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Molecular identification of pathogenic yeast from yoghurt

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ABSTRACT

Yoghurt is the most popular type of fermented milk in Egypt and around the world, and its sensory attributeshave a large effect on consumer acceptability. It is anutritiously balanced food containing most ofthe nutrients gift in milk but in a more assailable form.A total of 120 random samples of plain yoghurt (Companies and Dairy shops) and fruit yoghurt were purchased from different dairy shops and supermarkets from different placesinCairo Governorate.. Polymerase chain reaction was applied for identification of some isolated yeast specially Candida species, Yarrowia, Trichosporn, and Rhodotorula. PCR product of the isolate by ITS regions wassubjected to restriction enzymes (MspI). The internal transcribed gene regions were digested with restriction enzymes MspI (Thermo scientific) generated bands corresponded to the predicted size (330 – 600 bp). In conclusion; there is contamination in yoghurt samples by a wide variety of yeast. Candida was the most common genera isolated from yoghurt. Phenotypic method not enough to identify most yeast species. Genotypic method is the most accepted method for identification of yeasts into species and varieties.

1. INTRODUCTION

Yoghurt is the most common among the dairy products consumed around the world, and its sensory attributes, have a large effect on consumer acceptability (Saint-Eve et al., 2006) and it is anutritiously balanced food containing almost all the nutrients present in milk, but in a more assailable form (Olugbuyiro, 2011). Yoghurt is the most popular type of fermented milk in Egypt. The nutritive value of yoghurt is attributed to the fat, sugar and case in contents. Therefore, yoghurt is recommended for sick and convalescent people. It also inhibits the bacterial flora of intestine which may lead to constipation autointoxication and colitis, as well as, it helps in the absorption of calcium and phosphorus (Khan et al., 2008).

Yeasts are known to be the most important contaminants in some types of fermented dairy products, such as yoghurt and sour milk where yeasts are the major cause of spoilage because the low pH offers a selective environment for their growth (Flee, 1990). Typical defects are gas production, yeasty off-flavor and loss of texture and in pathogenic fungi, such as Candida species, secreted proteins play important roles in fungal pathogenicity and host immunity. The majority of those that have been specifically targeted for study are the hydrolytic enzymes of C. albicans, including the secreted proteinases, phospholipases and lipases (Hube and Naglik, 2001).

Molecular techniques have provided alternative methods for diagnosis and identification of pathogenic fungi, including Candida species (Yeo and Wong, 2002). Molecular identification methods are becoming popular due to their high accuracy, sensitivity (low false-positiverates), and specificity (low false-negative rates) for the identification and differentiation of C. albicans from other Candida species (Neppelenbroek et al., 2006). Therefore, the present investigation is aimed to isolation of different type of yeast from yoghurt, identification of isolated yeast by morphological, biochemical and using RapID yeast plus and molecular identification of some isolated yeast by using conventional PCR

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of One hundred and twenty random samples, forty of plain yoghurt (dairy shops), twenty of plain yoghurt (companies) and sixty of fruit yoghurt were collected from different markets in Cairo Governorate. The yoghurt packs were intact and within the valid date of consumption. The samples were transferred to the laboratory for mycological examination.

2.2. Yeast isolation and identification:

The examined samples were prepared according to the technique recommended by APHA (2002).

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The isolates were picked up by sterile loop from primary culture and were sub-culturedon to Sabouraud dextrose agar slopes and identified according to Lodder and Krieger Van Rij (1970).
Morphological examination was carrying out by studying the morphological characters of the isolates by macroscopic and microscopic examination according to Finegold and Martin (1982).Germ tube test was performed according toKoneman et al. (1992).

2.3. RapID identification kits were used for identification of yeasts (Espinel-Ingroff et al., 1998)
RapID identification kits: RapIDTM Yeast Plus System is a qualitative micro method employing conventional and chromogenic substrates for the identification of medically important yeast, yeast-like and related organisms isolated from human clinical specimens. A complete listing of the organisms addressed by the RapID Yeast Plus System is given in the RapID Yeast Plus Differential Chart. Identifications are made using individual test scores from RapID Yeast Plus panels in conjunction with other laboratory information to produce a pattern that statistically resembles known reactivity for taxa recorded in the RapID System database. These patterns werecompared through the use of the RapID Yeast Plus Differential Chart, or by derivation of a microcode and the use of ERIC (Electronic RapID Compendium) database.

2.4. Prevalence of proteolytic activity of pathogenic species:
Casein activity of isolates: on modified Czapek agar (MCA) medium according to El-fadaly et al. (2015). One ml of sterilized skim milk was added to Czapek agar (MCA) medium. All plates were spot inoculation by tested yeasts isolated strains. After incubation at 25˚C, the plates of casein were flooded with copper sulfate (10%). The results were recorded by measuring the diameter of growth of clean zone.

