



# Discriminatory ability of WL Nutrient Agar for yeasts associated with olive environments: a binary scoring and machine learning approach

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## ABSTRACT

Wallerstein Laboratory Nutrient Agar (WLNA) is widely used to differentiate yeast colonies in wine and brewing microbiology, but its discriminatory ability for species associated with table olives and olive oil production environments has never been formally evaluated. We assessed colony features of 227 ascomycetous yeast isolates from these matrices — representing twelve species, predominantly *Pichia membranifaciens*, *P. manshurica*, *P. kudriavzevii*, *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Candida boidinii* — using a binary scoring scheme applied to thirteen characters related to colony colour, shape, appearance and odour and four related to cell shape. Species identities were confirmed by MALDI-TOF mass spectrometry and ITS sequencing (overall agreement: accuracy = 0.90).

Initial machine learning classification (CART, Random Forest, SVM-linear, SVM-RBF) on colony features yielded modest performance (accuracy 0.51–0.65), with reliable discrimination limited to *S. cerevisiae* and *Nakazawaea molendinolei*. A reproducibility study involving ten operators of varying skill levels identified the root causes: colour and elevation characters contributed disproportionately to inter-operator disagreement while *Pichia* spp. showed inherently high intraspecific variability. Revised models excluding characters contributing most to disagreement and pooling *Pichia* species at genus level achieved improved performance (Random Forest: accuracy = 0.72), with acceptable classification of *S. cerevisiae*, *N. molendinolei*, and *Pichia* spp.

These results demonstrate that WLNA provides reasonable accuracy in the discrimination of some key species when using robust morphological characters, supporting its use as a low-cost screening tool prior to molecular identification, and establishes a methodological framework that is directly transferable to the evaluation of other differential media.

## 1. Introduction

Yeasts and yeast-like fungi play a significant role in food fermentation and spoilage (Bokulich and Bamforth, 2013; Hernández et al., 2018; Imre and Crook, 2025; Liu et al., 2016; Tofalo et al., 2020) and in human health, encompassing both probiotic (Tamang and Lama, 2022) and pathogenic species of food safety relevance (Bays et al., 2024; WHO, 2022). Because of this, enumeration, isolation and identification of yeasts in foods are of utmost importance (Kovács et al., 2025) and several selective and differential media have been developed and evaluated (Beuchat, 1993). This is especially true for the wine and brewing industries, in which wild yeasts are involved in both fermentation and spoilage (Kish et al., 1983; Lin, 1975; Longley et al., 1980; Morneau

et al., 2011; Rodrigues et al., 2001; van der Aa Kühle and Jespersen, 1998). Wallerstein Laboratory Nutrient Agar (WLNA), with or without selective supplements (Beuchat, 1993; Kovács et al., 2025; Maio et al., 2016; Maoloni et al., 2020; Morneau et al., 2011; Pallmann et al., 2001; Polizzotto et al., 2016; Russo et al., 2020; Simões et al., 2021; van der Aa Kühle and Jespersen, 1998) has been used extensively to distinguish the colony features of wine and brewery yeasts, and, occasionally, in other food matrices (Maoloni et al., 2020). To our knowledge, this medium has never been applied to the enumeration or isolation of yeasts from olive oil, table olives, and related environments (Arroyo-López et al., 2012; Ciafardini and Zullo, 2018).

Yeast colonies on WLNA can be differentiated on the basis of colour, due to the pH-dependent reaction of the bromocresol green indicator, as

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well as on the basis of shape, margin, elevation, consistency and surface texture. Several authors have provided photographs or descriptions of the colonies of selected species on this medium (Pallmann et al., 2001; Polizzotto et al., 2016; Simões et al., 2021). Formal vocabularies for the description of colony morphology exist (Kurtzman et al., 2011; Walker, 1998) and colony morphology phenotypes are included as a class in the Ontology of Microbial Phenotypes ([http://purl.obolibrary.org/obo/OM\\_P\\_0000100](http://purl.obolibrary.org/obo/OM_P_0000100)) (Chibucos et al., 2014; Siegle et al., 2019). Nevertheless, informal descriptions remain common in practice, and to our knowledge no formal evaluation of the discriminatory ability of WLNA has been carried out.

Automated image analysis for colony differentiation is an active research area (García-Soriano et al., 2021; Pintavirooj et al., 2026; Rattray et al., 2023; Rodrigues et al., 2022a, 2022b; Tang et al., 2014; Yoon et al., 2015) and has been applied to yeasts (Ruusuvaari et al., 2014). However, visual determination of colony morphology by trained or untrained personnel remains the norm in routine laboratory practice.

