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Analysis of Fungal Diversity in Ready-to-Eat Pizza and Effectiveness of Pulsed Ultraviolet-Light Treatment for Inactivation of Mold on Agar Surface

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Abstract

Fungal contamination is a significant issue in the food production industry; therefore, identification and characterization of food spoilage fungi would allow for early intervention to limit the amount of fungal contamination, particularly in cereal-based industries. In the present study, culture-dependent and culture-independent methods were applied to study the microbiota of ready-to-eat pizza. The study was pursued by evaluating the effectiveness of a broad-spectrum pulsed ultraviolet light for the decontamination of *Penicillium roqueforti* (a dominant spoilage mold in bakery products) on the surface of solid agar as a representative of a flat food surface. The average population of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), molds and yeasts (M+Y) on naturally spoiled pre-cooked pizza were 6.7 ± 0.5, less than 2.3, 2.8 ± 0.6 and 5.4 ± 0.4 log10 CFU g^{-1}, respectively. Cloning and sequencing identified at least 5 genera and species of fungi (*Penicillium* spp., *Saccharomyces* spp., *Rhodotorula mucilaginosa*, *Monascus fuliginosus*, *Galactomyces geotrichum*) from 2 phyla (Ascomycota and Basidiomycota). Pulsed light process parameters evaluated were treatment time (1, 3, 5, 7 and 10 min) and voltage input (500, 750 and 1,000 V) at 5 cm distance from the pulsed UV-light. An increase of the input voltage of the lamps and of the duration of the treatment resulted in a higher inactivation of *P. roqueforti*. The population of *P. roqueforti* was reduced after 10 min of exposure to pulsed light by 3.74, 5.36 and 6.14 log10 CFU ml^{-1} at 500, 750 and 1,000 V, respectively. The inactivation kinetics was best described by the Weibull model (2 parameters) with the smallest root mean squared error (RMSE) and $R^2 \geq 0.92$. The results of this work show that pulsed light is a promising technique for fungi elimination or decontamination in the bakery industry.

Keywords: Cloning; Food safety; Kinetics; Mold; Pulsed UV-light; Ready-to-eat pizza

Introduction

Bakery products remain an important component of a balanced diet and pizza in particular continues to be one of the most widely consumed fast foods in the world. Physical, chemical and microbial spoilage are of concern in most cereal-based products including pizza. Spoilage caused by fungi is considered to be the greatest factor in limiting shelf-life and the major cause of economic losses in the bakery industry. Ready-to-eat food products have become increasingly popular due to their convenience and improved flavor.

Ready-to-eat tomato pizza consists of oven baked white bread topped with tomato sauce and can be consumed without additional cooking. In food products that have been pre-cooked such as ready-to-eat pizza dough, fungi may be inactivated during baking, however, the food product may become contaminated again after cooking. Two types of fungi that are frequently found in ready-to-eat pre-cooked pizza dough belong to the *Penicillium* and *Aspergillus* genera [1]. Recently, molecular methods have provided another means for the detection, quantification, identification, and characterization of microorganisms in food products. These methods are now considered an indispensable tool that permits a more accurate description of the microbial ecology of food than culture-dependent methods alone [2,3]. Polymerase chain reaction (PCR) using domain or taxon-specific probes in combination with separation techniques such as DGGE and molecular cloning have revolutionized our understanding of the composition of food microbiota [4].

Pulsed ultraviolet-light is a unique emerging technology used mostly for surface decontamination. This non-thermal sterilization technology uses intense flashes of broad-spectrum radiation ranging from UV light (200 nm) to infrared radiation (1,000 nm) [5]. This novel technique is capable of reducing microbial populations of both vegetative cells and spores of bacteria, molds and yeasts [6]. Pulsed UV-light has been investigated as a means of extending the shelf-life of baked goods, seafood, meat products, and fruits and vegetables [7]. The ability of pulsed UV-light to kill fungal spores is well documented and studies have shown a 60-99% reduction of viable spores of *Aspergillus flavus, Aspergillus niger, Penicillium corylophilum* and *Eurotium rubrum* after only a 15 s exposure to pulsed UV-light [8]. Pulsed UV-light has also been successfully used to significantly reduce suspensions of *Botrytis cinerea* and *Monilia fructigena* conidia and to inactivate *Saccharomyces cerevisiae* in non-alcoholic beer [9,10]. In a study by Jun et al. [11] on corn meal, a 100 s treatment time, 3 cm of distance from the UV strobe with 3,800 V input gave a 4.93 CFU g-1 log10 reduction to the spore of *A. niger*.