2.5. Molecular identification by using conventional PCR:
DNA extraction from isolates was done according to QIAamp DNeasy Plant Mini kit Plant Mini kit Catalogue no.69104. It was\ used for PCR amplification of internal transcribed spacers (ITS) (ITS1 and ITS4) primers prepared by Sigma Company were used
ITS1 F-5´-TCCGTAGGTGAACCTGCGG-3'
ITS4 R-5'--TCCTCCGCTTTATTGATATG -3'
PCR reaction was performed in a Thermal cycler (BIO-RAD S1000 USA). The reaction mixture of DNA samples were tested in 50 µl. reaction volumes in a 0.2 ml. Eppendorf tube, containing 25 µl PCR Mix which was composed of: (10X buffer, 10mM d NTPs mixture, Taq polymerase), 1 µl of each primers, 2 µl target DNA, complete to a final volume of 50 µ with sterile molecular water. Protocol for amplification conditions of PCR products using ITS. initial step at 94°C followed by 35 cycles at 94 °C for 1 min., 56°C for 1 min. and 72 °C for 1 min. and a final extension step at 72 °C for 10 min.

2.6. Restriction enzymes MspI:
PCR product of the isolate by ITS regions subjected to restriction enzymes (, MspI) the internal transcribed gene regions were digested with restriction enzymes MspI (Thermo scientific), according to the manufacturer's instructions as follow.
The digests were separated on 1.5% agarose gels. The molecular sizes of the ITS digests were determined by comparison with a DNA molecular marker using AlphaView – AlphaImager Hb Version: 3.4.0.0. (Image acquisition and analysis software).

3. RESULTS

Incidence of yeast isolated from plain and fruit yoghurt examined samples
The results showed in table (1) declared that the incidence of yeast, in fruit yoghurt samples were 6 out of 60 (10%) , in plain yoghurt companies samples were 8 out of 20 (40%) and for plain yoghurt dairy shops samples were 33 out of 40 (82.5%) .

Table 1 Incidence of yeast isolated from different examined yoghurt samples

Type of yyo samples	No. of samples	No. of isolated yeast	
		No.	%
Plai n yoghurt(dairy shops)	40	33	82.5
Plain yoghurt (companies)	20	8	40
Fruit yoghurt (companies)	60	6	10

Incidence of yeast genera isolated from different examined yoghurt samples.
The results achieved in table (2) concluded that the predominant isolated (11) strains were Candida spp., Hanseniaspora spp., Zygosaccharomyces spp., Saccharomyces spp ,Rhodotorula spp Torulopsis spp , Brettanomyces bruxellensis, schizosaccharomyces pombe trichosporn spp., Debaryomyces spp. and Yarrowia lipolytica with prevalence of 23 (38.33%) , 20 (33.33%),9 (15%),8 (13.33%), 6 (10%), 5 (8.33%), 5 (8.33%), 4 (6.66%), 3 (5%), 3 (5%) and 1 (1.6%)in plain yoghurt (shops and companies), respectively. In fruit yoghurt isolated genera were Debaryomyces spp and Torulopsis spp prevalence of 3 (5%) of each.

Identification of yeast by RapID identification kits.
Fig. (1) and Fig. (2) showed identification of Rhodotrulla rubra and Yarrowia lipolytica respectively.

Fig. 1 RapID identification kits for Rhodotrulla rubra

Fig. 2 RapID identification kits for Yarrowia lipolytica

Prevalence of proteolytic activity of pathogenic species.

Proteolytic activity of pathogenic species was indicated by the clear zones of hydrolysis (degradation of milk protein around the colony) were measured and recorded. The results were shown that isolates of pathogenic spp. (51.35%) were positive for proteinase enzyme that result showed in table (3) and diameter of Proteolytic zone of pathogenic species arranged between 13-30 (Table 4 , Fig.3)

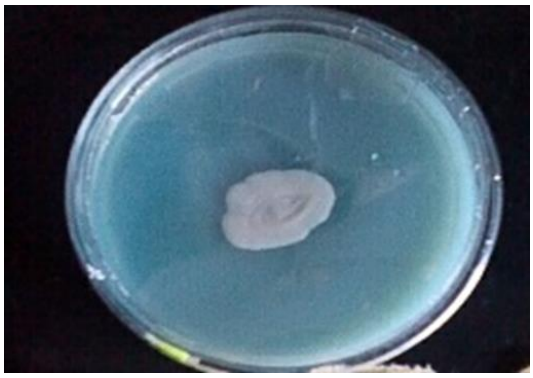

Fig. 3 Candida guiliermondii show zone of hydrolysis on (MCA) medium

Molecular identification of some pathogenic yeast:

PCR system was used for the identification of some isolated yeast. It identified (7) strains were Candida parapsilosis,Yarrowia lipolytica,Rhodotrulla rubra, Candida guiliermondii, Candida albicanis, Candida norvegenisis and Trichosporn beigelii broad range of clinically relevant yeasts. The primer used gives anamplicon (330-600 bp) from all isolates tested.(Fig. 4 &5).

