On Measuring, Modelling and Validating Growth of Surface Molds through Image Analysis in Industrial Salami Ripening

Michele Miccio*[a], Michela Fraganza[a], Giovanni Cascone[a], Carlo Diaferia[b], Massimo Ferrara[c], Donato Magistà[c], Giancarlo Perrone[c], Massimiliano Dodaro[d], Franco Longo[d], Lucia Seta[d]

[a]Università di Salerno, Dipartimento di Ingegneria Industriale, Via Giovanni Paolo II 132, 84084 Fisciano (SA).
[b]SSICA – Stazione Sperimentale per l'Industria delle Conserve Alimentari, V.le Tanara 31/A, 43100 Parma.
[c]CNR – Istituto di Scienze delle Produzioni Alimentari (ISPA), Via Amendola 122/O, 70126 Bari.
[d]Salumificio Dodaro SpA, Strada Provinciale 19, km 217+400, Località Cammarata, 87019 Spezzano Albanese (CS).
mmiccio@unisa.it

This paper reports the development and the results of a procedure aimed at measuring, modelling and validating the growth of surface molds in industrial salami ripening by relying on techniques of image analysis. The sausages under investigation were inoculated with fungal starters and ripened in a test carried out at SSICA (Parma) under closely monitored and controlled conditions in a pilot-scale ripening chamber based on the "Air flow from bottom upward" technology. The work has been carried out within the R&D PON01_01409 "Safemeat" project.

Among the various investigations, digital images were purposely acquired in a standardized way throughout the experimental test of sausage ripening. The graphical procedures here discussed involve a bit of image pre-processing, a digital image analysis work and some data post-processing. A pre-processing software introduces a black background around each photographed sausage. The open-source ImageJ software is used for recognizing and measuring the gut area covered by molds as a whole, distinguishing each individual mold colony, measuring its surface area and counting the overall number of colonies. Further data post-processing provides results in terms of percent surface covered by molds, number of mold colonies per unit gut surface and size distribution of colonies as a function of their individual area. Microbiological analyses confirmed that the fungal population established on the salami casing immediately after the surface inoculum was exactly corresponding to the mold starters.

The developed methodology and the encouraging results obtained so far promise to be a rather simple and cheap way to control the onset and progress of the fungal colonization in industrial ripening chambers.

1. Introduction

Salami are cured traditional foods in different geographical parts of the world. They have been extensively studied in the areas of food science and technology (e.g., Katz and Stinsky, 1987) as well as of engineering design and simulation (Grassi and Montanari, 2005; Cascone et al., 2015).

The operating conditions (i.e., T, RH, air speed and ventilation pattern) during the industrial ripening allow the surface growth of a fungal population (Baldini et al., 2006), commonly composed of the Penicillium and Aspergillus species, which play an important role on the final quality of products.

The use of fungal inoculation during meat processing is a known practice (Spotti et al., 2008). When performed on fresh salami, the superficial fungal inoculum introduces an additional value (e.g., attractive surface appearance, easy detachment of the casing, etc.), and also works as a protective measure against growth of competitive molds able to produce even mycotoxins (Ferrara et al., 2016a; Magistà et al., 2016).

Several studies have been made on surface molds from microbiological and toxigenic viewpoints (Ferrara et al., 2016b). Nevertheless, to date no experimental investigations have been carried out about quantification and monitoring of surface mold through image analysis techniques.

As a part of PON "Safemeat" research project, aimed at the development of "innovative" methods for salami production with low animal fat content and addition of functional starters, a set of experimental tests on salami ripening has been recently carried out at semi-industrial scale, under closely monitored and controlled conditions, with a comprehensive plan of investigations, including sausage sampling, during the ripening time (PON "SafeMeat", 2015). The consequent experimental results form the basis of the present work.

