3.5 Antimicrobial effect of air treatment by hydrogen peroxide aerosolization*
319
320 Air disinfection through aerosolization of hydrogen peroxide allowed to significantly improve the
321 quality of air in a way similar to ozonation. In the packaging room, following the post-treatment air
322 sampling, bacteria and yeasts were not detected during the 5 weeks of investigation, whereas some
323 moulds (12 CFU/25 plates, Table 2) were identified. The latter mainly consisted of *Alternaria alternata*
324 (75 %). The effectiveness of this disinfection method in reducing viable airborne microorganisms was
325 ascribed also to the reduced drop size of aerosol particles, which disperse well and settle within short
326 time. Burfoot et al. (1999) supported that these size levels give good coverage and the mist clears from
327 the air quickly enough not to pose major impact on factory operations.
328 In the cheese making room on weekly basis, the air nebulization was carried out after the daily
329 sanitation program. The preferred approach to avoid airborne contamination consists in reducing the
330 generation of aerosol. Holah et al. **(**1995) reported that, in food production areas, cleaning operations
331 could be a major source of aerosols including microorganisms. Among cleaning operations, also low-
332 pressure hosing (500–600 kPa) was reported to produce a high particle flux nearby the jet, leading to large
333 increases in the number of airborne droplets (Burfoot et al., 2003). In our investigation, low-pressure
334 equipment was adopted for distribution of foam sanitizers and for washings of open plant surfaces. We
335 judged this sanitation procedure to minimally contribute to aerosol generation, due to the adoption of the
336 low water pressure (100–200 kPa) of the piping system. The microbiota resulting in the cheese making
337 room after air treatment consisted of only moulds (Table 2). Main residual moulds were *Cladosporium*

herbarum (62 %), *Penicillium* spp. (27 %) and *Alternaria alternata* (5 %). We ascribed this pattern to the room recontamination from outdoor air. Really, the most common fungal genera of outdoor air of rural areas are *Cladosporium*, *Penicillium*, *Alternaria* and *Aspergillus* (Kaarakainen et al., 2008; Kasprzyk and Worek, 2006). Microbial resistance to disinfectants is receiving large attention in food industry due to their frequent use (Bore & Langsrud, 2005). We considered this factor of scarce relevance because hydrogen peroxide-based biocides are not reported as prone to microbial resistance. Furthermore, it is known that microorganisms are more resistant to sanitizers when attached to a surface (Bagge-Ravn, 2003). We verified that the occurrence of *Cladosporium herbarum* and *Alternaria alternata*, revealed after both techniques of air disinfection (Table 2), was not related to resistance phenomena. Above fungi were isolated and tested for resistance to both ozonation and hydrogen peroxide aerosolization. As expected, no isolates were observed after both air treatments. Overall, we ascribed the microbial air re-contamination to other contributing factors, among which the unrestricted airflow and the access of employees.

4. Conclusion and perspectives

The results of this study suggest that the periodic monitoring of air quality is a helpful tool to verify the compliance of dairy environment to specific hygienic requirements or, if necessary, to follow up with corrective actions. The typical sanitation procedure, based on cleaning and disinfection of surfaces, may be boosted by the adoption of a complementary air disinfection protocol. The exploitation of this strategy allows manufacturers to better comply with the provisions laid down in the Regulation 852/2004 on food hygiene and balance out potential failures in hygienic design of structure and equipment. In light of results obtained, both ozonation and chemical aerosolization characterized for their effectiveness and wide antimicrobial spectrum, fulfilling the considerable and current interest in localized air control in indoor processing locations. These techniques, largely automated, could be used as terminal disinfection

step integrating the periodic cleaning procedures, thus contributing to the fulfilment of the microbial reduction targets, in particular in high-care areas. Issues to be properly taken into account are represented by the sizing of delivery systems. More research is needed, in the case of aerosolization, to measure the corrosion of equipments over time as a function of the chemical adopted.

Declarations of potential conflicts of interest

None.

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Captions to Figures

Fig. 1 – Layout of dairy factory (1 to 5 identify the location of air sampling sites).

