



Innate pathogenic traits in oral yeasts

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Abstract

The normal flora community plays a significant role in both healthy and patient individual's bodies. It has improved its ability to change from beneficial to opportunistic organisms causing crucial infections in immunocompromised patients. This study was conducted to identify the community of yeast species, and to investigate potential virulence factors. Saliva samples were collected from healthy individuals, yeast species were isolated and identified using both traditional and advance molecular genetics technique. The results revealed that *Candida albicans* was the dominant isolate, while *Meyerozyma caribbica* was the least. The phylogenetic tree was constructed for 13 species using the Neighbour-Joining method, which revealed six separated clades. The significant virulence factors, biofilm formation, phospholipase and proteinase activities, were accomplished. The results showed that 95.8% of the isolates formed a biofilm, about 82.6% of them were shown strong biofilm activity. The enzymatic activity revealed that 56.25% of the tested isolates were positive for phospholipase, while 43.75% were positive for proteinase activity. The results showed that *C. albicans* and non-*albicans* were organisms that coexist in the oral cavity, however they possess virulence factors. *C. albicans* was the most popular species, while *C. dubliniensis*, *C. parapsilosis*, *P. kudriavzevii*, *P. kluyveri*, *K. marxianus*, *M. guilliermondii* and *M. caribbica* were showed smaller percentages respectively.

Keywords

Candida species, ITS region, Normal flora, Phylogenetic tree, Yeast

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Cover Page Footnote

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1. Introduction

The community of harmless microorganisms that exist in a human body are called normal flora (microbiota) [1]. Microbial flora comprise a wide range of microorganisms including bacteria, yeasts, molds and protozoa that inhabit or colonize the host body [1]. The diversity of microbial flora within the human body is affected by host-related factors such as age, health, physiological status, host body location (skin, oral cavity ... etc) and sexual behaviour as well as environmental-related factors such as nutrition, and geographic area [2]. They offer significant benefits to the host body such as production of vitamins or stimulation of the host's immune defences [2,3]. Yeasts convert to opportunistic pathogens when oral homeostasis is disrupted and where the immune system is deficient [4,1]. The medical significance of oral *Candida* species has been reported on many occasions causing particular infections called oral Candidiasis, while Invasive *Candida* spp. isolate from blood causing candidemia [5,6]. Such microflora reported to be involved with the oral cavity health, for instance they associate with dental plaque, dental caries [7]. Oral candidiasis have been divided into primary and secondary candidiasis depending on the site of infection [8]. The normal flora that possess virulence factors attributed to the yeast ability in altering their behaviour from useful to opportunistic [1,9]. The virulence factors enhance the ability of microbes to penetrate body layers or to overcome the immune defenses, such as pigments and enzymes (proteinase and phospholipase), siderophore, biofilm formation. etc [10]. The transformation of normal flora from friendly commensals to pathogenic has been addressed previously, however there was no previous study conducted to identify microflora in healthy individuals from Iraq. The pathogenicity of yeast species is multifactorial, therefore such traits were encouraging to investigate the potential virulence factors to enhance microflora alteration to pathogenic agents.

The Human Microbiome Project (2007) was established to investigate and characterise the microorganism community of the human body [7]. Diverse microbial communities inhabit the oral cavity and play a significant role in the healthy human body [7]. Yeasts include a variety of genera such as *Candida* and *Cryptococcus* [8,9]. The incidence of infections increased particularly with the breakout of Human

Immunodeficiency Virus (HIV) infection [11]. The diagnosis of pathogens is an essential step in successful treatment [12]. Strategies of fungal identifications were developed from primary simple methods to advance accurate methods [5]. Various criteria were considered as standard to characterise fungal species starting with obvious morphological or physiological criteria [13]. Commercially, chromogenic media were utilised to distinguish between *Candida albicans* and non-*albicans* species such as CHROM agar. This media possesses particular traits to distinguish among species based on colony colour appearance [5]. Advanced diagnostic approaches were developed to overcome the limitations of the traditional approaches such as accuracy [14]. DNA barcodes are considered a modern precise approach that allow quick characterisation and determination of cryptic fungal species [15]. To achieve consistency and a precise identification, DNA barcodes are required to be specific for a single species and constant within each species [16]. The Internal Transcribed Spacer (ITS) is the most common barcode used to identify fungal species [17]. The ITS region involves two non-coding and variable regions, ITS1 and ITS2, that are separated by a highly conserved 5.8 gene. The ITS region is flanked by 18S (small subunit SSU) at the 5' of ITS1 and by 28S (Large subunit LSU) of the 3' of ITS2 ribosomal DNA (rDNA) [18]. The International Society for Human and Animal mycology (ISHAM) was founded in 2015 along with the ITS DNA barcode database to offer high quality of controlled data consisting of primary fungal DNA barcode sequences [18,19]. This database contributes efficiently to the accurate identification of approximately two thirds of up to 700 common pathogenic fungal species [18,19].

