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ORIGINAL ARTICLE

Growth characterization and predictive behavior of *Eurotium* species in a feedstuff matrix

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Abstract Animal feeds are characterized by low water activity values. Nevertheless, fungal contamination with *Eurotium* species are quite common, causing nutritional depletion, spoilage and economic losses.

The aim of this work was to assess *Eurotium amstelodami*, *E. chevalieri*, *E. repens* and *E. rubrum* growth in a feed matrix at different conditions of water activity (0.71–0.97) and temperature (5, 15, 25, 30 and 37 °C).

It was found that *Eurotium* species are able to grow in a wide range of water activity and temperature in a short period of time (7 days) and faster than in synthetic media. Rosso and probabilistic models were applied in order to determine the limiting and optimum growth conditions as well as growth probability at certain combinations of environmental factors. Both models provided an accurate fit to the cardinal parameters and good performance for growth/no growth cases.

This is the first report assessing the growth parameters of *Eurotium* species directly in animal feed. Data obtained in the present study is useful to predict and avoid *Eurotium* species growth in animal feed.

PALABRAS CLAVE
Eurotium;
Matriz alimento balanceado;
Factores ambientales;
Actividad de agua;
Modelado

Caracterización del crecimiento y comportamiento predictivo de especies de *Eurotium* en matriz de alimento balanceado

Resumen Los alimentos balanceados se caracterizan por tener bajos valores de actividad de agua. Sin embargo, la contaminación por hongos con especies de *Eurotium* es bastante común y causa agotamiento nutricional, deterioro y pérdidas económicas.

El objetivo de este trabajo fue evaluar el crecimiento de *Eurotium amstelodami*, *E. chevalieri*, *E. repens* y *E. rubrum* en una matriz de alimento balanceado a diferentes condiciones de actividad de agua (0,71-0,97) y temperatura (5, 15, 25, 30 y 37 °C).

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Se determinó que las especies de *Eurotium* pueden crecer en un amplio rango de actividades de agua y temperatura en un corto período de tiempo (7 días), y a mayor velocidad que en medio sintético. Se utilizaron los modelos de Rosso y probabilísticos para determinar las condiciones de crecimiento limitantes y óptimas, así como la probabilidad de crecimiento en ciertas combinaciones de factores ambientales. Ambos modelos proporcionaron un ajuste preciso a los parámetros cardinales y una buena *performance* para los casos de crecimiento/sin crecimiento.

Este es el primer trabajo que evalúa los parámetros de crecimiento de las especies de *Eurotium* directamente en alimento balanceado. Los datos obtenidos en el presente estudio son útiles para predecir y evitar el crecimiento de especies de *Eurotium* en este tipo de alimentos.

Introduction

Feedlot practices were introduced in Argentina in the 90s mainly for economic reasons. Nowadays, this practice still continues and at the same time feedstuff production has globally increased. A recent feed survey indicates that worldwide feed production has exceeded a billion metric tons[4]; 10.6% of feed production in the Latin American region is represented by Argentina[34]. Particularly, more than 14 million tons were produced in 2015, while the most recent estimates suggest that around 25 million tons will be produced by 2020[27]. Animal feeds are intended for cattle, dairy cattle, porcine, poultry, aquaculture, rabbit and chinchilla, and more recently buffalo. Moreover, an important growth has been registered in the pet food industry in the last years. In addition to industrial feed production, small farmers also elaborate their own feed. However, in some circumstances, lack of quality assurance of raw material, improper technical production process or inappropriate storage conditions can lead to fungal spoilage development and mycotoxin production. In addition, sometimes due to lack of knowledge or negligence, despite the fact that visible fungal growth is observed, feeds are mixed with non-contaminated feed in order to dilute its contamination, thus economic losses are avoided. Therefore, feed quality decreases and becomes a risk for animals, and consequently for the complete food chain[37].