In recent surveys, we have isolated ascomycetous yeasts from table olives and fermentation brines (Parente et al., 2026), as well as from olive oil and associated environments (Giavalisco et al., 2022). WLNA was used systematically for colony purification, and we observed that the main species recovered (*Pichia membranifaciens*, *P. manshurica*, *P. kudriavzevii*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Wickerhamomyces anomalus*) appeared to show differences in colony features, prompting a formal evaluation of discriminatory power of this medium. The present study therefore tested the hypothesis that colony features on WLNA, assessed under conditions representative of routine microbiological practice, enables preliminary grouping of yeast species associated with table olives and olive oil. To this end, we: (i) developed a binary scoring scheme to standardize description of colony features and cell morphology, (ii) used multivariate exploratory methods and machine learning to test the hypothesis that species-level discrimination of the main species found in table olives and olive oil was possible, (iii) quantified inter-operator agreement using Cohen's  $k$ , with  $k > 0.6$  defined as substantial agreement.

## 2. Materials and methods

### 2.1. Strains

The list of isolates (227) and their sources is shown in Supplementary Table 1.

Yeast strains were isolated from Yeast Glucose Chloramphenicol Agar (Kovács et al., 2025) during surveys on the microbiology of intermediates and by-products of extra-virgin olive oil production (39, Giavalisco et al., 2022) or table olives (188, Parente et al., 2026). Work cultures were maintained on Yeast Peptone Dextrose Agar (YPD) slants (Kurtzman et al., 2011) while stocks were maintained frozen ( $-80^{\circ}\text{C}$ ) in 11% non-fat dry milk.

### 2.2. Identification

Identification was performed using MALDI-ToF (194 isolates) and/or ITS1-5.8S-ITS2 region of the ribosomal RNA operon (105 isolates). The Extended Direct Transfer™ Standard Operating Procedure for MALDI-ToF Biotyper Sirius (Bruker Co, Billerica, MA, USA), with Bruker Daltonics Flex Control 3.4 software and MBT Compass Library 2023, was used as the primary identification tool for isolates from the Parente et al. (2026) study and as a confirmatory tool for isolates from Giavalisco et al. (2022).

Sequencing the ITS1-5.8S-ITS2 region of the ribosomal RNA operon was carried out on purified DNA extracted from cell pellets obtained from 2 ml of YPD 24 h broth cultures, which were washed twice in sterile saline; lysis and purification were carried out using the Qiagen DNeasy PowerLyzer Microbial kit (QIAGEN GmbH, Hilden, Germany). PCR

amplification was carried out as described in Granchi et al. (1999). Amplicons were purified using Qiagen QIAquick PCR Purification Kit and sent for Sanger sequencing (Mix2Seq kit) to Eurofins Genomics (Ebersberg, Germany). Species assignment was performed using pairwise sequence alignment (<https://theyeasts.org/alignment>). This was the primary approach used for the identification of isolates in the Giavalisco et al. (2022) study but was also used to obtain an identification whenever MALDI-ToF bio-typing failed and to confirm MALDI-ToF identification for selected strains.

Eventually, both MALDI-ToF and ITS identifications were obtained and compared for 80 isolates.

### 2.3. Observation of colony features and cells morphologies

To observe colony and cell features, the isolates were streaked on WL Nutrient Agar (WLNA) plates adjusted to pH 5.5, which were incubated at  $25^{\circ}\text{C}$  for 5 days in aerobiosis. The plates were stored at  $4^{\circ}\text{C}$  if necessary and colony morphology was recorded within 24 h, a practice which was found not to affect colony colour in a detectable way. Observations were carried out directly on plates under a laminar flow hood (Steril VBH 48/MP/99, Milan, Italy) with stainless steel internal surfaces with a 54W T5 HO/850 fluorescent tube (colour temperature 5000 K, illuminance: 1400 lux, measured at the work surface with a calibrated luxmeter), in order to replicate the ordinary working conditions during isolation tasks. Colony features were assessed by examination of the entire plate, with scoring based on well-isolated, fully developed colonies appearing in the final streak sections. Examples of typical colony and cell morphologies are shown in Fig. 1: photos of colonies were taken using a Nikon D810 digital camera with a 105 mm focal length with a white (5500 K) led lighting on a white background.

Photo of yeast cells were taken using wet mounts of cells picked from a well isolated colony suspended in sterile saline using a Zeiss Axioskop 50 microscope at 400x magnification (phase contrast, bright field or dark field) and captured using a XCAMLITE4K48MPA camera (ToupTek Europe, Bury St Edmunds - Suffolk UK) with MII ImageView x86 v 4.11 software.