To increase our knowledge of the microbial community and its prophylactic strategy against developing opportunistic infections, this study was set out to isolate and identify some yeast species found in the oral cavity of healthy volunteers, while determining the ability of yeast isolates to convert into opportunistic pathogens. The virulence factors were investigated including biofilm formation and hydrolytic enzymes. To the best of our knowledge, this study is the first research conducted in the Iraq \ Basrah province focused on yeast species as normal flora in contrast to other previous studies that dealt with pathogenic isolates meanwhile considered yeast isolates from healthy volunteers as a control [20].

2. Materials and methods

2.1. Participants and ethical approval

All of the participants were from Basrah province \ Iraq, consisting of students and employers from the University of Basrah. Completed questionnaires were obtained from all of the healthy donors in this study (Appendix A). The participants were questioned regarding their health status, age, gender, medication and if there was a family history of fungal infections. This study deals with healthy individuals, and was supported with the written agreement from all of the participants. We were ethically committed not to breach the privacy of the participants or harm them physically.

2.2. Sample collection and yeast isolation

Saliva samples were obtained from 30 healthy individuals; 1 ml was taken from each participant and transferred directly to a sterilised plastic tube [13]. The samples were collected between November 2018 and March.2019. The saliva samples were diluted with sterilised distilled water at a ratio of 1:1. Following this, 300 µl of diluted saliva was then cultured into Sabouraud Dextrose Agar (SDA) plates and incubated for 48 h at 37 °C. The plates were scrutinised daily and the growing colonies were then picked up and sub-cultured onto fresh SDA plates. The colonies were purified by streaking twice onto SDA plates and preserved in slant culture for further analysis.

2.3. Morphological and molecular identification

Chromogenic Brilliant Candida Agar was used for primitive detection, while the molecular genetic approach used for isolates characterisation of yeasts species.

2.3.1. Morphological identification

The primitive identification of the isolates was carried out by using chromogenic media called (Brilliant candida agar). The media were obtained from a commercial supplier (Thermo Fisher Scientific,USA),. Yeasts isolates were sub-cultured onto agar in the aseptic condition and incubated at 37C° for 48 h. Plates were examined daily and results were recorded according to manufacturer instructions.

2.3.2. Molecular identification approaches

2.3.2.1. *DNA isolation.* The genomic DNA of yeast colonies was obtained using the Presto™ Mini gDNA Yeast Kit (Geneaid) according to the manufacturer's instructions (Geneaid, Taiwan). The genomic DNA was stored at a deep freeze of –80 °C for further analysis. ITS region was amplified using universal primer sequences of ITS1: F-5-TCC GTA GGT GAA CCT GCG G-3 and ITS4: R-5-TCC TCC GCT TAT TGA TAT GC-3 [21]. The PCR reaction mixture and condition were performed following the protocol described by Mirhendi et al. [21].

The amplified fragments were separated on agarose gel and visualised using a UV transilluminator and fragment sizes were measured in comparison with a 1-kb ladder (Promega). The positive PCR products were then sent to Macrogen for sequencing (Macrogen company, Korea) (<http://dna.macrogen.com>).

2.3.2.2. *Sequence analysis and phylogenetic tree construction.* The amplified fragments of ITS regions were sent for sequencing (Macrogen company, Korea). Later, the Basic Local Alignment Search Tool (BLAST) was used to compare the homology of ITS region with the counterpart region deposited in the public database at the Gene Bank.

To construct the phylogenetic tree, FASTA files were prepared from the candidate sequence and several sequences from pre-identified strains. Multiple sequence alignment was carried out using the muscle alignment method via the Mega X software [22]. The resulting sequences were exported in the mega (Meg) format and analysed to construct the phylogenetic tree using the Neighbour-Joining method (Fig. 1).