In order to further understand the potential of pulsed UV light on mold decontamination, the response of *Penicillium roqueforti* to pulsed UV-light treatment need to be understood through modeling.

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The inactivation of microorganisms via UV light has been shown to follow first-order kinetics in solution [12], but it has also been reported to display a sigmoidal shape with a shoulder and/or a tail [13]. The aim of the present study is to analyze the abundance and composition of the microbial community found on ready-eat-to-eat pizza, with a focus on molds, and to determine the proper pulsed UV treatment necessary to construct inactivation model kinetics of a spoilage mold.

Materials and Methods

Sampling

Pre-cooked pizza samples that had been naturally contaminated with spoilage molds were obtained from a pizza-manufacturing company in the city of Montreal, QC, Canada. The samples consisted of baked white bread covered with tomato sauce. Samples for enumeration and identification purposes were collected at two different time intervals with 10 samples in June and 10 in September of 2013. Samples collected in June were used for the microbial analysis and those collected in September were used in order to create the 18S clone library.

Preparation of pizza samples for microbial enumerations

All of the spoiled pizzas were aseptically removed from their individual package and weighed. A 25 g amount of each sample was then placed in a sterile stomacher bag containing 225 ml of buffered peptone water (Don Whitley Scientific, WY, UK) (in the proportion with pizza samples at 10:1). The mixture was then blended in a Stomacher 400 Lab Blender (Seward medical, London, UK) for 2 min or until homogenized. From the resulting mixture, serial decimal dilutions (10-1 to 10-10) were conducted in buffered peptone water and the dilutions were spread-plated in triplicate onto appropriate media, as described below.

Microbial enumerations

To determine the total population of mesophilic aerobic bacteria (MAB) and mesophilic anaerobic bacteria (MANB), Petri plates containing Plate Count Agar (PCA, Oxoid, Hampshire, UK) were prepared. Plates were incubated at 35°C for 48 h under aerobic (MAB) or anaerobic (MANB) conditions. Anaerobic incubation was carried out in a Forma anaerobic system, model 1025/1029 (Thermo Scientific, Marietta, Ohio, USA) [13]. In order to enumerate populations of lactic acid bacteria (LAB), MRS Agar and M17 agar (Oxoid, Hampshire, UK) were prepared with a lactose solution (5% v/v) according to Terzaghi and Sandine [14]. Plates were incubated at 37°C for 48-72 h. Molds and yeasts (M+Y) were enumerated on Potato Dextrose Agar (PDA, Oxoid, acidified to pH 3.5 with a solution of 10% of tartaric acid). Plates were incubated at 24°C for 3-5 days. Colonies were counted with the aid of a Quebec Colony Counter (Reichert Technologies, Depew, NY, USA) and results expressed in terms of CFU g^{-1} food product.

DNA extraction

Packaged precooked pizza samples with signs of spoilage were aseptically opened and one gram of each sample (taken from a spoiled portion of the sample) was weighed and placed into a sterile stomacher bag. Sterile 0.1% peptone water (1 ml) was added to each sample and the contents homogenized in a Stomacher 400 Lab Blender (Seward Medical, London, UK) for 2 min. Total DNA was subsequently extracted using a Power Food Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. The concentration of eluted DNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA extracts were stored at -20°C until analysis as recommended by manufacturer. Although DNA extraction was from each of the 10 samples, insufficient DNA was extracted from two samples leaving a total of 8 samples used for fungal 18S sequencing.