Animal feeds are composed of the necessary nutrients that animals need at every stage of their growth to reach a proper development and maximal growth in a profitable period of time for producers. Several parameters are involved and considered to define the quality of feed pellets such as formulation, durability, starch gelatinization and protein content, particle size, exposure to high pressure steam and proper cooling, among others[17]. In addition, the final product stability is regulated by a_w. Therefore, a_w is the most critical parameter to be taken into consideration since variations tend to increase fungal proliferation[11,29,46].

Fungal contamination in raw materials and animal feed has been widely reported worldwide. In fact, most of them emphasize mycotoxigenic genera, mainly *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*[5,16,26,37,42]. In addition, based on the findings reported by Hassan et al.[26], mixed cereals used as animal feeds are contaminated with high levels of mycotoxigenic fungi and mycotoxins. Moreover, several cases of human and animal intoxication and death caused by food and feedstuff contaminated with mycotoxins were also informed in the recent years[18,21,33,38].

The presence and development of fungi cause changes in food and feed properties involving nutritional and organoleptic quality aspects. Due to their xerophilic physiology *Eurotium* species are able to grow at a_w values as low as 0.65[12,28]. Furthermore, these species are also able to produce a large variety of secondary metabolites[10,22,32,43]. Therefore, it is of relevance to study the behavior of *Eurotium* species in order to prevent their growth in animal feed, and their potential production of secondary metabolites.

In recent years mathematical modeling has gained importance since they are considered helpful tools applied to avoid fungal growth and increase food shelf-life.

Previously, we have reported a high incidence of *Eurotium* species in raw materials and animal feedstuff and an initial study of the effect of a_w and temperature on the growth of *Eurotium* species was performed on synthetic media[23]. However, considering the high incidence and magnitude of damages that this genus can cause, it is more accurate to study the effect of environmental parameters in a more realistic context thus, in this case in a feed matrix.

Therefore, the aims of this work were (i) to study the effect of a_w and temperature on the growth of *Eurotium* species in a feed matrix, and (ii) to obtain predictive mathematical models for each species: the cardinal model of Rosso for secondary kinetic growth, and the probabilistic model for growth/no-growth interface.

Materials and methods

Fungal isolates

Four *Eurotium* species previously isolated from rabbit and chinchilla feed were used in the present study. *Eurotium amstelodami*, *E. chevalieri*, *E. repens* and *E. rubrum* strains were previously identified by scanning electron microscopy and DNA sequencing and characterized by

secondary metabolite profiling, and growth on synthetic media[22,23].

Experimental design and data generation

A full factorial design was used to study the growth of four *Eurotium* species in a feed matrix. The effect of nine a_w and five temperatures were evaluated. The a_w assayed were in the range 0.71–0.97 (0.71, 0.73, 0.75, 0.79, 0.83, 0.87, 0.91, 0.95, 0.97), and the incubation temperatures were 5, 15, 25, 30 and 37 °C.

Additional a_w values were evaluated for some of the species: *E. amstelodami* at 0.77 and 0.81 a_w at 15, 25, 30 and 37 °C; *E. repens* at 0.85 and 0.89 a_w and 5, 15, 25 and 30 °C.

The maximum a_w that the feed could reach without showing excess of water was 0.97 a_w corresponding to a moisture content of 60%. Above this value there was surrounding water.

Feedstuff media and water activity adjustment

Commercial a package of rabbit and chinchilla feed was purchased from a local store. The total content was subdivided into 1 kg samples, each of which was placed in plastic bags and the total number of bags were placed into the original package made of a double layer of wood paper, and then closed again. Immediately, the bag sample was sent to the Semi-Industrial Irradiation Plant (PISI) in the Ezeiza Atomic Center (Buenos Aires province, Argentina) where it was irradiated with 35kGy of gamma irradiation in order to achieve sterilization. Initial moisture content and a_w were $11.06 \pm 0.43\%$ (humid basis) and 0.687 ± 0.014, respectively. Then, an adsorption isotherm was built. First, small samples (50-100 g) were adjusted to the required a_w, and then were scaled to 1 kg sample size. Each kilogram of sample was put into a 3 liter glass jar previously sterilized in autoclave at 121 °C for 15 min and dried in a drying oven at 105 °C for 3 h. Later, appropriate volumes of sterile distilled water were added in aseptic conditions, mixed and stored at 4 °C for 7 days with periodic mixing. Moisture content was determined according to the AOAC Official Method[6] and a_w was measured with LabSwift-aw Novasina. Each determination was done by triplicate, jars were vigorously shaken and samples were taken from different points. The feedstuff media were aseptically transferred into the Petri plates, a thin layer of feed was placed into them (10–15 g), and previous to inoculation the plates were placed at each final incubation temperature for 24 h in order to reach the incubation temperature[40].