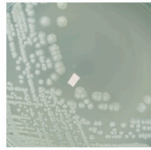
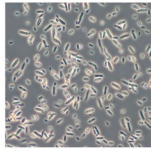
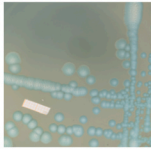
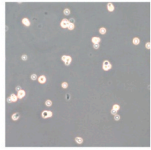
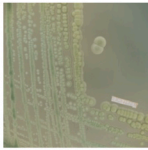
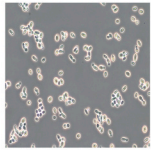
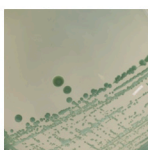
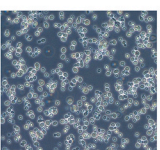
Two approaches were used to evaluate the discriminatory ability of WLNA. In the first, a single experienced operator scored all isolates using the binary scheme described in Table 1, and the resulting data were used for exploratory multivariate analysis and machine learning classification (Section 2.5). American Society for Microbiology (ASM) colony morphology protocol (<https://asm.org/asm/media/protocol-images/colony-morphology-protocol.pdf?ext=.pdf>) was used as a reference for terminology, which was modified to include some specific aspects which were highly typical of selected yeast colony features (a fermentative odour resembling dirty socks or sweat). In the second, a subset of strains was scored independently by ten operators of varying skill levels to assess inter-operator reproducibility (Section 2.5).

### 2.4. Microbiological media and ingredients

All microbiological media and ingredients were obtained from Thermo Scientific Oxoid (Basingstoke, United Kingdom). All reagents were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

### 2.5. Statistical analysis

Exploratory statistical analysis (non-monotonic Multidimensional scaling and hierarchical clustering of the Jaccard distance matrix and Kohonen Self Organizing Maps) of colony features or to colony and cell features were carried out using functions of R v 4.6.1 (R Core Team, 2026), and packages vegan (Oksanen et al., 2026), ggtree (Shuangbin et al., 2022), kohonen (Wehrens and Kruisselbrink, 2018). Supervised machine learning was carried out using classification trees, support vector machines and Random Forest algorithm with package caret (Kuhn, 2008). Models were first trained with a set of 10 colony features

| Species and strain                                                                                                                                                                                                                                                                                                | Colony morphology on WL Nutrient Agar                                             | Cell morphology (phase contrast 400x)                                             |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| <i>Candida boidinii</i> (Y6).<br><b>Colony</b> (size 2-5 mm):<br>• <b>colour:</b> white to pale green;<br>• <b>margin:</b> undulate<br>• <b>surface:</b> dull<br>• <b>profile:</b> crateriform, with wrinkles<br>• <b>odour:</b> -<br><b>Cells:</b> long cells, no apical buds, occasionally forms pseudomycelium |  |  |
| <i>Debaryomyces hansenii</i> (M055Y).<br><b>Colony</b> (size 2-4 mm):<br>• <b>colour:</b> blue green<br>• <b>margin:</b> lobate<br>• <b>surface:</b> dull<br>• <b>profile and elevation:</b> smooth, convex<br>• <b>odour:</b> -<br><b>Cells:</b> small, globose                                                  |  |  |
| <i>Kluyveromyces lactis</i> (M207Y)<br><b>Colony</b> (size 2-4 mm):<br>• <b>colour:</b> pale green to yellowish green<br>• <b>margin:</b> entire<br>• <b>surface:</b> dull<br>• <b>profile and elevation:</b> smooth, convex<br>• <b>odour:</b> -<br><b>Cells:</b> small, distinctly ellipsoidal, sub-apical buds |  |  |
| <i>Lachancea fermentati</i> (Y24AV):<br><b>Colony</b> (size 2-4 mm):<br>• <b>colour:</b> mid-green<br>• <b>margin:</b> entire<br>• <b>surface:</b> shiny<br>• <b>profile and elevation:</b> smooth, flat<br>• <b>odour:</b> -<br><b>Cells:</b> small, globose, apical buds                                        |  |  |