PCR amplification of fungal 18S rDNA

Fungal (molds and yeasts) 18S rRNA genes were amplified from extracted DNA from spoiled pizza samples using primers NS1-F and NS6-F (Integrated DNA Technologies, Toronto, ON, Canada; Table 1). All PCR reactions were prepared in a dedicated PCR laminar flow cabinet (Esco Technologies Inc., PA, USA). PCR reaction mixtures and thermal cycling conditions were as described in Wu and Blomquist [15]. Briefly, each PCR reaction consisted of: 1×buffer (Denville Scientific, Saint-Laurent, QC, Canada), 500 µM of each primer, 1.5 U of Hot Start Taq DNA polymerase (Denville Scientific, Saint-Laurent, QC, Canada), 200 mM of each dNTP (Bioshop Canada Inc., Burlington, ON, Canada), 1-5 ng of template DNA extracted from the spoiled samples, and molecular grade water (Sigma-Aldrich, Oakville, ON, Canada) in a total volume of 50 µl. PCR amplifications were conducted using a Veriti Thermocycler (Applied Biosystems, Foster City, CA, USA) with the following temperature cycling profile: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 50 sec, and extension at 72°C for 90 sec, followed by 5 min at 72°C for the final extension. A positive control with DNA extracted from *Rhizoctonia solani* and a negative control consisting of the reaction mixture without DNA were used in each PCR run. PCR products were run at 75V for 85 min on a 1.5% agarose gel stained with ethidium bromide and visualized using a Red AlphaImager (Protein simple, Santa Clara, CA, USA). A Gene Ruler 100-bp DNA ladder (Thermo Fisher Scientific Inc.) was used to estimate the size of the products.

Cloning and sequencing of fungal 18S rDNA PCR products

PCR products were purified using a SpinSmart PCR purification kit (Denville Scientific Inc., Metuchen, NJ, USA) and cloned into a pGEM-T Easy vector (Promega, Madison WI, USA) according to manufacturer's protocol. Four to six white colonies (positive transformants) for each sample were selected and screened by PCR for the presence of an insert using primers M13-F and M13-R (Table 1). Each PCR reaction contained 1× PCR buffer, 200 nM of each primer (Integrated DNA Technologies, Toronto, ON, Canada), 80 µM dNTPs (Bioshop Canada Inc., Burlington, ON, Canada), 2 U Hot-Start Taq DNA polymerase (Denville Scientific, Saint-Laurent, QC, Canada), and molecular grade water (Sigma-Aldrich, Oakville, ON, Canada) in a total volume of 50 µl. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min, and then 10 min at 72°C for the final extension. PCR products were verified for the correct insert length on a 1.5% agarose gel.

PCR products containing the desired insert were selected for sequencing. In total, 28 clones were sequenced from 8 samples. Sequencing was carried out at the McGill University and Genome Québec Innovation Centre (Montreal, QC, Canada) using a 3730 xl DNA Analyzer system (Applied Biosystems, Carlsbad, CA, USA). Sequences were checked for putative chimeras using DECIPHER [16] and chimeras were removed before comparing sequences with those in the Ribosomal Database project (RDP; http://rdp.cme.msu.edu) and BLASTn (http://blast.ncbi.nlm.nih.gov). Sequences were submitted to the GenBank database under accession numbers KJ958135 to KJ958162.

Primer name*	Sequence [5′ → 3′]	Target	Amplicon size [bp]	References
NS1-F	GTAGTCATATGCTTGTCTC	Universal for fungi	1400	[42]
NS6-R	GCATCACAGACCTGTTATTGCCTC	Universal for fungi	1400	[42]
M13-F	GTAAAACGACGGCCAG	Plasmid linker region	1663	[24]
M13-R	CAGGAAACAGCTATGAC	Plasmid linker region	1663	[24]

*Primer set NS1-NS6 was used for the generation of PCR products for TA cloning. Primer set M13 was used for the sequencing of PCR products cloned with the Easy T vector system.

Table 1: Oligonucleotide primers used in this study.

Preparation of inoculum for pulsed UV light experiments

As *Penicillium* spp. were identified using DNA sequencing, Penicillium *roqueforti* ATCC 10010 (Cedarlane Corporation, Burlington, Ontario, Canada) was used throughout pulsed UV light experiments. *P. roqueforti* was grown in Potato Dextrose Broth (PDB, Difco, Spark, MD, USA) with shaking at 180 rpm at 24°C for 4 to 7 days. Cultures were maintained by spreading 0.1 ml of liquid culture on PDA. After incubating the PDA plates at 24°C for 7 days, the spores were harvested by adding 5 ml of Tween 80 (Acros Organic, New Jersey, USA) onto the surface of PDA, and then filtered by using 4 layers of cheese cloth. The resulting inoculum solution had 2.74×10^{10} CFU ml^{-1} and this cell suspension was serially diluted up to 1:100,000. An inoculum of 0.1 ml of each dilution (1:100, 1:1,000 and 1:100,000) was spread in triplicates on PDA plates.