Inoculum, inoculation and incubation

Heavy sporulating cultures of each strain were obtained after seven days of growth at 25 °C on Czapek yeast extract agar with 20% of sucrose (CY20S). Conidia were collected from the plate surface and suspended in 5 ml of a water/glycerol solution adjusted to the required a_w level and containing 0.05% v/v of Tween 80. Conidia concentration was determined with a Neubauer chamber and adjusted to a final concentration of 10^5 conidia/ml. Petri plates containing the feed substrate were centrally inoculated with 1 μl of the conidia suspension using a calibrated loop. Petri plates with the same a_w were enclosed in polyethylene bags with plastic glasses containing water/glycerol solutions of the same a_w in order to keep constant the relative humidity inside the bags and plates; water/glycerol solutions were renewed weekly. Incubation temperatures were 5, 15, 25, 30 and 37 °C and the experiment had a duration of 40 days and a maximum of 90 days for the most unfavorable conditions. For each combination of a_w*T*species ten plates were used. Additional non-inoculated plates with adjusted a_w were placed into the corresponding bags to asses any a_w deviation during the study.

Growth assessment

Plates were observed twice a day with a binocular magnifier until growth was detected. Then, two perpendicular diameters of the colony were measured daily without opening the plates, until the colony reached the edge of the plate or until the end of the experiment.

Growth data treatment and mathematical analysis

Data treatment

Colony diameters were plotted against time, then linear regression was applied to obtain the growth rate (mm/day) as the slope of the line. All obtained data for each pair of (a_w, T) conditions and replicates were transformed to obtain homogeneity of variance. All data were square root-transformed.

Secondary model

Secondary models are used to describe the influence of environmental factors on fungal growth[39]. These models are of great importance and fundamentally very useful because they can be used to predict the microbiological shelf life of a food product[35]. In particular, in the Rosso cardinal model all of the studied parameters have a physiological meaning[19]. The data set obtained from this study was tested with other secondary models (data not shown), however the best fit was obtained with the Rosso cardinal model.

Then, the influence of a_w and temperature on fungal growth was studied. The Rosso cardinal model was applied to describe the effect of both environmental parameters on the radial growth rate. Each model performance was assessed by the root mean square error (RMSE) and coefficient of determination (R^2).

The equation describing the model is:

$$\mu_{max}^{0.5} = (\mu_{opt} * ((a_w - a_{w\,max}) * (a_w - a_{w\,min})2)/ ((a_{w\,opt} - a_{w\,min}) * ((a_{w\,opt} - a_{w\,min}) * (a_w - a_{w\,opt}) - (a_{w\,opt} - a_{w\,max}) * (a_{w\,opt} + a_{w\,min} - 2 * a_w))) *((T - T_{max}) * (T - T_{min})2)/((T_{opt} - T_{min}) * ((T_{opt} - T_{min}) *(T - T_{opt}) - (T_{opt} - T_{max}) * (T_{opt} + T_{min} - 2 * T))))^{0.5}$$

The equation was fitted using the nls function (nonlinear least squares) from the R Stats Package, in R (version 3.4.0).

The parameter $a_{w\,max}$ was fixed at 0.97 since it was the maximum value that the feed can reach by addition of water without having free water/surrounding water. It is significant to mention that above 0.95 a_w values there are notorious changes in feed appearance (pellet disintegration). Moreover, according to Sautour et al.[41] $a_{w\ max}$ should be set to one only for non-xerophilic fungi.