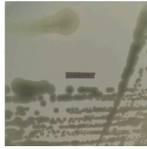
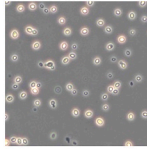
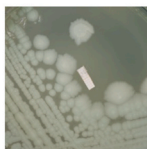
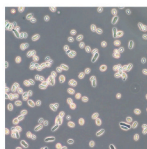
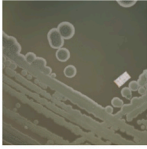
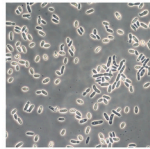
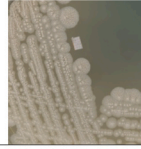
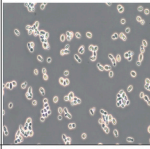
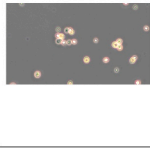
|                                                                                                                                                                                                                                                                                                                                           |                                                                                      |                                                                                      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>Nakazawaea molendinolei</i> (Y35AV):<br><b>Colony</b> (size 2-6 mm):<br>• <b>colour:</b> white to green<br>• <b>margin:</b> entire<br>• <b>surface:</b> shiny, slimy<br>• <b>profile and elevation:</b> smooth, flat<br>• <b>odour:</b> -<br><b>Cells:</b> small, ellipsoidal, apical buds                                             |   |   |
| <i>Pichia kudriavzevii</i> (M060Y):<br><b>Colony</b> (size 2-8 mm):<br>• <b>colour:</b> cream white to very pale green<br>• <b>margin:</b> undulate<br>• <b>surface:</b> dull, granular<br>• <b>profile and elevation:</b> convex, wrinkled<br>• <b>odour:</b> -<br><b>Cells:</b> elongated, apical or subapical buds                     |   |   |
| <i>Pichia manshurica</i> (M045Y):<br><b>Colony</b> (size 2-5 mm):<br>• <b>colour:</b> pale green to dark green<br>• <b>margin:</b> undulate<br>• <b>surface:</b> dull, granular<br>• <b>profile:</b> crateriform, with wrinkles<br>• <b>odour:</b> distinctive sweaty sock odour.<br><b>Cells:</b> elongated, apical or subapical buds    |   |   |
| <i>Pichia membranifaciens</i> (M096Y):<br><b>Colony</b> (size 4-6 mm):<br>• <b>colour:</b> white to yellowish<br>• <b>margin:</b> undulate<br>• <b>surface:</b> dull, granular<br>• <b>profile:</b> crateriform, with wrinkles<br>• <b>odour:</b> distinctive sweaty sock odour.<br><b>Cells:</b> elongated, pseudomycelium often evident |   |   |
| <i>Wickerhamomyces anomalus</i> (M093Y):<br><b>Colony</b> (size 4-6 mm):<br>• <b>colour:</b> pale green to blue green<br>• <b>margin:</b> undulate<br>• <b>surface:</b> dull, granular<br>• <b>profile:</b> crateriform, with wrinkles<br>• <b>odour:</b> -<br><b>Cells:</b> globose to ellipsoidal                                       |  |  |

Fig. 1. Verbal description of yeast colony and cell morphology on WL Nutrient Agar.

Table 1

Binary coding of colony features of yeasts on WL Nutrient Agar and cell shape observed under a microscope. See Fig. 1 for examples.

| Feature                                       | Binary character   | Outcome                                                                                               |
|-----------------------------------------------|--------------------|-------------------------------------------------------------------------------------------------------|
| Prevalent colony colour (>80% of the surface) | Col_pale_green     | 1 if pale or pastel green, 0 otherwise                                                                |
|                                               | Col_mid_green      | 1 if mid-intensity green, 0 otherwise                                                                 |
|                                               | Col_blue_green     | 1 if blue-green, 0 otherwise                                                                          |
|                                               | Col_dark_green     | 1 if dark green or olive, 0 otherwise                                                                 |
|                                               | Col_cream_yellow   | 1 if creamy yellow, 0 otherwise                                                                       |
|                                               | Col_cream_white    | 1 if cream or white, 0 otherwise                                                                      |
|                                               | Col_gray           | 1 if gray, 0 otherwise                                                                                |
| Colony margin                                 | Plain_edge         | 1 if plain/entire, 0 otherwise                                                                        |
| Colony surface aspect                         | Rough_surf         | 1 if not smooth (granular, wrinkled, etc.), 0 if smooth                                               |
|                                               | Shiny_surf         | 1 if shiny, 0 otherwise                                                                               |
| Colony surface shine                          |                    |                                                                                                       |
| Colony side view                              | Convex             | 1 if convex or umbonate; 0 if flat                                                                    |
| Colony surface profile                        | Elevation          | 1 if the colonies are umbonate or crateriform; 0 if their elevation is uniform when viewed from above |
|                                               |                    |                                                                                                       |
| Odour                                         | Fermentative_odour | 1 if, upon opening the plate, a distinct sweaty/dirty sock odour is perceived, 0 otherwise            |
|                                               |                    |                                                                                                       |
| Cell shape                                    | Globose_ellips     | 1 if globose to slightly ellipsoidal (length: width ratio $\leq 2$ ); 0 otherwise                     |
|                                               | Apical_bud         | 1 if buds consistently apical, 0 otherwise                                                            |
|                                               | Pseudomycelium     | 1 if there is clear occurrence of pseudomycelium, 0 otherwise                                         |
|                                               | Long cells         | 1 if ratio of length to width of the cells is $> 4$ , 0 otherwise                                     |