UV inactivation of *P. roqueforti*

The pulsed-UV apparatus consists of a metal housing surrounding a treatment chamber made of polished stainless steel (20 cm wide × 14 cm deep × 12 cm high) and equipped with an upper Xenon lamp. PDA plates inoculated with *P. roqueforti* spores were positioned on the tray at 5 cm from the UV lamp in the treatment chamber. At this distance, the effect of treatment time (1, 3, 5, 7 and 10 min) and input voltages of 500, 750 and 1,000 V were investigated. Following UV treatment, all Petri plates were incubated for 4 days in the dark at 24°C and the impact of UV treatment on the inactivation of *P. roqueforti* was determined by enumerating the colonies on each PDA plate. The log10 reduction was calculated by subtracting the log10 value of the control from that of treated sample.

Development of log-linear model

Traditionally, microbial inactivation resulting from application of both thermal and non-thermal processes has been described by the log-linear equation [17]. This model (Equation 1) follows the rules of first-order inactivation kinetics to describe microorganism destruction [18].

$$\log\left(\frac{N}{N_o}\right) = -kt \tag{1}$$

Where N is the number of microorganisms at time t (CFU ml^{-1}), N0 is the initial number of microorganisms (CFU ml^{-1}), t is the treatment time (sec or min), k is the first-order extinction coefficient (s^{-1} or min^{-1}). From this equation, the classic "D-value" or the time necessary for a 1 log10 reduction can be calculated as the reciprocal of the first-order rate constant. The log-linear equation is simply appropriate for linear inactivation curves and research findings suggest that many inactivation curves are non-linear [17].

Weibull model

The second model used in the current study is the Weibull model (Equation 2) which is offered as a simple model for the description of microbial inactivation by thermal and non-thermal treatment methods [18]. In the case of UV-light treatment, inactivation curves are generally sigmoidal and exhibit concavity or convexity behaviors through downwards or upwards as a function of inactivation time or UV dose [19]. The Weibull model usually describes this behavior accurately. This model is composed of two parameters (α and β) given in the following correlation [17,18]:

$$\log\left(\frac{N}{N_o}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \tag{2}$$

Where N is the number of microorganisms (CFU ml^{-1}), N_0 is the initial number of microorganisms (CFU ml^{-1}), t is the treatment time (sec or min), α is the characteristic time (s or min) and β is the shape parameter (unit less). Many survival curves exhibit concavity, either downwards or upwards and the β parameter is used to describe this concavity. If β<1, the curve displays upward concavity. This can be a sign of stress adaptation of surviving microorganism. If β>1, the curve displays downward concavity that shows considerable damages of pulsed UV-light in the cells. In addition, by using α and β, reliable life (tR) (indicating the time necessary for 90% reduction in the number of microorganism) can be calculated [18]. This parameter corresponds to the D-value for the first log reduction (Equation 3).

$$t_R = \alpha(2.303)^{\frac{1}{\beta}} \tag{3}$$

Where α is a characteristic time and β is the shape parameter.

Statistical analysis

Analysis of variance was performed using PROC GLM in SAS (SAS Inst., Inc., Cary, NC, USA) to compare the effects of different applied pulsed-light intensity (voltage input) and treatment time (number of pulses). All treatments were done in triplicate and results were considered significant at the α=0.05 level.

Results and Discussion

Enumeration of microbial populations

In this work, the microbial diversity of 10 spoiled precooked pizza samples was studied. The samples were examined for the presence of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), molds and yeasts (M+Y) (Table 2). MAB counts varied from 6.5×10^5 (sample 8) to 2.2×10^7 (sample 5) CFU g^{-1} pizza product, with 4 samples having concentrations of approximately 10^6 CFU g^{-1} pizza product. MANB counts ranged from less than 101 to 2.7×10^2 (sample 5) CFU g^{-1} pizza product. These numbers are considerably lower than the MANB counts of 1.6×10^4 CFU g reported by Rodriguez et al. [20] in study of the shelf life of pre-baked pizza dough. In all precooked ready-to-eat pizza samples, counts of LAB ranged between 1.2×10^2 (samples 3 and 4) and 1.6×10^4 (sample 7) CFU g^{-1}. This is in agreement with a LAB concentration of 10×10^4 CFU g^{-1} reported by Rodriguez et al. [20] in prepackaged pizza dough.