Fig. 2 – Box and whiskers plots of airborne microbial loads in different environments of the dairy factory. The box represents the interquartile range (25^{th} – 75^{th} percentiles), the horizontal line the median, and the whiskers the range. Asterisks are outlier values (> 1.5 box lengths from the 75^{th} percentile).

Fig. 3 –View by phase-contrast microscopy of some moulds revealed in the air of dairy factory.

Fig. 4 – Relative distribution of moulds genera in the environment of the dairy factory.

Highlights

- Air acts as a vehicle of microbial contamination in dairy industry
- Monitoring of air bioload is of major concern in critical areas
- *Cladosporium*, *Alternaria*, and *Penicillium* are frequent spoilage contaminant
- Ozonation and hydrogen peroxide aerosolization inactivated air microorganisms
- After air disinfection, residual fungi derived from environmental re-contamination

Figure 1

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Figure 1

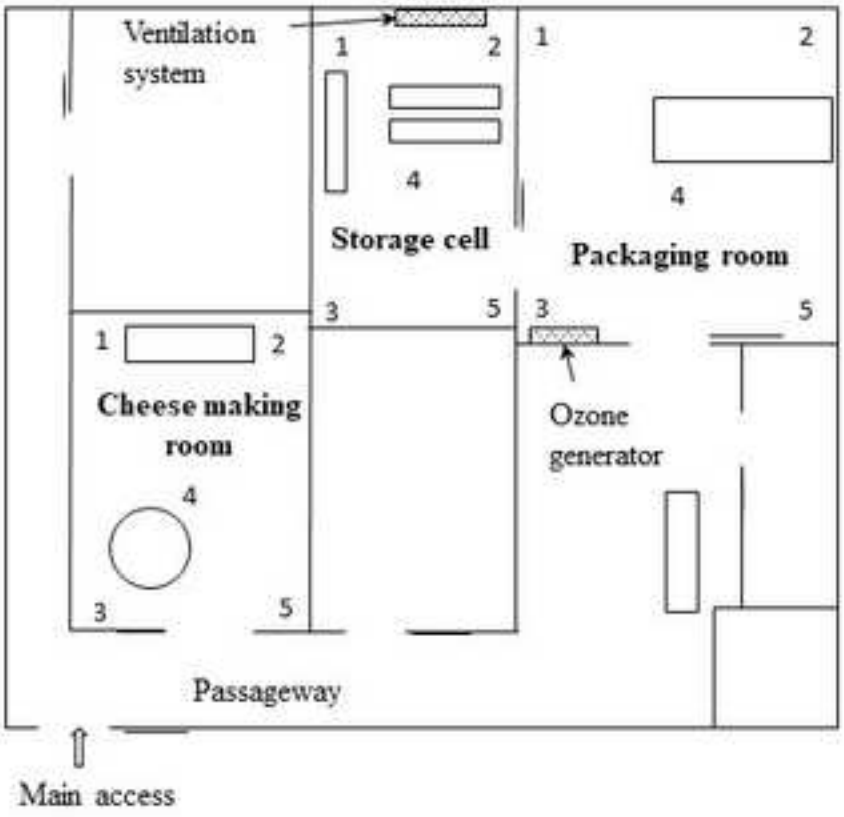

Figure 2

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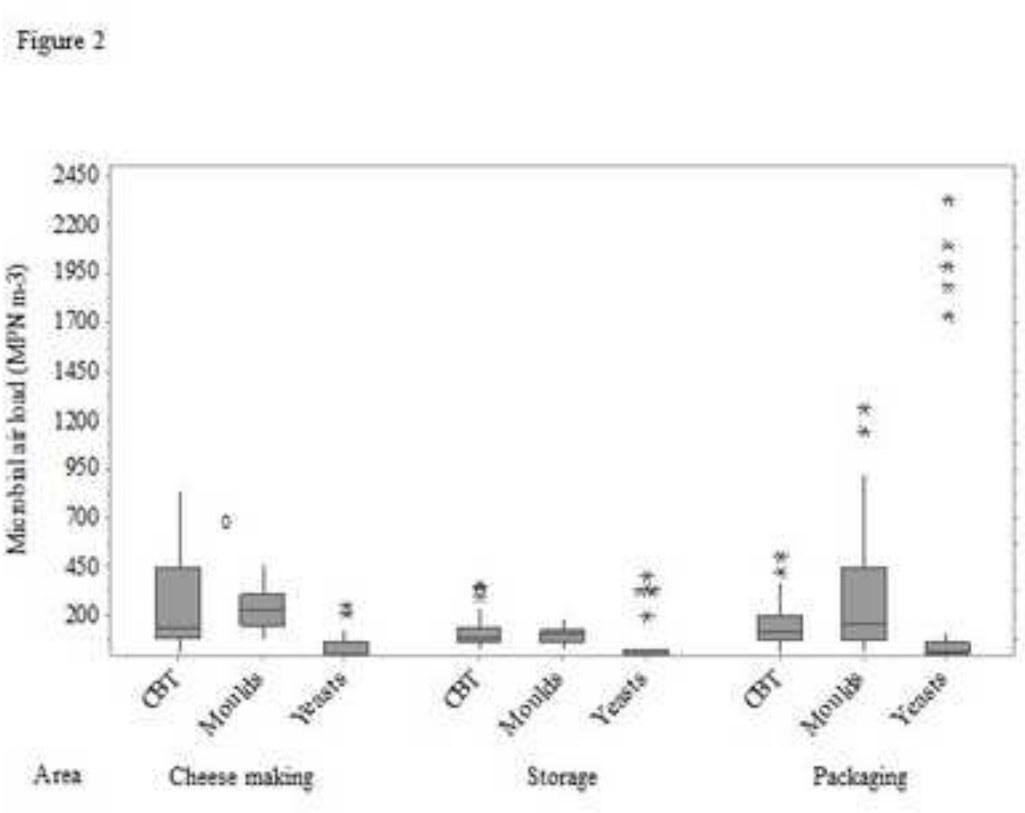

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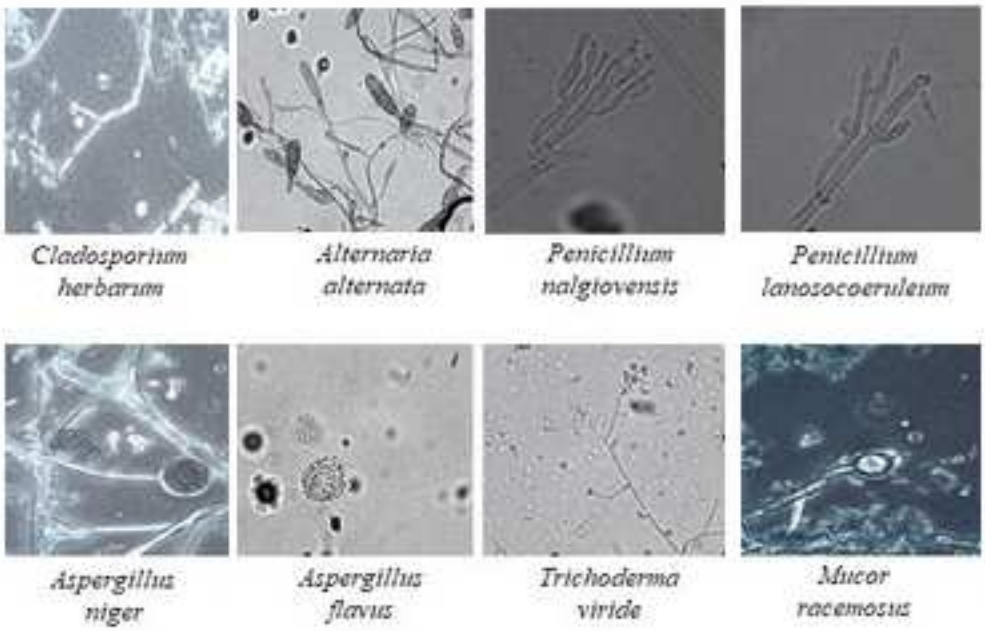