2. Materials and methods

2.1 Samples under investigation

The tested sausages come from the same ripening test carried out in a pilot, ascending-flow pilot chamber that is located in the SSICA labs at Parma (Italy), has a volume of about 10 m^3 and max load capacity of 300 kg (PON "SafeMeat", 2015). The salami under investigation were manufactured starting from a traditional recipe (i.e., "salsiccia dolce", 43-45 mm natural gut diameter) by the Dodaro SpA company (Spezzano Albanese, Italy). For handling and working simplicity, the shape imprinted to the fresh sausage has been that of a pseudo-cylindrical "stick" rather than the typical "horseshoe" shape of the Calabrian sausage. The *Lactobacillus plantarum* strain 187 functional starter, isolated and characterized by the University of Foggia (PON "SafeMeat", 2015), was added to the fresh sausage paste. A batch of 250 kg of fresh sausages was produced by Dodaro SpA and then transferred to SSICA under refrigerated conditions (4-8 °C). At the 7th day of test running in the SSICA pilot chamber, a sub-lot of the sausages was inoculated on the outer surface with fungal starter strains, i.e., *Penicillium nalgiovense* (Perrone et al., 2015) and *Penicillium salamii* (Magistà et al., 2016), provided by the CNR-ISPA (Bari, Italy). In parallel, the non-inoculated sausages were ripened as "control samples" (PON "SafeMeat", 2015).

The SSICA3-L3 samples investigated here come from the same rack No. 1, same level (L3), same support "stick", during the ripening test No. 3 carried out by SSICA in Parma from March 17 through April 23, 2015, for a total duration of 37 days, with a final salami weight loss of about 47%.

Among the various investigations, the above test made available digital images of salami and microbiological determinations of surface mold concentration (PON "SafeMeat", 2015). Photos show always the same sausage sample (SSICA3-L3) for approximately ¾ of its length at increasing ripening time.

2.2 Fungal starters

The fungal inoculum of *Penicillium nalgiovense* ITEM 15292 and *Penicillium salamii* ITEM 15302 starters was prepared by scraping conidia from pure cultures of the strain grown on PDA (Potato Dextrose Agar) plates.

Sausages were inoculated with a conidial suspension at a concentration of 10^4 colony-forming units (CFU)/mL. The concentration of conidia adhering to the gut surface after inoculation has been estimated as described in the following. Three samples of inoculated sausages were shipped to CNR-ISPA immediately after inoculum. Each inoculated sausage was individually dipped as received in a sterile plastic bag with a known amount of sterile distilled water, for one minute. The conidia concentration was determined by plating aliquots of appropriate 10-fold serial dilutions from the respective solutions on Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, Oxoid, UK), and incubating them at 25 °C for 3–5 days. The volumetric fungal concentration (CFU/mL) was then converted in surface concentration (CFU/cm^2) using the estimated surface area of each sausage and expressed as log CFU/cm^2. The isolated fungal colonies were grown in purity, and their identification was carried out by morphological methods (Perrone et al., 2015).

2.3 Image analysis procedures

Figure 1 – An example picture of a sausage placed for its length against two orthogonal rulers.

Digital images of the same sausage have been acquired in a standardized way under repeatable conditions in subsequent days during the ripening test at the SSICA labs. A Canon PowerShot G7 has been used as camera with a resolution of 3648x2736 pixels. Each picture has been taken over a white paper by positioning the sausage for about ¾ of its length against two orthogonally placed rulers (see Figure 1).

Original images have been rastered and processed using Adobe Photoshop© software to get a black homogeneous background, then submitted with ImageJ software (ImageJ, 2017) to different procedures for image processing, which are briefly outlined in the following.

2.3.1 RGB color threshold method

1. ImageJ automatically recognizes the portion of the pic under examination with its original colors, which is to be measured, and calculates its total area in terms of number of pixels;

2. the operator adjusts the threshold levels in the RGB color space by choosing the lowest threshold and the highest one so that the area covered by molds in the pic under examination (white surface in the original photo) appears red-colored in the processed image;

3. ImageJ automatically highlights each circumscribed area (red-colored) by a closed (yellow) line;

4. ImageJ automatically measures in pixels the total surface in all circumscribed areas (red-colored) and exports the result in pixels into an MS Excel© table;

5. the operator calculates the % surface covered by molds as the ratio of the previously identified areas.

2.3.2 Binary image auto-threshold method

a. the operator loads the pic under examination with its original colors to the desktop, makes a working copy of it and converts the working copy to grayscale (8 bit in our case);

b. ImageJ automatically recognizes the portion of the working pic that is to be measured, and calculates its total area in terms of number of pixels;

c. the operator triggers an "auto-thresholding" action by which the working copy of the pic is moved from a grayscale format to a binary one (i.e., black and white);

d. ImageJ automatically counts the white pixels, which are to be referred to mold-covered surface in the original pic and exports the result in pixels into a MS Excel© table;

e. the operator calculates the % surface covered by molds as the ratio of the previously identified areas.