Samples	Microbial populations [log_{10} CFU g^{-1}]*			
	MAB	MANB	LAB	Y+M
1	6.8	1.6	2.5	5.3
2	7.1	<1	3.0	5.2
3	6.1	<1	2.1	4.9
4	6.0	1.8	2.1	4.6
5	7.4	2.4	2.9	5.2
6	5.9	<1	2.1	5.4
7	6.8	<1	4.2	5.6
8	5.8	<1	2.9	6.0
9	6.5	1.8	3.0	5.9
10	7.2	1.2	2.7	5.8
Average	6.6 ± 0.5		2.8 ± 0.6	5.4 ± 0.4

*Mean values of the CFU g^{-1} for each sample, analyzed in triplicate, are reported. Refer to materials and methods for media specifications and incubation conditions. MAB: Mesophilic Aerobic Bacteria; MANB: Mesophilic Anaerobic Bacteria; LAB: Lactic Acid Bacteria; Y+M: Yeasts and Molds; log_{10}: Logarithm to the base 10; CFU: Colony Forming Units.

Table 2: Microbial populations in spoiled pre-formed pizza. Data are the results of the microbiological analysis of 10 samples.

However, Ricciardi et al. [21] observed LAB counts of 10^7 to 10^8 CFU g^{-1} in fermented bread, an expected result given that LAB are used to produce this particular fermented product.

The concentration of molds and yeasts ranged from 3.95×10^4 (sample 4) to 9.17×10^5 (sample 8) CFU g^{-1} in pizza products (Table 2). These counts are comparable with those recorded by Rodríguez et al. [22] of 3.78 to 6.09 log10 CFU g^{-1} in sliced wheat flour bread (with preservative) after 18 days of storage at room temperature. Yeast numbers are particularly dependent on the inoculation ratio of the dough. It was shown by Hammes et al. [23] that regardless of the inoculum's origin, yeasts in dough may originate from the flour or other ingredients in the dough and from the bakery environment.

Fungal diversity

To our knowledge, this is the first study to investigate the fungal (molds and yeasts) microbiota in packaged precooked ready-to-eat pizza using culture-independent analysis. The near full-length fungal 18S rRNA gene (≈ 1,400 bp) was amplified using universal fungal 18S primers, cloned, and then sequenced. A total of 28 PCR products from these clones were sent for sequencing and the average length of each sequence was 753 bp, all of which sharing 99 to100% similarity with sequences found in public databases. Sequencing of these clones revealed the presence of at least 5 genera of fungi from 2 phyla (Table 3).

Among the 28 clones sequenced, 7 clones (clones 9 to 14, and 26) from 4 samples were closely related to the *Penicillium* genus (Table 3). *Penicillium* is a genus of ascomycetous fungi that can be found on foodstuffs, leather, and fabrics. They are of economic importance in the production of antibiotics (e.g. penicillin), organic acids, and cheeses [24]. However, the presence of *Penicillium* spp. in food is also a spoilage issue. Some species produce toxins and may render food inedible or even dangerous for consumption. Hammes et al. [23] reported that *Penicillium* spp., such as *P. notatum*, *P. expansum,* and *P. viridicatum*, were the predominant spoilage molds in bakery products with a high water activity (>86%). Nielsen et al. [25] also indicated that *Penicillium roqueforti* is the major contaminant of rye bread. Rodriguez et al. [22] investigated the effect of modified atmosphere packaging on the shelf life of sliced wheat flour bread and identified *Penicillium* spp. as the most important spoilage agent in this product.

A total of 6 clones (clones 2, 3, and 15 to 18) from 4 samples were identified as either *Saccharomyces cerevisiae* or *Saccharomyces sp.* WW-W23 (Table 3). *S. cerevisiae*, also known as the baker's yeast, has a long history of use in food processing and production. It has been used for centuries as a leavening agent for bread and as a fermenter of alcoholic beverages. With its prolonged use in industrial applications, this yeast has also been the model for various studies on the principles of microbiology [26]. The presence of *S. cerevisiae* in the product may be due to contamination of the bakery environment with commercial baker's yeast. The investigation of the microbiota of sourdough bread by culture-dependent and culture-independent methods has revealed that the majority of all yeasts isolated from this product are *S. cerevisiae* [27]. *S. cerevisiae* has also been found to be dominant among yeasts in studies of microbial characterization of sourdough for sweet baked products [28].