Growth boundary model

Probabilistic models are used when the aim is to determine if growth can happen or not under specific conditions[19]. The information provided by these models is very useful because it allows to predict and avoid fungal (or bacterial) growth or even toxin production.[19] Then, this model instead of determining growth rate, determines whether or not growth occurs.

The growth/no growth boundaries at different a_w, temperature and time were built according to the linear logistic regression analysis. A value of 1 was assigned when growth was registered and 0 value when no growth occurred. A total of ten replicates were used.

The equation for the logistic regression model is:

$$\text{Logit}\,P = \ln(P/(1-P)) = b_0 + b_1 a_w + b_2 T + b_3 t + b_4 aw^2 + b_5 T^2 + ¦b_6 a_w T + b_7 t^2$$

where P is the probability of growth (range 0–1), b_i are the coefficients to be estimated, t (days) is the incubation time, a_w is the water activity of the feed medium, and T is the incubation temperature (°C).

The equation was fitted using the glm function (generalized linear models) from the R Stats Package, in R (version 3.4.0).

The predicted growth/no growth boundaries interfaces for $p = 0.5$ were calculated and plotted with Microsoft Excel 2007 Solver and Octave (GNU Project, Version 4.2.1).

The predictive power/capacity and the goodness of fit of the logistic models were assessed by pseudo R^2 indices (McFadden and Nagelkerke), percentage of concordance and accuracy[3,14].

Performance of probability models at probability level of 0.50 was additionally evaluated by comparison with independent data obtained from studies carried out in analog media[1,2,24,30,44].

Results

Growth description in feed

Growth of *E. amstelodami*, *E. chevalieri*, *E. repens* and *E. rubrum* was studied at different a_w and temperatures.

E. amstelodami grew in the range 0.77–0.97a_w at 25 and 30 °C. At 15 °C the range was 0.81–0.97a_w; and at 37 °C the range was shorter, from 0.91 to 0.95 a_w. The minimum growth rate was 0.19 mm/day at 0.77a_w and 25 °C, while the maximum growth rate was 18.3 mm/day at 0.91a_w and 30 °C. High growth rates were observed in the range 0.87–0.95 a_w in the temperature interval of 15–37 °C. Nevertheless, optimal a_w and temperature were 0.91 and 30 °C, respectively.

E. chevalieri growth was observed in the range 0.79–0.97a_w from 15 to 37 °C and at 0.75a_w at 30 °C. The lowest growth rate was 0.13 mm/day at 0.75a_w and 30 °C, while the highest growth rate was 23.6 mm/day at 0.91a_w and 30 °C. High growth rates ranging from 10.5 to 15.8 mm/day were also observed in the intervals of 0.83–0.91a_w at 25–37 °C temperature range.

For *E. repens*, growth was registered from 0.91 to 0.97 a_w at 5, 15, 25 and 30 °C. Minimum growth rate was 0.29 mm/day at 0.97a_w and 5 °C, whereas maximum growth occurred at 0.91a_w and 25 °C (20.16 mm/day). At 15 and 30 °C, high growth rates also occurred at both 0.91 and 0.95a_w (10.2 mm/day at 15 °C and 16.7 mm/day at 30 °C; 9.3 mm/day at 15 °C and 6.3 mm/day at 30 °C, respectively).

With regard to *E. rubrum*, growth was observed in the interval of 0.75–0.97a_w. At 0.75a_w only grew at 25 °C, and growth at 5 °C only occurred at 0.91a_w. From 0.79 to 0.97a_w growth took place at 15, 25 and 30 °C. The optimal a_w values ranged from 0.83 to 0.95 at 25 and 30 °C with growth rates above 10 mm/day, except at 0.95a_w and 25 °C. The maximum growth rate was 15.9 mm/day (0.91a_w at 25 °C) and the minimum growth rate was 0.16 mm/day (0.75a_w and 25 °C). No a_w upper limit of growth was observed.

It has been observed that the rate of growth in all species studied decreased at a_w above 0.91 at all the temperatures tested.