2.3.3 Watershed segmentation method

- the operator loads the pic under examination with its original colors to the desktop, makes a working copy of the pic, assigns a metric scale (cm) to it by direct comparison with the rulers and then converts the working copy to grayscale (8 bit in our case);

- ImageJ automatically recognizes the portion of the working pic that is to be measured, and calculates its total area in cm^2;

- the operator triggers an "auto-thresholding" action by selecting one of the following methods (Default, Otsu, Minimum or Intermodes); the working copy of the pic is moved from a grayscale format to a binary one (i.e., black and white);

- the operator triggers a "watershed" segmentation algorithm by which mold colonies are recognized as "discrete particles"; hence, particles are numerated, those ones that touch are automatically separated or cut apart, yielding their overall number;

- the operator triggers an "analyze particles" action by means of the Fiji software plug-in, which yields the surface area in cm^2 and the Cartesian coordinates in cm of each mold colony;

- the operator triggers a "summarize" action by which the previous results are logged and exported into a MS Excel© file for further data processing.

3. Results and discussion

3.1 Analysis of images

Figure 2 shows the first outcome of the image analysis on the SSICA3-L3 sample as the percentage of the gut surface covered by molds. As expected, the figure shows a trend to a natural growth of the molds with the

time progress, after an induction time. Obviously, the growth rate is initially high (see the days between 10 and 14) and then declines (i.e., after the day 27), determining a gradual trend for the mold-covered surface to approach a plateau toward the end of ripening. The different methods present a scatter in measured values that appears to become larger after the induction time. All in all, the final value settles in the 55-65% range of the entire surface and, therefore, does not correspond to the whole coverage of the salami, although purposely treated on the surface by molds starters at the 7th day. As a support to these observations, it should be noted that salami inoculated by the starter molds have visually shown for the whole test a more widespread and uniform fungal presence on the casing with respect to the corresponding non-inoculated "control samples" (PON "SafeMeat", 2015).

The induction time turns out in the order of 3 days (i.e., 10th ripening day), as confirmed both by direct visual observations during the test and from other digital images taken during the first days following the inoculum (data not shown), but demonstrating no growing of molds on a visible scale.
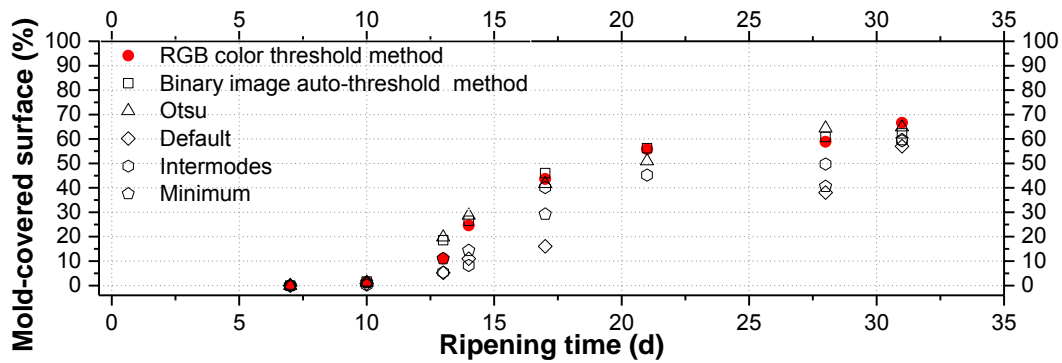

Figure 2: Time profile of percent surface covered by molds from the different image analysis methods