2.4. Physiological characterisation

The characterisation of isolates involved in investigating the isolate ability to form biofilm, the measurement of activities for both phospholipase and proteinase are described as follows.

2.4.1. Biofilm formation

All yeast isolates were tested for biofilm formation according to the method described by Saxena et al. (2014) using Congo red agar medium [23]. The isolates were inoculated onto prepared media in duplicate and incubated for 48 h at 37 °C. Positive results were

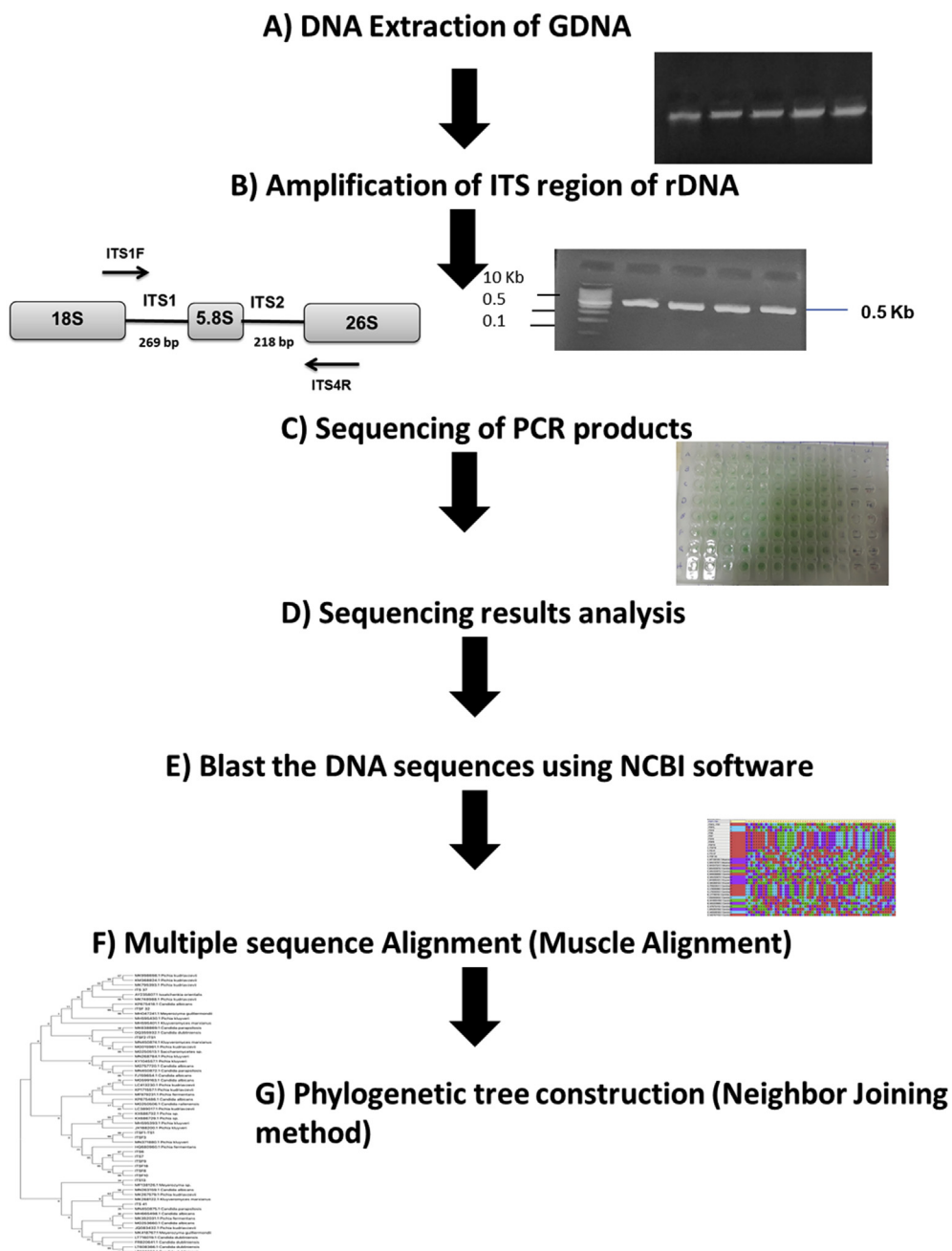


Fig. 1. A schematic diagram representing the steps in molecular identification of the yeast isolates and construction of the phylogenetic tree. The genomic DNA was obtained from the purified yeast isolates, the ITS conserved region of the rDNA was amplified using ITS1 and ITS4 primers and then the sample was sent for sequencing for further analysis. Bioinformatic software was utilised to analyse the results and to identify the yeast isolates by aligning them with the public database on the BLAST website. The phylogenetic trees were constructed using the Mega X software.

distinguished by the colonies' colour. Later on, the activities of isolates were categorised as either weak, moderate or strong according to the colonies' colour grade. Weak positive isolates appeared as pink and negative isolates were either white or light pink.