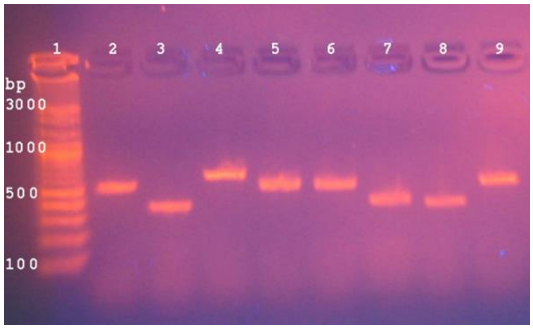

Fig. 4 Ethidium-bromide stained 1.5% agarose gel with ITS-1/4 amplicons. Lane1: Molecular weight marker. Lane2: *Candida parapsilosis* (520 bp). Lane3: *Yarrowia lipolytica* (350 bp). Lane4: *Rhodotrulla rubra* (600 bp). Lane5*: Candida gulliermondii* (516 bp). Lane6: *Candida albicanis* (535 bp). Lane7: *Candida norvegenisis* (488 bp). Lane8: *Trichosporn benigeli* (330 bp). Lane9: *Candida guiliermondii*(516 bp).

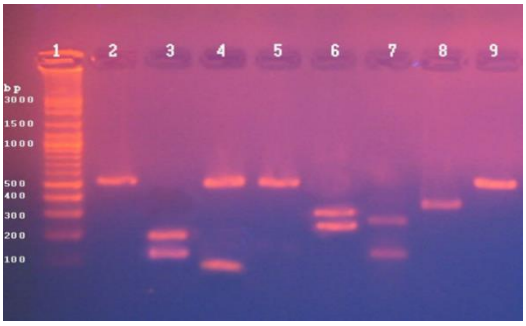

Fig. 5 Ethidium-bromide stained 1.5% agarose gel digested with *MspI*. Lane1: Molecular weight marker. Lane2: *Candida parapsilosis*(520 RF). Lane3: *Yarrowia lipolytica* (150-200 RF). Lane4: *Rhodotrulla rubra* (100-500 RF). Lane 5*: Candida gulliermondii* (516 RF). Lane6: *Candida albicanis* (238-297 RF). Lane7: *Candida norvegenisis* (139-524 RF). Lane8: *Trichosporn benigeli* (330 RF). Lane9: *Candida guiliermondii*(516 RF).

Comparative methods for identification of different yeast isolates:

Identification of yeasts by conventional method was not enough to achieve an accurate result and used molecular identification was important to give accurate result by using PCR(Table 5).

4. DISCUSSION

During milking, yeast contamination originates in most cases from the floors, litter, feed, and air, and only less frequently from the milking machine or udders affected with mastitis (Büchl and Seiler, 2011). Yeasts are the main spoilage organisms found in cultured milk/yogurtdue to the higher acidity in these products (Mayoral, 2005). Despite technological advances, fungal spoilage was still a main issue in the dairy industry(Garnier et al., 2017). In the current study, we identified yeasts from yoghurt examined samples through mycological and molecular identifications to investigated some pathogenic yeast isolated from yoghurt from different dairy shops and supermarket in Cairo governorate in Egypt.

Result cleared that fruit yoghurt samples were 6 out of 60 (10%) are positive yeast, also result cleared thatin plain yoghurt companies samples were 8 out of 20 (40%) samples were positive yeast and result of plain yoghurt dairy shops samples were 33 out of 40 (82.5%) samples were positive yeast. This result agreed with El-Bakri and EL-Zubeir (2009), who recorded that the plain yoghurt samples showed higher contamination with yeast compared with fruit yoghurt. While, Šalomskien and Mačionien (2009), mentioned that addition of fruit gives more yeasts population on fruit yoghurt products.