(after excluding variables with near-zero variance the following

characters were retained: “Plain\_edge”, “Convex”, “Elevation”, “Size\_large”, “Col\_pale\_green”, “Col\_cream\_yellow”, “Col\_cream\_white”, “Col\_gray”, “Rough\_surf”, “Fermentative\_odour”) and 7 species for which at least 10 isolates were available (*C. boidinii*, *N. molendinolei*, *P. kudriavzevii*, *P. manshurica*, *P. membranifaciens*, *S. cerevisiae*, *W. anomalus*). Model performance was evaluated using leave-one-out cross-validation (LOOCV) applied to the full dataset of known-species isolates; out-of-fold predictions were pooled across all iterations to construct a single confusion matrix and to derive per-class statistics (sensitivity, specificity, precision, F1) and AUC values.

To evaluate how repeatable was the description using binary characters, a subset of strains (29) was used in a further experiment in which, after providing scoring instruction, the same plates were scored by 10 operators, including 3 well trained researchers and 7 graduate or undergraduate students. The results were analysed using Procrustes analysis with package vegan and used for prediction of species assignment using the previously trained models.

Finally, after removing the features which were more variable in the repeatability experiment and/or by pooling together all *Pichia* species the machine learning models were re-trained and evaluated.

### 3. Results and discussion

We evaluated selected colony features on WL Nutrient Agar (WLNA) and the cell morphology of 227 yeast isolates obtained from olive related environments (table olives and their brines, intermediates and wastes of extra-virgin olive oil – EVOO- production).

A list of all isolates and their sources is available in [Supplementary Table 1](#). For 231 isolates an identification was available with one or both of the two methods used (partial sequencing of the ITS1 – 5.8S–

ITS2 regions and MALDI-ToF). We evaluated how consistent the results of the two methods were for isolates (80) for which identification with both methods was available. Indices derived from the analysis of the confusion matrix are available in [Supplementary Table 2](#). The overall accuracy was 0.90 while Cohen's kappa coefficient was 0.89, indicating almost perfect correspondence ([Landis and Koch, 1977](#)). In fact, both precision (the proportion of predictions which were correct) and accuracy (the proportion of positive predictions which were correct) were 1 for the majority of classes. The MALDI-ToF procedure yielded no identification for all members of genus *Nakazawaea* and for *C. diddensie*, while mismatching identifications were obtained for one *Schwanniomycetes etchellsii*, and one *P. membranifaciens*. The lack of identification for *Nakazawaea* is due to the absence of this genus in the instrument database. The value of MALDI-ToF in yeast identification is well known ([Agustini et al., 2014](#); [Kovács et al., 2025](#); [Quintilla et al., 2018](#)) and this method, whenever the mass spectra are available in data bases, is as good as identification based on marker genes sequencing.

The relative abundance of species in our data set reflects well the importance of different yeast species in table olive fermentations and in olive oil environments, as pointed out by recent literature ([Parente et al., 2026](#); [Ricciardi et al., 2025](#); [Zullo and Ciafardini, 2020](#)).

Initially, colony features were scored by a single operator. Because plates used for scoring the morphology were streaked for isolation, the number of scorable colonies was inherently variable. Scoring therefore did not involve systematic enumeration of a fixed number of colonies - as in automated image analysis - but rather an overall judgment formed from the range of colonies observed, with emphasis on well-isolated colonies. Operators were instructed accordingly, and this approach was adopted deliberately to reflect conditions of routine laboratory practice rather than those of standardised sensory evaluation or image analysis pipelines. Colony consistency (buttery, creamy, waxy, viscid), which is frequently used in descriptions of yeast colonies on WLNA ([Pallmann et al., 2001](#); [Simões et al., 2021](#)) was not included in the feature set because in a set of preliminary experiments multiple operators were unable to score this feature reproducibly. On the other hand, almost all *P. manshurica* and *P. membranifaciens* isolates produced a very intense and distinctive sweaty/dirty sock odour on WLNA. This feature, which could be attributed to the production of volatile phenols ([Saez et al., 2011](#)), was included in the analysis.