2.4.2. Phospholipase activity test

To investigate the ability of isolates to synthesise phospholipase, the procedure of Samaranyake et al. (1984) was used [24]. Two replicates for each strain were conducted. The activity of phospholipase was

calculated by securitising the precipitation zone (P_z) around the colony. The calculation was carried out by measuring the ratio of colony diameter to the diameter of colony plus the precipitation zone. The results were read as follows: no activity ($P_z = 1$), weak positive ($P_z = 0.7–0.99$), moderate activity ($P_z = 0.5–0.69$) and strong activity ($P_z < 0.5$).

2.4.3. Proteinase activity

The ability of yeast isolates to produce proteinase was evaluated according to the method of Aoki (1990) using bovine serum albumin (BSA) agar [25]. The inoculum of each isolate (10 μ l of 1×10^6 cfu/ml) was deposited onto plates in duplicate and incubated at 37 °C for 7 days. The appearance of a clear zone around the colonies indicated proteinase activity. The P_z value was measured in a similar manner to the procedure mentioned above.

3. Results

3.1. Demography of participants

Thirty healthy individuals among the employers and students of the University of Basrah participated in this study. They were 20 males and 10 females donated one-ml of saliva before having breakfast. Their ages ranged from 19 to 39 years old. All smoked whereas the females did not, they were all healthy individuals, took no medication except for one participant on antibiotics and there was no family history of fungal infections with the exception of one. There was a mixture of dietary systems including both vegan and omnivorous. This information was obtained from the participants via the questionnaire form (Appendix A). All the consents were taken from the healthy participants before collecting the samples.

3.2. Yeast isolates identification

3.2.1. Plate-based method (primitive identification)

Based on the colony appearance (colour and texture) on the brilliant candida agar and according to the manufacturer's instruction isolates were identified. The 35 isolates with a green colour were considered to be *C. albicans* or *Candida dubliniensis*. The rest of the 13 isolates resulted in either a beige, such as *C. glabrata*, *Candida kefyr*, *Candida parapsilosis*, *Candida lusitanae* and purple colony were undefined species.

3.2.2. Molecular genetic identification

After the preliminary phenotypic identification, results found the plate-based method is inadequate to identify all yeast species, this promote the authors to extend their work and use advance molecular approach for accurate characterization. The genomic DNA of all 48 yeast isolates was obtained and the ITS regions were amplified using primer pairs ITS1-ITS4 [21]. The PCR products with a size of about 500 bp were sent for sequencing (Fig. 1). Isolates were identified by comparing the ITS region sequence with sequences deposited in the public database at the GeneBank. The ITS region sequences of all isolates were homology to the deposited sequences. The percentage of identity was 100% as global similarity, therefore there was no need to deposit the same sequences in the public database. Following this, 8 different species were categorised into 4 genera as shown in Table 1.

3.3. Phylogenetic construction of the strains

To determine the relations among the yeast isolated in this study, a Neighbour –Joining phylogenetic tree was constructed based on the ITS1 and ITS2 region sequences of rDNA for 13 representative strains out of various species (Fig. 1). Based on the results from the nucleotide BLAST and phylogenetic tree construction of the strains, the samples were categorised into 6 clades as shown in Fig. 2. The first clade includes strain ITS 37, which shows a close relation with *P. kudriavzevii*. Similarly, the second clade includes strain ITSF32 that corresponds with *M. guilliermondii*, while there is a close association between the third clade that includes ITSF2 and *Kluyveromyces marxianus*. Both ITSF1 and ITSF3 are associated with the fourth clade and show 100% similarity with *Pichia kluyveri*. The fifth clade is represented by six strains, namely ITSF6, ITSSF7, ITSF9, ITSF18, ITSF18 and

Table 1

The incidence of the species isolated from healthy individuals.