Figure 3 shows another output of the image analysis in terms of "discrete particles" (number of colonies) per unit sausage surface [cm^{-2}] as a function of the ripening time. In this case the growth pattern of the number of colonies is non-monotone, as roughly indicated by the plotted trend curve, and is passing through a maximum at a time (i.e., day 24) that is about 2/3 of the ripening test length (i.e., 37 days). After the inoculum, the growth rate in the number of colonies is obviously high. Later, when the colonies become large enough in size, face some spatial interference on the gut surface and begin to merge each other, the number of "discrete particles" as detected by the image analysis method is to be interpreted as the number of "emerging colonies". This latter first levels off and then decreases (see Figure 3). Again, in many cases the different methods present a considerable scatter in measured values.
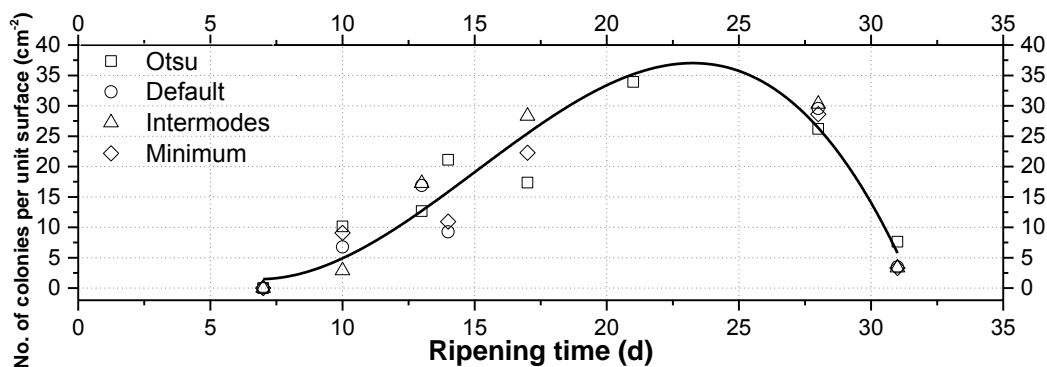

Figure 3: The number of mold colonies per unit surface [cm^{-2}] as a function of the ripening time as obtained by the different image analysis methods

Figure 4 shows the time evolution of the size distribution of mold colonies. This latter is referred to as p(A) and was defined at each time as

$$p(A) = \frac{1}{N_{tot}} \frac{N(A + \Delta A) - N(A)}{\Delta A} \quad [cm^{-2}] \tag{1}$$

where N_{tot} is the overall number of mold colonies on the gut surface at time t; $N(A + \Delta A) - N(A)$ is the number of mold colonies in the surface range between $A \; and \; (A + \Delta A)$ at time t; ΔA is a surface size interval, which is taken as a suitably small value with respect to the largest colony surface measured at time t. Actually, p(A) has been calculated at each ripening time after a suitable processing of output data generated by the above mentioned "Watershed segmentation method".
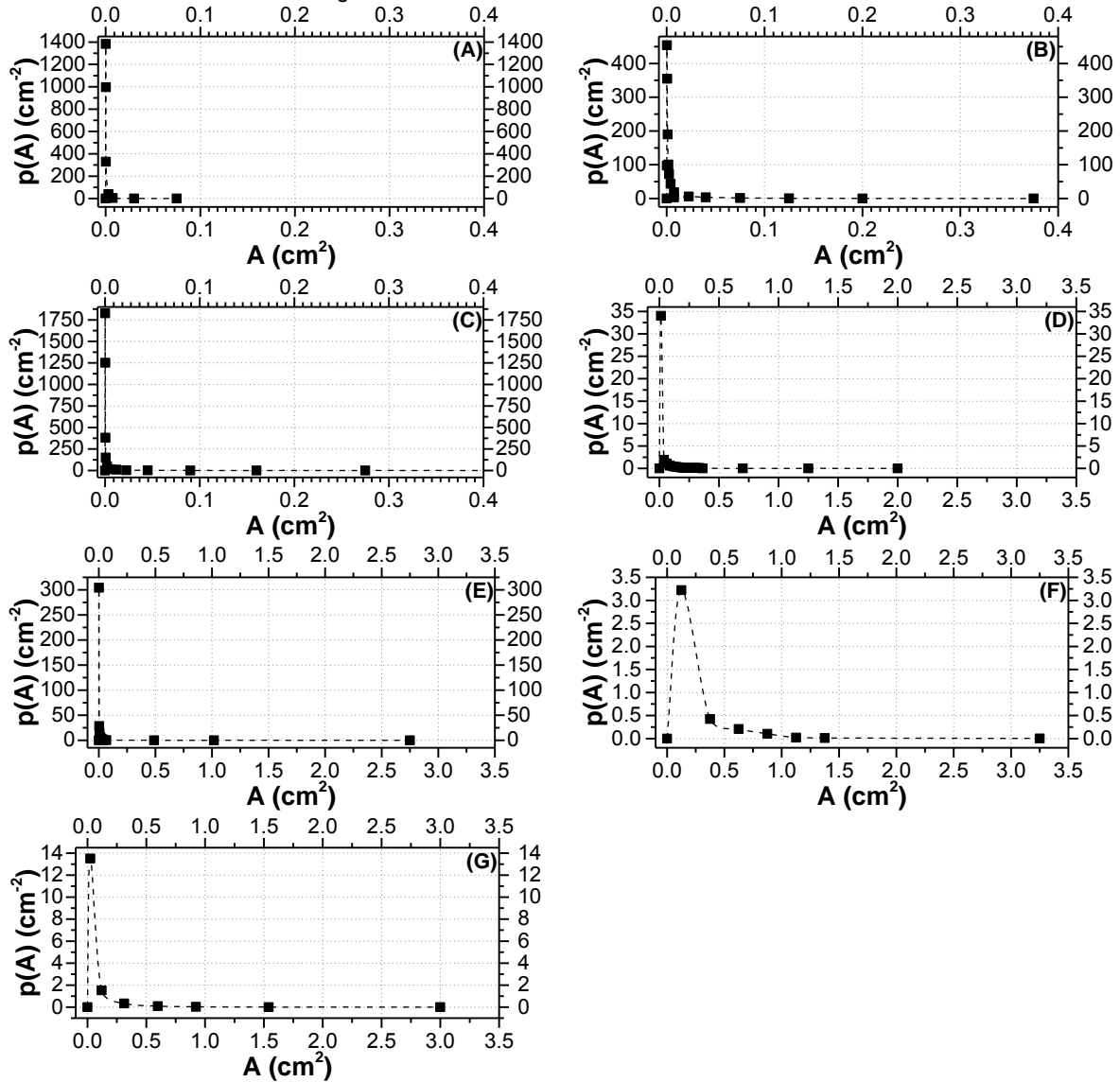