A selection of images for most frequent species is available in [Fig. 1](#), while a larger selection of colony and cell morphology images is available on Zenodo (<https://doi.org/10.5281/zenodo.19679768>). Due to the complexity of verbal descriptions and to facilitate data analysis we decided to simplify the description and convert it in binary characters, as shown in [Table 1](#). Cell morphology in wet mounts was also recorded and binarized according to the scheme presented in [Table 1](#).

Interestingly, some of the species which are most abundant in table olive fermentations ([Parente et al., 2026](#); [Ricciardi et al., 2025](#)), namely *W. anomalus*, *P. membranifaciens*, *P. manshurica* and *C. boidinii*, had similar colony appearance, with granulated colony surface and irregular edges. All four species have been documented as biofilm formers ([Kovács et al., 2023](#); [Moreno-García et al., 2021](#); [Zepeda-Giraud et al., 2016](#)), and form thick biofilms on the surface of liquid media when grown in static conditions (data not shown). *C. boidinii*, in particular, has been shown to produce a sticky extracellular matrix that facilitates adhesion to olive surfaces in poly-microbial biofilms ([Arroyo-López et al., 2012](#); [León-Romero et al., 2015](#)). We hypothesise that the granulated surface texture and irregular colony margins observed on WL Nutrient Agar reflect the production of this extracellular matrix at the colony level, suggesting that colony morphology on this medium may serve as a simple proxy for biofilm-forming capacity in olive-associated yeasts. This interpretation warrants further direct experimental verification.

An exploratory approach, including Multidimensional Scaling (MDS), Hierarchical Cluster Analysis and Kohonen Self Organizing Maps was initially used on the Jaccard's coefficient matrix. The results for

colony features are shown in [Supplementary Figs. 1–3](#). Stress of the MDS configuration for 3 dimensions was 0.101, but two dimensions were already sufficient to obtain a stress close to 0.2. With all three multivariate methods some species had very similar colony features for all isolates (*S. cerevisiae*, *N. molendinolei*, *L. fermentati*), most of which also formed pure or almost pure clusters (see [Supplementary Figs. 2 and 3](#)), while others, including species which are prevalent in table olives, like *C. boidinii*, *P. kudriazevii*, *P. manshurica*, *P. membranifaciens*, *W. anomalus*, had very variable features and very rarely formed monospecific cluster.

When cell morphology was added to the analysis the results improved only marginally. The MDS configuration for the first two dimensions is shown in [Supplementary Fig. 4](#), while a dendrogram is shown in [Supplementary Fig. 5](#). Although observation of cell morphology would be a natural step in an isolation and characterization experiment, we decided to exclude it from further statistical analysis, since it did not add any value to the discrimination based on colony morphology.

Our next step was to test four machine learning algorithms (Classification trees with CART, Random Forest – RF; Support Vector Machines – SVM – Linear, and Support Vector Machines – SVM – Radial Basis Function – RBF) on a reduced data set including only the species for which at least 10 isolates were available (*C. boidinii*, *N. molendinolei*, *P. kudriazevii*, *P. manshurica*, *P. membranifaciens*, *W. anomalus*). Accuracy and kappa, measured on the test set, are shown in [Table 2](#). Combined confusion matrices are shown in [Supplementary Table 3](#), while per-class performance indices are shown in [Supplementary Table 4](#).

The performance of all models was fair to moderate on the basis of the Cohen's kappa scale ([Landis and Koch, 1977](#)). The two algorithms which performed better were Random Forest (RF) and Classification and Regression Trees (CART).

Specificity (0.97–0.99) was very high for *S. cerevisiae* and *N. molendinolei*, although precision was lower (0.64 and 0.97 for the Random Forest model for *S. cerevisiae* and *N. molendinolei*, respectively). The models were usually unable to discriminate members of the genus *Pichia*, and its performance on *Wickerhamomyces* and *C. boidinii* was also poor. This substantially reduces the value of WLNA for the study of yeast species in table olives and olive oil related environments, although this group of species could be easily discriminated from *S. cerevisiae* and *N. molendinolei* and from other species (*K. fragilis*, *L. fermentati*) which were not included in the training set (see [Supplementary Figs. 1–3](#)). In a way, this is not surprising, because WLNA was originally designed to discriminate yeasts in winery or brewery environments ([Pallmann et al., 2001](#); [Polizzotto et al., 2016](#)).