Secondary model

Table 1 shows the cardinal values of environmental factors obtained using the secondary cardinal model. The RMSE was calculated to assess the performance of the predictive models; in the calculation no-growth conditions next to the growth ones were included[31]. In general terms, the models showed a good fit with R^2 ranging from 0.885 to 0.950. Nevertheless, the RMSE values were above 1, indicating a poor fit to experimental data. Despite the fact that all the RMSE values were higher than 1, there was high agreement between the parameters estimated by the model and the experimental data. For $a_{w\,min}$, $a_{w\,opt}$ and $a_{w\,max}$, the model provided values that fell within the experimental domain.

For three out of four species there was agreement between the experimental $a_{w\,opt}$ and $a_{w\,max}$ and those estimated by the model (0.91 and 0.97, respectively). In addition, for T_{opt} and T_{max}, the model also showed high concordance with experimental data.

With regard to T_{min} for *E. repens* and *E. rubrum*, both species were able to grow at 5 °C. The estimated value was below 5 °C (2.78 °C) for *E. repens*, whereas for *E. rubrum*, T_{min} was estimated slightly above 5 °C.

With respect to optimal growth rate (μ_{opt}), there was high agreement for three out of four species. Nevertheless, there was a significant difference for *E. repens*, where the estimation of the model turned out to be twice as that of the experimental value.

Growth/no-growth interface model

Probabilistic models were built for each of the *Eurotium* species at 7, 15 and 40 days. Table 2 shows the coefficients obtained for each model.

Developed models include linear, quadratic and interaction terms. There were two exceptions since terms were

Table 1 Experimental and estimated cardinal values of water activity and temperature, growth rate and coefficients

Species	Parameter	Estimated value ± SE	Experimental data	R^2	RMSE
E. amstelodami	μ_{opt} (mm/day)	17.76 ± 0.912	18.3	0.908	1.614
	$a_{w\ max}$	0.974 ± 0.001	0.97		
	$a_{w\ opt}$	0.913 ± 0.003	0.91		
	$a_{w\ min}$	0.757 ± 0.002	0.77		
	T_{max} (°C)	38.93 ± 0.483	37		
	T_{opt} (°C)	30.41 ± 0.597	30		
	T_{min} (°C)	5.09 ± 0.436	15		
E. chevalieri	μ_{opt} (mm/day)	24.88 ± 6.450	23.6	0.950	2.021
	$a_{w\ max}$	0.978 ± 0.001	0.97		
	$a_{w\ opt}$	0.909 ± 0.002	0.91		
	$a_{w\ min}$	0.744 ± 0.002	0.75		
	T_{max} (°C)	37.20 ± 0.402	37		
	T_{opt} (°C)	34.99 ± 2.127	30		
	T_{min} (°C)	5.39 ± 0.403	15		
E. repens	μ_{opt} (mm/day)	10.21 ± 1.275	20.2	0.885	2.139
	$a_{w\ max}$	0.990 ± 0.006	0.97		
	$a_{w\ opt}$	0.928 ± 0.010	0.91		
	$a_{w\ min}$	0.897 ± 0.002	0.91		
	T_{max} (°C)	36.96 ± 0.249	30		
	T_{opt} (°C)	24.44 ± 0.603	25		
	T_{min} (°C)	2.78 ± 1.810	5		
E. rubrum	μ_{opt} (mm/day)	14.50 ± 0.605	15.9	0.896	1.687
	$a_{w\ max}$	0.972 ± 0.0006	0.97		
	$a_{w\ opt}$	0.911 ± 0.003	0.91		
	$a_{w\ min}$	0.727 ± 0.002	0.75		
	T_{max} (°C)	37.04 ± 0.029	37		
	T_{opt} (°C)	26.17 ± 0.497	25		
	T_{min} (°C)	5.38 ± 0.339	5		