Species	Number of strains	Percentage of strains (%)
<i>C. albicans</i>	31	64.5
<i>C. dubliniensis</i>	4	8.3
<i>C. parapsilosis</i>	1	2.1
<i>P. kudriavzevii</i>	4	8.3
<i>P. kluyveri</i>	3	6.3
<i>K. marxianus</i>	2	4.2
<i>M. guilliermondii</i>	2	4.2
<i>M. caribbica</i>	1	2.1
–	48	100

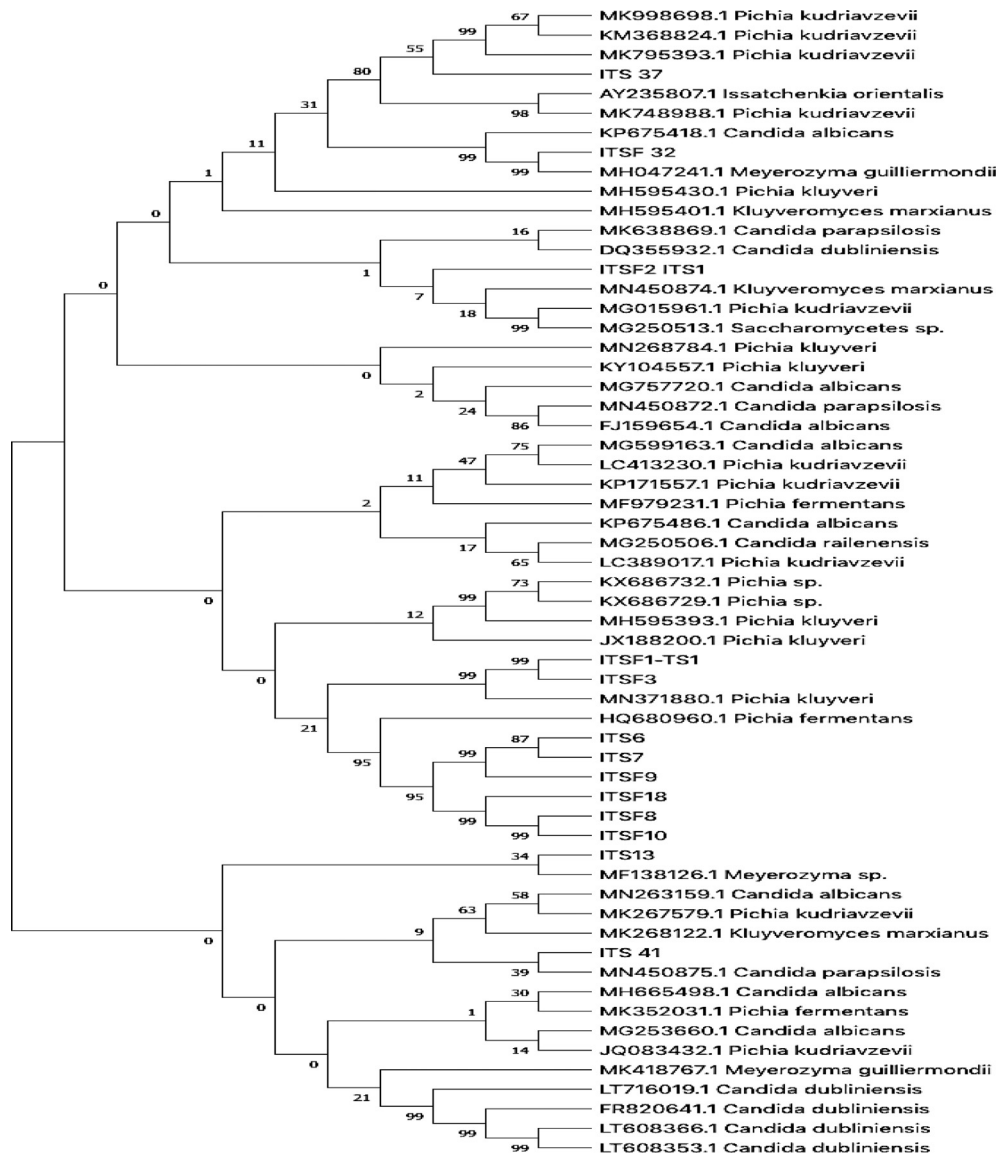


Fig. 2. The phylogenetic tree construction of 13 fungal strains (These 13 isolates were the common strains achieved from our study, we excluded the rest strains that showed the same strain sequence analyses). The tree was constructed using the Neighbour-Joining method [26]. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) has been shown next to the branches [27]. The tree is constructed to scale, with the branch lengths in the same unit as the evolutionary distance used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [28]. The rate variation among the sites was modelled using gamma distribution (shape parameter = 0.5). Evolutionary analyses were conducted in MEGA X software (ITSF NO = the number of strains).