Rhodotorula mucilaginosa was the third most frequently fungus identified in this study, with 5 clones (clones 21 to 25) from 3 samples sharing high similarity (100%) with this species (Table 3). This microorganism is an anamorphic genus of heterobasidiomycetous yeasts, normally found in air and soil. *Rhodotorula* species can also be isolated from human skin, stool, food and fruits and produce pink, orange and red pigments [29]. Red yeasts are the predominant yeasts recognized in many studies of food products and are primarily members of the genera *Rhodotorula, Rhodosporidium and Sporobolomyces* [30]. Although most species of the genus *Rhodotorula* are nonpathogenic, some of them are infectious [31]. *Rhodotorula* is characterized by the absence of ballistoconidia, no fermentation ability, no starch-like compounds, and no xylose in whole-cell hydrolyzates [32].

Three clones from sample 3 shared 99-100% similarity with the yeast *Monascus fuliginosus* (Table 3). Species of this genus are commercially important in the production of various Asian fermented foods, e.g. red-rice and food-colouring pigments [33]. *Monascus fuliginosus van Tieghem* is a cosmopolitan fungus found in soil, soya, sorghum, tobacco, rice, oat and silage [34].

Clone 27 had a high level of identity (99%) with *Galactomyces geotrichum*. Galactomyces is an arthroconidial ascomycetous genus [35,36]. *G. geotrichum* is considered a plant pathogen and is a common post-harvest fungus disease of citrus known as sour rot [37].

A single clone from sample 1 shared 99% similarity with *Hordeum jubatum* (foxtail barley). This plant is a short-lived perennial plant species indigenous to Western North America that has become naturalized in Eastern North America. It is most prevalent in soils with a high water table and high salinity [38]. The presence of *Hordeum jubatum* in the product can be due to contamination of the wheat, which is the main pizza dough ingredient, with foxtail barley seeds from soil.

Pulsed UV light and microbiological analysis

As sequences closely related to *Penicillium* spp. were predominant in the samples analyzed, *Penicillium roqueforti* was used to estimate the reduction of mold spores on the Potato Dextrose Agar surface following exposure to pulsed UV light. It was found that an increase in treatment time significantly ($p<0.05$) enhanced *P. roquefortii* inactivation at all three voltages assessed (Figure 1). There were significant ($p<0.05$) differences between all treatment times (1, 3, 5, 7 and 10 min) when 500 V was applied. For treatments using 750 V, a significant difference ($p<0.05$) was demonstrated between 1 to 3 and 5 to 7 min. When 1,000 V was used, UV exposure times of 1 to 3, 3 to 5 and 7 to 10 min showed a significant effect ($P<0.05$) on the inactivation of *P. roqueforti*.

Clone	Sample	Phylum	Closest match	Similarity [%]	Accession number	Sequence size of the clones [bp]
Clone 1	1	*Tracheophyta*	*Hordeum jubatum*	99	AF168852	781
Clone 2	2	*Ascomycota*	*Saccharomyces cerevisiae*	99	AY99853.1	800
Clone 3	2	*Ascomycota*	*Saccharomyces* sp. WW-W23	99	DQ345280.1	563
Clone 4	3	*Ascomycota*	*Monascus fuliginosus*	99	HM188430.1	800
Clone 5	3	*Ascomycota*	*Monascus fuliginosus*	100	HM188430.1	734
Clone 6	3	*Ascomycota*	*Monascus fuliginosus*	100	HM188430.1	561
Clone 7	4		Uncultured fungus clone	99	JN054689.1	561
Clone 8	4		Uncultured fungus clone	99	JN054689.1	849
Clone 9	5	*Ascomycota*	Penicillium expansum	99	JX470345.1	759
Clone 10	5	*Ascomycota*	Penicillium expansum	100	JX470345.1	734
Clone 11	5	*Ascomycota*	Penicillium freii	99	JX470344.1	816
Clone 12	6	*Ascomycota*	Penicillium freii	99	JX470344.1	809
Clone 13	7	*Ascomycota*	*Penicillium* sp. HSL	99	JX910356.1	745
Clone 14	7	*Ascomycota*	*Penicillium* sp. HSL	99	JX910356.1	798
Clone 15	7	*Ascomycota*	*Saccharomyces cerevisiae*	100	KF447113.1	808
Clone 16	8	*Ascomycota*	*Saccharomyces cerevisiae*	99	KF447113.1	828
Clone 17	8	*Ascomycota*	*Saccharomyces cerevisiae*	100	KF447113.1	766
Clone 18	5	*Ascomycota*	*Saccharomyces cerevisiae*	99	KF447113.1	797
Clone 19	6		Uncultured fungus clone	99	KC337083	814
Clone 20	7		Uncultured fungus clone	99	KC337083	826
Clone 21	7	*Basidiomycota*	*Rhodotorula mucilaginosa*	100	KC186124	803
Clone 22	1	*Basidiomycota*	*Rhodotorula mucilaginosa*	100	KC186125	803
Clone 23	1	*Basidiomycota*	*Rhodotorula mucilaginosa*	100	KC186126	802
Clone 24	1	*Basidiomycota*	*Rhodotorula mucilaginosa*	100	KC186127	803
Clone 25	2	*Basidiomycota*	*Rhodotorula mucilaginosa*	100	KC186128	766
Clone 26	2	*Ascomycota*	*Penicillium* sp. ljg1	99	KC833482	691
Clone 27	4	*Ascomycota*	*Galactomyces geotrichum*	99	JQ668740	529
Clone 28	4		Uncultured eukaryote	99	JX394808	728