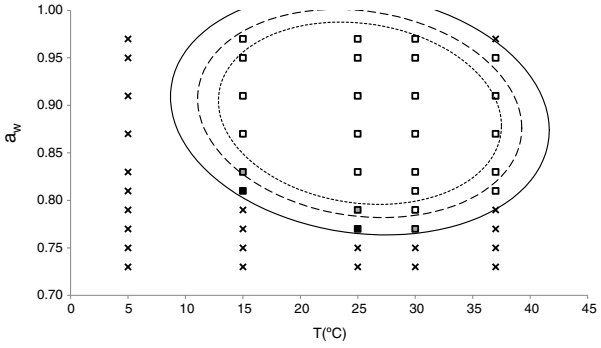

Figure 1 Growth/no growth interface of *E. amstelodami* after 7, 15 and 40 days of incubation in a feed matrix. *P*(0.50): short dashed line: at 7 days; long dashed line: at 15 days; solid line: at 40 days; white square: growth at 7 days; gray square: growth at 15 days; black square: growth at 40 days; cross: no growth.

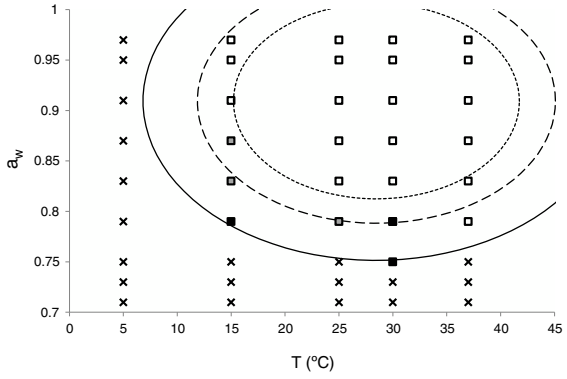

Figure 2 Growth/no growth interface of *E. chevalieri* after 7, 15 and 40 days of incubation in a feed matrix. *P* (0.50 short dashed line: at 7 days; long dashed line: at 15 days; solid line: at 40 days; white square: growth at 7 days; gray square: growth at 15 days; black square: growth at 40 days; cross: no growth.

not significant (p-value > 0.001): a_w*T for *E. chevalieri* (p-value = 0.0079), and a_w^2 for *E. rubrum* (p-value = 0.452). Despite the fact that coefficients to assess predictive power and goodness of fit were accurate in both cases, the terms a_w*T and a_w^2 were suppressed in each respective case in order to improve model fit and avoid overfitting (backward elimination of terms).

Figures 1–4 show plots of probability of growth (p = 0.50) for temperature and a_w at 7, 15 and 40 days of incubation for each species. As it can be seen from the figures, growth occurred at almost all suitable conditions during the first 7 days. In general, a high percentage of logistic model agreement with the experimental data was found. The models

Table 2 Estimated parameters by secondary logistic regression models

Species	Variable	Estimated value	Standard Error
E. amstelodami	Intercept	−481.10000	14.66
	t	0.25680	0.01026
	a_w	1010.00000	31.45
	T	2.70700	0.10007
	a_w^2	−550.10000	17.43
	T^2	−0.03314	0.001079
	$a_w{*}T$	−1.16900	0.07698
	t^2	−0.00296	0.0001321
E. chevalieri	Intercept	−454.000	17.180
	t	0.435	0.018
	a_w	953.100	37.210
	T	1.594	0.054
	a_w^2	−524.000	20.840
	T^2	−0.028	0.001
	t^2	−0.004	0.000
E. repens	Intercept	−3220	172
	t	0.211	0.015
	a_w	6840	365
	T	−1.63	0.312
	a_w^2	−3640	195
	T^2	−0.0355	0.00166
	$a_w{*}T$	3.10	0.344
	t^2	−0.0017	0.00015
E. rubrum	Intercept	−51.7	2.208
	t	0.1992	0.01027
	a_w	39.45	2.091
	T	2.114	0.0874
	T^2	−0.03568	0.00110
	$a_w{*}T$	−0.613703	0.06746
	t^2	−0.002345	0.00013

p-value < 0.001.