ITSF10, which are close to *P. fermentans*. The last clade represented by the ITSF41 strain is closely related to *C. parapsilosis*.

3.4. Determination of virulence factors

3.4.1. Biofilm formation

The results found that 95.8% of isolates were formed biofilm, while only 4.2% revealed an inability.

In addition, among the 46 biofilm positive isolates, 82.60% and 17.4% showed strong and weak biofilm activity respectively (Fig. 3).

3.4.2. Enzyme activity (phospholipase and proteinase)

The activity of phospholipase and proteinase were also investigated. The findings proposed that 56.25% of the isolates were positive for phospholipase activity, and out of them, 22.% showed weak activity, while

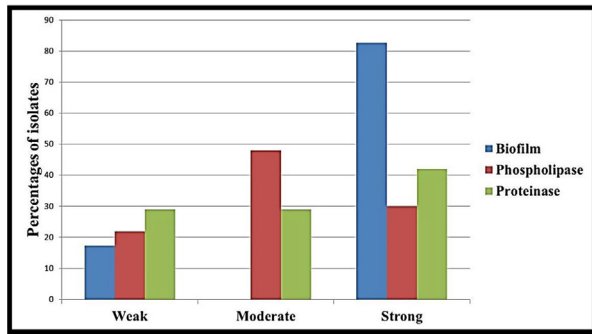


Fig. 3. The percentage of yeast isolates that showed positive activity for biofilm, phospholipase and proteinase production.

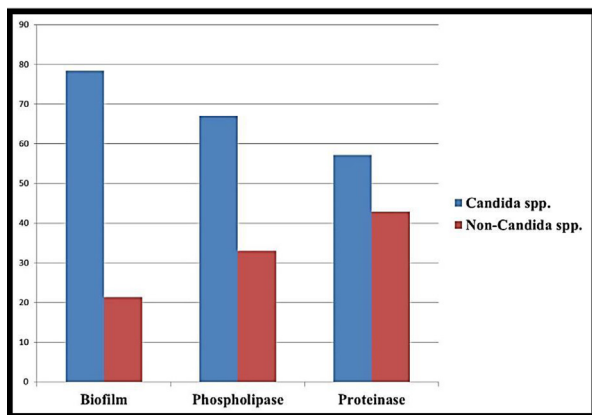


Fig. 4. The percentage of *Candida* spp. and non-*Candida* isolates that showed positive activity for biofilm, phospholipase and proteinase production.

48% and 30% showed moderate and strong phospholipase activity respectively. Following on from this, 43.75% of the isolates appeared to show positive proteinase activity among which 29%, 29% and 42% were

indicated weak, moderate and strong proteinase activity respectively (Fig. 3).

3.4.3. The differences between *Candida* spp. and other yeast species

The results were analysed further to compare the activities of *Candida* spp. and non-*Candida* spp. (other yeast species) in terms of the formation of biofilm and the enzyme activities for both phospholipase and proteinase. Most *Candida* spp. isolates formed a biofilm totalling 78.3% while the percentage was 67% for phospholipase and 57% for proteinase respectively. In contrast, only 21.3%, 33% and 42.9% of non-*Candida* spp. isolates appeared to show biofilm, phospholipase and proteinase activity respectively (Fig. 4).

Six strains *Pichia kudriavzevii*, *P. kluyveri*, *K. marxianus*, *C. parapsilosis*, *Meyerozyma guilliermondii* and *Meyerozyma caribbica* had negative proteinase activity while two of them, *K. marxianus* and *P. kudriavzevii*, reported negative phospholipase activity. All strains formed a biofilm with the exception of *K. marxianus*. Table 2 displays the activity of isolates.

4. Discussion

This study was identified the common yeast species in the oral cavity of healthy individuals and focused on significant virulence factors by investigating the physiological properties that alter their friendly existence in the host body to one that is pathogenic, including the formation of biofilm and the enzymatic activities for both phospholipase and proteinase. Also, the study assessed the genetic relatedness by constructing phylogenetic trees.