To investigate why discrimination of some species was so problematic we carried out a reproducibility study using a subset of isolates which includes representatives of all species in our collection, and whose colony morphology was analysed by 10 operators, including skilled and unskilled ones, under the same environmental conditions and after a short training session in which the scoring matrix was illustrated, together with unmarked photographs showing specific colony features. MDS was used to obtain a graphical representation of the distance relationships among colony morphologies and a configuration plot is shown in [Supplementary Fig. 6](#). The operators' scoring for the same strain and for the same yeast species showed clear evidences of variability. We used Procrustes analysis to analyse the disagreement

**Table 2**

Performance of four machine learning algorithms in correctly assigning a yeast species based on colony morphology on WL Nutrient Agar. (Classification trees with CART, Random Forest – RF; Support Vector Machines – SVM – Linear, and Support Vector Machines – SVM – Radial Basis Function – RBF).

| Model      | Accuracy | Kappa |
|------------|----------|-------|
| CART       | 0.577    | 0.490 |
| RF         | 0.653    | 0.582 |
| SVM linear | 0.485    | 0.366 |
| SVM RBF    | 0.510    | 0.396 |

with the operator who performed the scoring in the first set of experiment and to identify which characters gave the highest contribution to the disagreement. The Procrustes residual analysis is shown in [Supplementary Fig. 7](#): there were no significant differences based on skill levels.

The characters which contributed most to the residuals are shown in [Fig. 2](#) which displays the contribution of individual features to inter-operator disagreement. Bar length represents the mean binary variance ( $p \cdot (1-p)$ ) of scores averaged across all strains and operators, a measure of the average intensity of disagreement for each feature. Colour encodes the number of strains for which meaningful disagreement occurred (variance > 0.10), capturing the breadth of disagreement. This analysis quantifies disagreement across all operators simultaneously and complements the pairwise Procrustes analysis, in which each operator's configuration was compared to the reference operator (RO) who performed the original assessment.

Characters related to colony elevation and those related to the perception of the colour of the colonies (especially for pale colours) were the most variable. The latter was due to two different problems: the colour of the colonies did sometime develop over time and varied between isolates of the same species and, although care was used in adjusting illumination, even the same operator was sometimes unable to be reproducible in scoring some faint colours. This also confirms the importance of standardization of illumination conditions in colony feature scoring.

The effect of such variability on the predictive ability of the machine learning models on the new data set was disastrous. The results for the two best models are shown in [Supplementary Table 5](#). At the very best, the proportion of correct predictions did not exceed 0.7 and was well below 0.5 for many operators. It is interesting to note that, even for the operator who scored colony morphology in the first experiment, success rate was low.

These results clearly confirm the limitations of WLNA as a differential medium for yeast species from olive related environments.

However, some of these limitations can, indeed, be used to extract from the data at least the information which is reliable. We therefore retrained the models on the first data set with two modifications: a) *Pichia* species were pooled in a single category (Pi\_sp); b) features which were found to be most variable in the repeatability analysis ("Elevation", "Convex", "Col\_cream\_white", "Size\_large", "Col\_pale\_green") were removed.

The results for the two best models are shown in [Table 3](#). Overall accuracy significantly improved (0.71-0.72) while Cohen's Kappa, although still acceptable, only slightly improved. The combined confusion matrices are shown in [Supplementary Table 6](#) while per class performance is shown in [Supplementary Table 7](#). The reduced data set did not allow the discrimination of *C. boidinii*, but the assignment of *Pichia* spp. improved.

The new models were then tested on the data set obtained in the reproducibility study. The results, in terms of successful predictions of species identity, are shown in [Supplementary Table 8](#) and show a significant improvement over results obtained with the original model, although differences among operators are evident.

To our knowledge, this is the first attempt at evaluating the repeatability of colony features descriptions. Although the use of trained panels and sensometric methods for assessing inter-operator agreement is well established in food sensory science ([Rossi, 2001](#); [Sipos et al., 2021](#)), no study has applied this framework to the evaluation of microbial colony features scoring. This study highlights the limitation of visual discrimination of colony morphology by human operators. Use of image analysis or sensors has been notably successful in the discrimination of colony morphology, in both bacteria ([Pintaviraoj et al., 2026](#); [Ratray et al., 2023](#); [Rodrigues et al., 2022a, 2022b](#); [Tang et al., 2014](#)) and yeasts ([Ruusuuvuori et al., 2014](#)), especially with two class – problems. However, the common practice in research and diagnostic laboratories is simply to rely on human operators observing and, possibly, scoring the colony morphology against a reference set of images, often with very little control of conditions which may affect the morphology of the colonies (including batch by batch variation of laboratory media and of duration of incubation, but also illumination conditions and background). Our study highlights the importance of training and of standardization of conditions used for observation of colony morphology and demonstrates how only strongly contrasted features