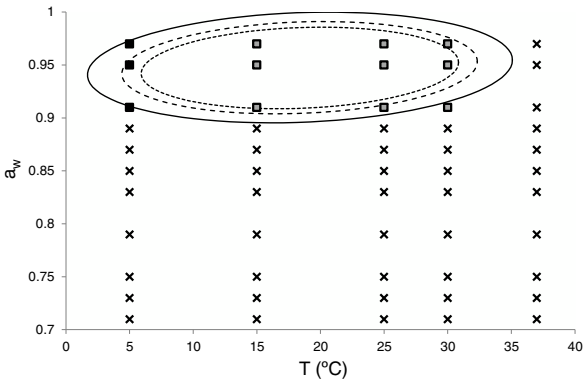

Figure 3 Growth/no growth interface of *E. repens* after 7, 15 and 40 days of incubation in a feed matrix.
P (0.50): short dashed line: at 7 days; long dashed line: at 15 days; solid line: at 40 days; white square: growth at 7 days; gray square: growth at 15 days; black square: growth at 40 days; cross: no growth.

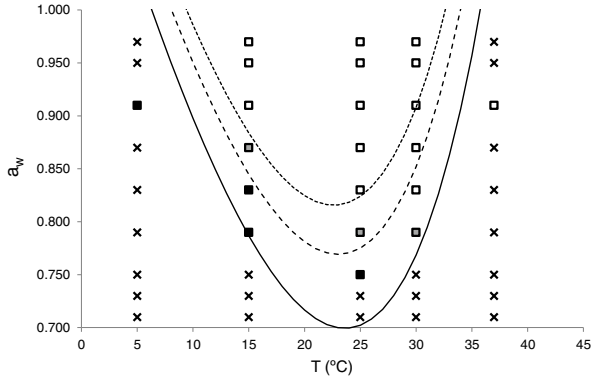

Figure 4 Growth/no growth interface of *E. rubrum* after 7, 15 and 40 days of incubation in a feed matrix.
P (0.50): short dashed line: at 7 days; long dashed line: at 15 days; solid line at 40 days; white square: growth at 7 days; gray square: growth at 15 days; black square: growth at 40 days; cross: no growth.

showed a concordance percentage above 95 and accuracy above 0.929 (Table S1, supplementary material).

Figures S1–S4 (supplementary material) show plot probability of growth vs. temperature for different values of a_w and incubation times of 7, 15 and 40 days. These figures were also constructed by using the equations obtained by the model, and are complementary to Figures 1–4.

It is important to notice that the probability curves are almost centered at the experimental optimal temperature of growth for each species (except for *E. repens*, whose probability curve is centered at a lower temperature), and show how the probability of growth increases with time. It is worth noting that for *E. chevalieri* and *E. rubrum* this probability increment is notorious when comparing figures for 7 and 40 days respectively (Figs. S2 and S4).

In addition, the validation of the models was also performed with external data from other authors[1,2,24,30,44]. This comparison is shown in Table S2 (supplementary material), where the probability of growth of the models is shown on the ''*P* model'' column. Despite the fact that some disagreements were observed (8 false-positives and 5 false-negatives out of a total of 70 samples), the models predicted acceptably considering that the validation points were located at the domain-model boundaries.

Discussion

Growth and probabilistic models were developed for *Eurotium* species isolated from animal feed. It was found that in most of the evaluated conditions of a_w and temperature, growth happened in a shorter period of time than in previously assayed synthetic media (MEA)[23]. Synthetic media are richer than natural feed and food, the latter provide a more real condition while synthetic media might induce an overestimated growth[7]. However, some discrepancies were found in the present study since in the feed matrix the temperatures required for growth at $0.75a_w$ were 25 and 30 °C (for *E. rubrum* and *E. chevalieri*, respectively), and no growth was detected at 15 °C, while in synthetic media the lowest a_w value at which growth occurred was 0.75 at 15 and 25 °C. In addition, the temperature range of growth in feed was wider than in synthetic media, while the a_w range of growth was more limited. Thus, we consider that the most appropriate choice is to perform the analysis in a culture media as close as possible to the original source from which molds were isolated. With regard to this topic, it was determined that the temperature and a_w at which fungal growth occurred in animal feed were more in accordance with several authors[1,13,15,47]. However, differences might be due to the nutritional composition of the substrates and geographical origin[7,20,36].