The investigation of yeast species in the oral cavity owing to the association of oral flora with oral diseases

Table 2

The activity of isolated species expressing important virulence factors by forming a biofilm, phospholipase and proteinase.

Number of yeast isolates (No.)	Biofilm formation		Phospholipase activity			Proteinase activity						
	Negative	Positive		Negative	Positive		Negative	Positive				
		W	S		W	M		S	W	M	S	
<i>C. albicans</i>	31	0	12	19	9	3	12	7	11	5	6	9
<i>C. dubliniensis</i>	4	0	0	4	2	0	1	1	3	1	0	0
<i>C. parapsilosis</i>	1	0	1	0	0	1	0	0	1	0	0	0
<i>P. kudriavzevii</i>	4	0	3	1	4	0	0	0	4	0	0	0
<i>P. kluyveri</i>	3	0	3	0	2	1	0	0	3	0	0	0
<i>K. marxianus</i>	2	2	0	0	2	0	0	0	2	0	0	0
<i>M. guilliermondii</i>	2	0	2	0	1	1	0	0	2	0	0	0
<i>M. caribbica</i>	1	0	1	0	1	0	0	0	1	0	0	0
Total	48	2	22	24	21	6	13	8	27	6	6	9

*W:weak; *M:moderate; *S:strong.

such as dental caries, diabetes, obesity and periodontitis [6,7,29]. However, there was no previous work that identified normal flora of oral cavity in Iraq. To ensure that the sample contains microbes from different parts of the oral cavity (tongue, cheek and teeth as well as pharynx), saliva was collected from healthy individuals [1].

C. albicans is the most dominant species in the samples. This results coincides with the finding of Rivera et al. [30]. The present study showed a slight variation in the incidence of *C. albicans* and *C. dubliniensis* in comparison with Rivera et al. [30] who recorded the frequency of *C. albicans* (52%) and *Candida dubliniensis* (7.5%) respectively isolated from 96 healthy adults in Colombia. Interestingly, there is a marked alteration in the incidence of both *C. albicans* and non-albicans *Candida* yeasts isolated from the oral cavity of healthy individuals based on several factors like the tool used for identification, geographical area of the study, oral hygiene care used and lifestyle [31,32]. The isolated species including *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *M. guilliermondii* were recorded as human pathogenic yeasts [33]. *P. kudriavzevii* was classified as a distinct environmental species that possesses an industrial value [34]. Nosocomial infections in a neonatal intensive care unit were attributed to this species [35]. Recently, Douglass et al. (2018) examined the environmental and clinical isolates of *P. kudriavzevii* and *Candida krusei* respectively. They proposed that there is no genetic discrepancy between them [36]. *P. kluyveri* was isolated from different environmental and clinical resources, while biotechnological and industrial significance was found for *K. marxianus* (*C. kefyri*) in addition to their existence in the oral cavity [31,34,37]. The closely linked species of *M. guilliermondii* and *M. caribbica* were distinguished genetically using the ITS-RFLP approach [38]. In this work, the results of sequencing and their alignment to the public database separated them into distinct species.

Phylogenetic analysis was carried out using the Neighbour-Joining method on the ITS regions based on the sequence of different *Candida* isolates. The results showed 6 distinct branches of our isolates in comparison with reference strains. The first branch includes the highest relation (99%) between ITS37 isolate with that of a *P. Kudriavzevii* MK795393.1 strain. The second branch displayed (99%) of rDNA ITS between the ITS32 isolate and KP675418.1 *C. albicans* and MH047241.1 *Meyerozyma guilliermondii* (*Candida guilliermondii* reference strains). The third branch involved ITSF2 isolate and showed partial matching

with *K. marxianus* (*C. kefyri*). A recent study conducted in Iran revealed *K. marxianus* as the second major non-albicans *Candida* isolated from the oral cavity of 150 Iranian patients suffering from haematological malignancies [39]. Gorkaska et al. [40], stated that *K. marxianus* as one of the leading non-*C. albicans* yeasts isolated from students in the faculty of Medical Science. The fourth branch performed a close relation between both ITSF1 and ITSF3 strains with 99% identity with MN371880.1 *P. kluyveri* respectively. Another branch showed high matching (95%) between the 6 strains of ITS6, ITS7, ITSF, ITSF18, ITS8 isolates and ITS10 with HQ6806901 *P. fermentans* reference strain. A study conducted by Thiyahuddin et al. [41], stated that there was a low incidence of *P. fermentans* from the saliva of older people. ITS13 strain showed a match with MF13816.1 *Meyerozymasp*. The last branch revealed a relationship between the ITS41 isolate with MN450875 *C. parapsilosis*.