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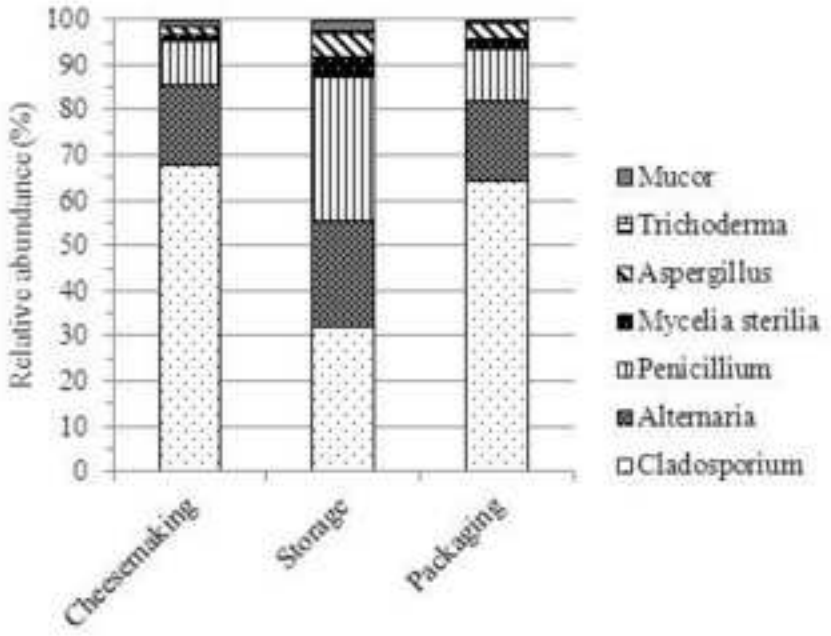

Table 1

Mean airborne counts (CFU m^{-3}; n = 5) of packaging area.

Microorganism	First sampling					Mean ± SD
	27th Oct	3rd Nov	10th Nov	17th Nov	24th Nov	
TBC	138[a]	265[a]	252[a]	284[a]	148[a]	217[A] ± 108
Moulds	412[b]	1006[a]	171[c]	499[b]	462[b]	510[A] ± 297
Yeasts	4[b]	22[b]	79[b]	4[b]	2004[a]	422[A] ± 813
Microorganism	Second sampling					Mean ± SD
	1st Dec	15st Dec	12th Jan	19th Jan	2nd Feb	
TBC	155[a]	98[a]	30[b]	44[b]	65[b]	78[B] ± 58
Moulds	63[b]	20[c]	148[a]	81[b]	73[b,c]	77[B] ± 51
Yeasts	6[c]	65[a]	8[c]	2[c]	32[b]	23[B] ± 26

Means with the same lowercase superscripts in the same row are not statistically different ($p < 0.05$). Means with the same uppercase superscripts in the same column are not statistically different ($p < 0.05$).

Table 2

Air microbial load (expressed as total colonies enumerated in 25 Petri dishes) before and after air disinfection treatments

Microorganism	Area					
	Cheese making		Packaging		Packaging	
	Blank[a]	H_2O_2[b]	Blank[a]	H_2O_2[b]	Blank[a]	O_3[c]
Bacteria	280	0	192	0	486	0
Moulds	261	55[d]	207	12[e]	1022	3[f]
Yeasts	47	0	56	0	756	7[g]

[a]: air load before aerosolization or ozonation; [b]: air load after hydrogen peroxide aerosolization; [c]: air load after ozonation.
[d]: *Alternaria alternata* (n = 3), *Cladosporium herbarum* (n = 34), *Mycelia sterilia* (n = 2), *Penicillium* spp. (n = 15), *Thricoderma viride* (n = 1); [e]: *Alternaria alternata* (n = 9), *Aspergillus* spp. (n = 1), *Cladosporium herbarum* (n = 1), *Penicillium* spp. (n = 1); [f]: *Alternaria alternata* (n = 2), *Cladosporium herbarum* (n = 1); [g]: *Bulleromyces albus* (n = 1), *Cryptococcus albidus* (n = 3), *Debaryomyces hansenii* (n = 2), *Sporobolomyces roseus* (n = 1).