In relation to the cardinal models, they provided accurate estimations for $a_{w\,max}$ and $a_{w\,opt}$, μ_{opt} (except for *E. repens*), T_{max} and T_{opt} for all the four species studied (except T_{opt} for *E. chevalieri*). On the other hand, the parameters $a_{w\,min}$ and T_{min} were underestimated in all cases with the exception of $a_{w\,min}$ for *E. chevalieri* which was closer to the experimental observation.

Rosso and Robinson[39] estimated *E. amstelodami* and *E. chevalieri* radial growth rate, $a_{w\,min}$ and $a_{w\,opt}$ from external data, which was obtained in a laboratory medium at 25 °C and glycerol as substrate to control a_w (among other solutes). These authors determined a radial growth rate of 2.52 mm/day, 0.647 $a_{w\,min}$ and $0.974a_{w\,opt}$ for *E. amstelodami*; while for *E. chevalieri* the radial growth rate was 2.664 mm/day, $0.705a_{w\,min}$ and $0.955a_{w\,opt}$. Once again, our findings differ widely since the radial growth rate in feed was about ten times higher than any informed value, $a_{w\,min}$ was higher than those and $a_{w\,opt}$ was lower than the values reported by the authors. However, Guynot et al.[25] reported a mean of maximum growth rates estimated by linear regression (at $0.90a_w$) comparable with the informed growth rates in the present study. In addition, Suhr and Nielsen[44] reported that *E. repens* was able to grow in rye bread medium at $0.80a_w$ (controlled by glycerol) and exhibited visible growth before 30 days. Moreover, these authors reported that the shortest time for spoilage was observed at the highest a_w (0.95) within a week[44]. In our study, *E. repens* was not able to grow below $0.91a_w$ in animal feed and all the four species were able to grow at the highest a_w (0.97) in less than a week's time.

The RMSE and R^2 were the parameters used to evaluate the goodness of fit and the fitting capacity of the models, respectively. The first parameter measures the deviation between observed and predicted values, while the second parameter indicates how useful the explanatory variables are in predicting the response variable[9]. A good fit is indicated by RMSE values close to zero, while a proper fitting capacity (R^2) is indicated by values close to one.

In the present work, despite the fact of having inoculated molds directly on the feed pellets, the obtained R^2 values were very high (>0.885), comparable to values usually obtained from well-defined synthetic media[45]. Otherwise, RMSE values were too high to indicate a proper fit ($2.139 > RMSE > 1.614$). Similarly, Basak and Guha[8] informed an R^2 range from 0.980 to 0.991 while RMSE ranged from 3.5 to 5.3 in a *Penicillium expansum* spore germination study.

Probabilistic models developed for each *Eurotium* spp. showed agreement between observed and predicted data. This agreement was supported by the high values of the obtained coefficients. The pseudo R^2 indices (McFadden and Nagelkerke) were accurate enough to indicate a good fit for each model. In addition, accuracy and concordance percentage for all developed models were also high, ranging from 95.58 to 97.81% and from 0.929 to 0.945, respectively. This agreement was also supported graphically, although probability models failed in some cases. Nevertheless, this kind of information can be usefully applied to prevent *Eurotium* spp. growth in animal feed during storage. As many of the conditions which favored growth occurred during the first 7 days, a safe storage from *E. amstelodami* growth will be achieved below $0.77a_w$ regardless of the temperature (p of growth < 0.10). To prevent *E. chevalieri* growth, a_w above 0.79 (p of growth < 0.10) should be avoided. In the case of *E. repens* a_w should be kept below 0.91, and below $0.73a_w$ for *E. rubrum*.

In conclusion, the predictive models applied in the present study were able to satisfactorily describe and predict the effect of a_w and temperature on growth and the probability of growth of *Eurotium* species in a feed matrix. Since these models were constructed in a real feed matrix they can be used as predictive tools to improve food safety, optimize food storage and prevent fungal spoilage. Thereby, important economic losses can be avoided and feed quality can be improved over time.

To our knowledge this is the first report assessing the growth parameters of *Eurotium* species directly in animal feed.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2020.09.006.

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