As mentioned in the literature, normal flora can affect the health of the host by altering into a pathogenic organism [3]. Pathogenic species possess virulence factors that are associated with enhancing pathogenicity and resisting harsh conditions such as the implication of biofilm resulting in the resistance of yeasts to antifungal drugs [42,43].

The determination of virulence factors of yeast isolates can explain their ability to convert into pathogenic agents through the adhesion, invasion and infection steps [10,42]. Hence, the present study evaluated the ability of yeast isolates to produce biofilm in addition to two types of extracellular enzyme that included phospholipase and proteinase. The majority of isolates (95.8%) formed biofilm. Our results found that this happened up to 82.6% in contrast with the 68.5% of *Candida* isolates that formed moderate to strongly [44]. It could thus be suggested that the difference between biofilm formations is related to the fact that our samples were collected from the oral cavity while the previous study deals with clinical isolates. Alrubayae et al., found that 79% of isolates form biofilm strongly while 21% of isolates showed weak activities [45]. The participants in our study were healthy versus to other studies that conducted on patients. There are different factors affecting the formation of biofilm such as the potential of species to form a biofilm, nutrients involved, their interaction with other microbial flora and the current physiological condition [46]. In our results, all of the strains formed a biofilm with the exception of *K. marxianus*. In contrast, the previous studies reported that *K. marxianus* is an etiological agent in immunocompromised patients and

that it forms a biofilm [47]. It could be concluded that species with less pathogenicity have less potency when they form biofilms [44]. It could be concluded that forming biofilm is multifactorial process that needs further analysis.

The activity of phospholipase and proteinase was also investigated. These enzymes refer to potential for pathogenicity that are associated with invasion and colonisation [42]. In our study, all of the strains produced phospholipase with the exception of *P. kudriavzevii*, *K. marxianus* and *M. caribbica*. Two strains, *C. albicans* and *C. dubliniensis*, appeared to show proteinase activity. A previous study found that hydrolytic enzymes synthesise strongly in *C. albicans* rather than in non-*albicans* species [48]. Our work compared the *Candida* species with other yeasts and found that the production of these enzymes in *Candida* species was higher than those in non-*Candida* species.

In general, our results encouraged us to extend our work in the future to determine the relationship between nutrition habits, and the viability and activity of yeast flora in oral cavity. Previous studies indicated the antifungal activities of plant extract such as Ankaferd Blood Stopper (ABS) against *Candida* species [49]. Also, Man et al. investigated the growth of *Candida* in the different concentrations of glucose and fructose [50].

5. Conclusion

This study is the first study investigating yeast microflora in Iraq. The results reveal that *C. albicans* is the most common popular species followed by *C. dubliniensis*, *C. parapsilosis*, *P. kudriavzevii*, *P. kluyveri*, *K. marxianus*, *M. guilliermondii* and *M. caribbica*. The appearance of uncommon species in the isolates may rely on the unhealthy life style of students that lead essentially to alter these isolates from beneficial to harmful organisms through the disruption of the body's homeostasis. These common yeast species inhabit the oral cavity of healthy individuals possesses the hidden ability that enhance their virulence to shift into a pathogenic organisms.

Author contributions

Ayat Al-laeiby conceived and designed this study in addition to performing isolation and molecular identification. Adnan A. Al-Mousawi constructed phylogenetic tree while Inaam M.N. Alrubayae carried out the physiological study. Abdullah Al-Saadoon provided valuable advice and guidance. Maysoon Almayahi contributed in the molecular genetics results

analysis. Ayat, Adnan, Inaam and Albdullah collaborated in the paper writing.

Conflict of interest

Authors declare that there is no conflict of interest regarding this work.

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AppendixA:

- Name:
- Gender: Male Female
- Age
- Are you smoker?
- How many cigarette did smoke per day?
- Do you have antibiotics? Any medication?
- Family history of fungal infection.
- Employer or student?

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