Figure 4:Time evolution of the calculated size distribution p(A) of mold colonies during the ripening test. (A) 10 days; (B) 13 days; (C) 14 days; (D) 17 days; (E) 21 days; (F) 28 days; (G) 31 days

All the size distribution curves show the same pattern in Figure 4 with a spike in the initial part indicating the presence of a very large number of colonies with a very small surface area with respect to the maximum A value detected on the sample. Of course, the height of the spike is larger, i.e., the number of colonies is huge, in correspondence of the early stages of mold growth, i.e., for a ripening time lower than about 15 days. Please notice that it was not possible to plot the size distribution curves with the same scales in Fig. 4A-G because of the noticeably different values on the x- and particularly the y-axis at the different ripening times. When approaching the end of ripening, e.g., day 28 (Fig. 4F) and day 31 (Fig. 4G), the mode of the distribution moves to the right and the spike appears both shallower in height and flatter at the base, indicating that mold colonies have reduced in number and consistently grown in size, with a number of them having a surface A>1 cm^2. By extension, if the sausage had been completely covered by molds, the size distribution curve would have turned in Dirac δ, i.e., an infinitely tall spike of zero width, positioned at an abscissa equal to the sausage external surface.

3.2 Microbiological analyses

The average fungal population determined on the salami casing immediately after the inoculum was 2.3 log CFU/cm^2. The identity of re-isolated pure colonies of P. *salamii* ITEM 15302 and P. *nalgiovense* ITEM 15292 was confirmed morphologically.

4. Conclusions

A new study methodology for fungal growth was successfully developed based on availability of digital images of sausage surface during an industrial-like ripening test. The developed methodology overcomes one of the limitations of the previous work (PON "SafeMeat", 2015), which was restricted to consider the mold-covered surface as a whole. Vice versa, the technique here proposed is able of counting the fungal colonies, discriminating their size and allowing their size distribution to be quantified and mathematically described.
The developed methodology and the results obtained promise to be very interesting in view of an application in the sausage ripening industry. Apart from a trained operator, a few simple and cheap tools are just required for the implementation of this technique: they are a commercial digital camera, an ordinary PC and open-source software for image analysis and spreadsheet elaboration. Therefore, once implemented, this technique can provide monitoring and quantitative assessment of the mold growth in quasi-real time during the first part (2/3 of duration) of ripening, and give the possibility of timely corrective actions in the operation of the industrial ripening chamber, if required.
As a follow-up of this work, additional knowledge is to be pursued to quantitatively assess how uniformly the mold colonies are distributed on the gut, i.e., preferentially or randomly positioned along the sausage main spatial coordinates. Moreover, the statistical significance of the obtained results is to be better assessed. Further development effort is in progress.

Acknowledgement

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