Table 3: Tentative identification of clones by sequencing and BLAST analysis. Clone sequences were compared to 16S rRNA gene sequences in the GenBank database with BLAST.

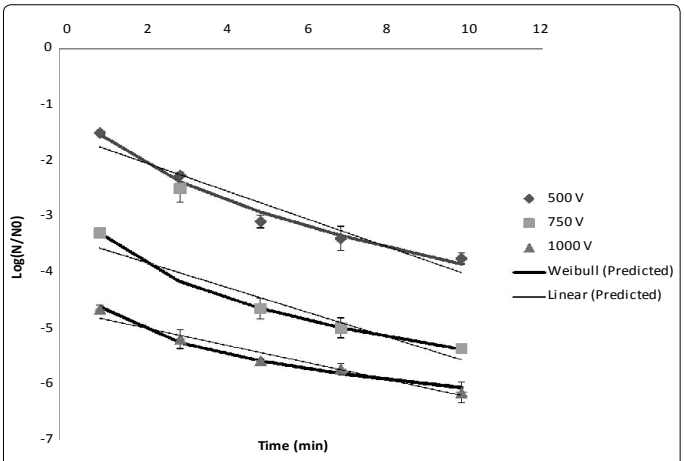

Figure 1: Inactivation of *Penicillium roqueforti* with pulsed UV light fitted with the first order and Weibull kinetics models. Inactivation was performed on Potato Dextrose Agar at three different voltage inputs (500, 750, and 1,000 V) and five different treatment times (1, 3, 5, 7, and 10 mins). Data shown are mean and standard deviation values.

Therefore, it can be noted that at a specific energy, the reduction in the population of *P. roqueforti* improved significantly with an increase in the treatment time.

According to our results, there was also a significant ($p<0.05$) improvement in the reduction of *P. roqueforti* when this mold was subjected to an increase in the voltage with a constant pulse number (or treatment time). After 10 min of pulsed UV light treatment, a log10 reduction of 3.74, 5.36 and 6.14 CFU ml^{-1} was obtained using 500, 750 and 1,000 V, respectively. Marquenie et al. [10] also reported a maximal inactivation of 3 and 4 log10 units for conidia of *Botrytis cinerea* and *Monilinia fructigena* in vitro after pulse UV treatment of 120 s at the fluence of 0.10 J cm^{-2}. The treated fungal populations of *Aspergillus niger* and *Fusarium culmorum* were reduced by 3 to 4.5 log10 orders after 1,000 light pulses of the 3 J UV intensity light [26]. The maximal log10 reduction was close to 1 with a pulsed UV-light fluence of 1.2 J cm^{-2} for *Aspergillus niger* inoculated in sugar syrup, whereas on agar, the log10 reduction for the same fungi was even lower than 1 for the same fluence [39,40].

Taking into account that the solid agar used in the experiments was artificially contaminated, the initial number of mold spores was higher than in industrial conditions. Therefore, it could be estimated that it is possible to achieve a complete decontamination by a 6.14 log10 CFU ml^{-1} reduction (the highest mold reduction achieved in this experiment). Therefore, the results of this work show that pulsed light can be considered a promising technique for fungi elimination or decontamination in the bakery industry.