**Table 3**

Performance of two machine learning algorithms in correctly assigning yeast isolates to a species or a species group based on selected colony features on WL Nutrient Agar. (Random Forest – RF, Classification trees with CART).

| Model | Accuracy | Kappa |
|-------|----------|-------|
| RF    | 0.719    | 0.585 |
| CART  | 0.714    | 0.576 |

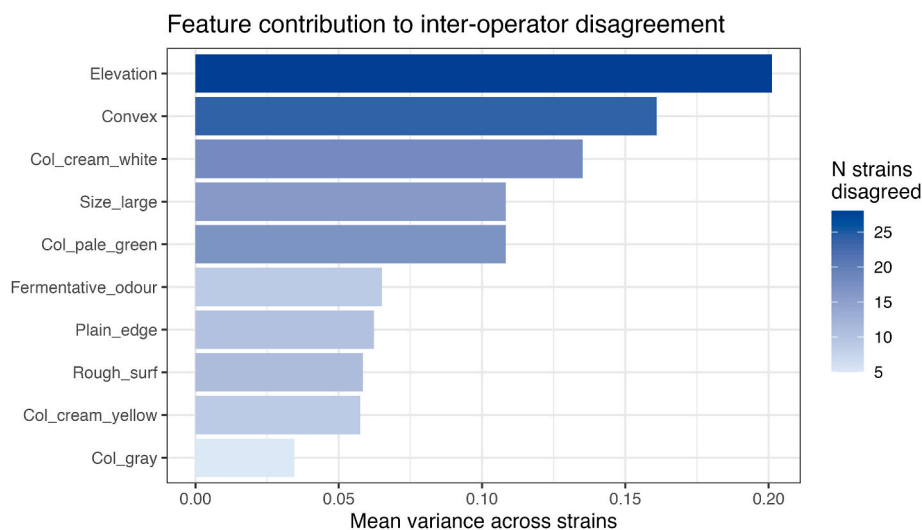


**Fig. 2.** Yeast colony features contributing to disagreement within operators when scoring yeast morphology on WL Nutrient Agar. Bar length represents the mean binary variance ( $p \cdot (1-p)$ ) of scores averaged across all strains and operators, a measure of the average intensity of disagreement for each feature. Colour encodes the number of strains for which meaningful disagreement occurred (variance > 0.10), capturing the breadth of disagreement. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

can be easily differentiated.

#### 4. Conclusions

This study provides the first systematic evaluation of the discriminatory ability of WL Nutrient Agar for yeast species associated with table olives and olive oil production environments, combining exploratory multivariate analysis, machine learning classification, and a reproducibility assessment.

Exploratory analysis revealed that, while some species produced highly distinctive and consistent colony morphologies, the majority of species prevalent in table olive environments showed considerable intraspecific morphological variability and overlapping features that severely limited species-level discrimination.

Machine learning classification on the full feature set yielded modest performance across all four algorithms tested, with Random Forest performing best. A reproducibility study with 10 operators suggested that skill level did not significantly affect reproducibility in scoring colony morphology, suggesting that the variability reflects inherent ambiguity in the scoring scheme rather than operator training. When models trained by a single expert operator were applied to scores obtained by other operators, predictive performance declined markedly, confirming that operator-induced variability represents a critical practical limitation of visual colony morphology assessment.

Taken together, these results indicate that WL Nutrient Agar retains limited but non-negligible value as a first-pass screening tool in olive yeast ecology - sufficient to flag the presence of easily recognisable species and to broadly separate some species or genera, thus facilitating their targeted isolation. Future work should explore the integration of automated image analysis to reduce operator-dependent variability, and the potential of a refined, image-anchored scoring scheme to improve inter-operator reproducibility without requiring specialist training.

#### CRedit authorship contribution statement

**Eugenio Parente:** Writing – original draft, Software, Project administration, Funding acquisition. **Rocchina Pietrafesa:** Writing – review & editing, Investigation, Data curation. **Vito Antonio Romano:** Investigation, Data curation. **Maria Grazia Labella:** Investigation, Data curation. **Annamaria Ricciardi:** Writing – review & editing, Supervision, Investigation.

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2026.105282>.

#### Data availability

I have provided links to data at the attach file step.

[Scripts and data for the “Discriminatory ability of WL Nutrient Agar for yeasts associated with olive environments: a binary scoring and machine learning approach” manuscript \(Original data\) \(Mendeley Data\)](#)

[Colonies and cell morphologies of yeasts from olives \(Original data\) \(Zenodo\)](#)

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