Inactivation kinetics model

The comparison of the predicted and experimental inactivation data is shown in Figure 1. It was observed that none of the inactivation curves (at different voltages) exhibited a linear trend. As indicated

by these results, the Weibull model was found to more accurately estimate the microbial reductions obtained during pulsed UV light treatments. Bialka et al. [17] also reported that first-order kinetics are not suitable for the estimation of microbial inactivation on berries treated with ozone or pulsed UV-light, but that the Weibull model can be successfully used to estimate the reductions of *E. coli* O157:H7 and Salmonella enterica on raspberries and strawberries. A modified Weibull model was chosen by Levy et al. [40] to fit microbial reduction curves for *Bacillus subtilis, B. atrophaeus, B. cereus, Geobacillus stearothermophilus*, and *Aspergillus niger* treated by pulsed UV-light on solid agar. Koseki and Yamamoto [41] showed that a linear model was not suitable to describe the reduction of *Escherichia coli* during high pressure processing. Their findings also demonstrate that the Weibull model as well as the modified Baranyi model could be used to accurately estimate this inactivation and were capable of fitting the tail of the survivor curve resulting from high pressure processing. The ability of this model to accurately estimate reductions of *P. roqueforti* can be seen in Figure 1.

Goodness-of-fit parameters of log-linear and Weibull models for pulsed UV-light treatment are shown in Tables 4 and 5, respectively. These parameters provide greater insight into the shape of the inactivation curves. Overall, the RMSE and R^2 values obtained for the Weibull model are less than and greater than, respectively, those obtained for the log-linear model, with the exception of 750 V due to the existence of an outliner in the results. The scale parameter α is usually considered as a measure of the organism resistance (kinetic parameter) to treatment and decreases with exposure time. For the Weibull model, this value was found to be decreasing when voltage increased. For the three voltages, β values were less than 1, which accounts for curve's upward concavity, but also indicated that the remaining cells are less susceptible to pulsed UV-light, perhaps due to lack of pulsed light penetrability. A trend can also be seen in the dose required for 1 CFU ml^{-1} log10 reduction (tR): values for tR decrease when a higher voltage is applied, which indicates the cells are inactivated quickly with higher voltages. In fact, pulsed UV light irradiation was very effective at inactivating the spores of *P. roqueforti*; however, the dose of energy per pulse and the position of xenon lamp will determine the lethality. Moreover, decontamination by pulsed UV-light has to account for possible interactions between the target surface and the microorganism. Therefore, further research on the influence of pulsed light on foods with more complex composition is needed to define the applicability of the technology in decontamination processes [42].

Based on culture-dependent analysis, the number of microbes in the precooked pizza samples in decreasing order of concentration was: Mesophilic aerobic bacteria (MAB), molds and yeasts (M+Y), mesophilic anaerobic bacteria (MANB), and finally lactic acid bacteria (LAB). Analysis of 18S rDNA PCR-amplified clones revealed fungi communities with low diversity in ready-to-eat pizza samples. The spoilage microbiota was mostly comprised of fungi with a high sequence similarity to the *Penicillium* mold genus and to the *Saccharomyces* and *Rhodotorula* yeast genera.

The most effective pulsed UV treatment for inactivating *P. roqueforti* was found to be 1,000 V for 10 min. The experimental data also show that inactivation of *P. roqueforti* by pulsed UV light does not follow first-order kinetics, but that the inactivation kinetics of the tested microorganism on solid agar was best described by the Weibull model with the smallest RMSE and $R^2 \geq 0.92$. The results of this work show that pulsed light can be considered a promising technique for fungi elimination or decontamination in the bakery industry.

Voltage	RMSE	R^2	K
500 V	0.29317	0.9219	0.2497
750 V	0.72810	0.7296	0.2965
1,000 V	0.13248	0.9584	0.9584

RMSE: Root Mean Square Error; R^2: Root square; K: First-order extinction coefficient.

Table 4: Goodness-of-fit parameters of the first order kinetics models estimating reductions of *P. roqueforti* on solid agar after treatment with pulsed UV-light.

Voltage	α	β	t_R	RMSE	R^2
500 V	0.04326	0.40072	0.34687	0.1374	0.9828
750 V	0.00260	0.30359	0.04063	0.8163	0.6601
1,000 V	2.2×10^{-9}	0.11851	2.51×10^{-6}	0.0824	0.9839

α: Characteristic time [s or min]; β: Shape parameter; t_R: Reliable life [indicating the time necessary for 90% reduction in the number of microorganism]; RMSE: Root Mean Square Error; R^2: Root square

Table 5: Goodness-of-fit parameters of Weibull models estimating reductions of *P. roqueforti* on solid agar after treatment with pulsed UV-light.

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