The high yeast incidence often indicates neglected hygienic measures during production and handling, contamination of raw material, unsatisfactory sanitation or unsuitable time and temperature during storage and/or production (Soliman and Aly, 2011).The high total yeast counts may be resulted from inadequate processing (Aly et al., 2010). From the results achieved in table (2) concluded that the predominant isolated (11) strains were Candida spp., Hanseniaspora spp., Zygosaccharomyces spp., Saccharomyces spp ,Rhodotorula spp Torulopsis spp , Brettanomyces bruxellensis, schizosaccharomyces pombe trichosporn spp., Debaryomyces spp. and Yarrowia lipolytica with prevalence of 23 (38.33%) ,

20 (33.33%), 9 (15%) , 8 (13.33%) , 6 (10%) , 5 (8.33%), 5 (8.33%) , 4 (6.66%), 3 (5%) , 3 (5%) and 1 (1.6%) in plain yoghurt (shops and companies) respectively

Table 2 Incidence of isolated yeast from examined yoghurt samples.

Isolated genera	Plain yoghurt (dairy shops and companies)		Fruit yoghurt	
	No.	%	No.	%
Brettanomyces bruxellensis	5	8.3	0	0
Candida spp	23	38.33	0	0
Debaryomyces spp	3	5	3	5
Hanseniaspora spp	20	33.33	0	0
Rhodotorula spp	6	10	0	0
Saccharomyces spp	8	13.33	0	0
Schizosaccharomyces pombe	4	6.66	0	0
Torulopsis spp	5	8.33	3	5
Trichosporn *spp.*	3	5	0	0
Yarrowia lipolytica	1	1.6	0	0
Zygosaccharomyces	9	15	0	0

Table 3 Prevalence of proteolytic activity of pathogenic species from examined yoghurt samples.

Strain	Total No. of isolates	No. of positive isolates	Percentage
Pathogenic *spp.*	37	21	56.75%

Table 4 Diameter of proteolytic zone of pathogenic species.

Yeast Strains	No. of examined strain	+Ve		No. of –Ve Strain(Non – proteolytic)
		+Ve	Zone (mm)	
Candidia spp iuuundefined genera Undefined genera Undefined generaUndefined genera Undefined genera	23	11	13-25	12
Rhodotorula spp	6	5	15-20	1
Torulopsis spp	8	5	24-30	3

Table 5 Comparative methods for identification of different yeast isolates.

Species of yeast	Conventional method of identification	RapID yeast system	PCR (Restriction enzyme)
C.parapsilosis	*C. parapsilosis*	*C.parapsilosis*	*C.parapsilosis*
Yarrowia lipolytica	Unknown	*Yarrowia lipolytica*	*Yarrowia lipolytica*
Rhodotrulla rubra	*Rhodotrullaspp*	*Rhodotrulla rubra*	*Rhodotrulla rubra*
C. guiliermondii	*Candida spp*	*C. guiliermondii*	*C. guiliermondii*
C. albicanis	*C. albicanis*	*C. albicanis*	*C. albicanis*
C. norvegenisis	Unknown	Unknown	*C. norvegenisis*
Trichosporn benigeli	Unknown	*Trichosporn benigeli*	*Trichosporn benigeli*

In fruit yoghurt isolated genera were Debaryomyces spp and Torulopsis spp prevalence of 3 (5%) in each other that similar to Sharma et al., (1993) , Spreer and Mixa (1998) , Rohm et al., (1992) and Mayoral et al., (2005) reported that the isolation of Rhodotorula spp., Candida spp. and Torulopsis spp. from locally produced yoghurt that suggests its high fungal contamination. El-Diasty and El- Kaseh (2008) examined a total of 40 of yoghurt samples which randomly collected. Yeasts were detected in 50 % of yoghurt samples. Species isolated were Candida spp., Rhodotorula spp., Torulopsis spp. and Saccharomyces spp. concerning proteolytic activity of isolated yeast, the result was shown that isolates of pathogenic spp. (56.75%) were positive for proteinase enzyme. Showed that table (3, 4) and figure (3).that similar to Deak et al., (2000) recorded that Yarrowia lipolytica and Candida zeylanoides have ability to produce proteases. El-Diasty and Salem (2009) showed that most isolates of Candida lipolytica, Candida parapasillosis Candida tropicalis, Rhodotorula spp. and Saccharomyces spp. exhibited a proteolytic activity with different strength. Candidiasis otherwise known as thrush is a fungal disease caused by yeasts of the genus Candida having nearly 200 species. Among them, six are most frequently isolated, while C. albicans is the most abundant and significant species C. tropicalis, C. glabrata, C. parapsilosis, C. krusei and C. lusitaniae have also been implicated as causative agents (Dhama et al., 2013). Yeasts are responsible for several types of infections including oral thrush, vaginitis, urinary tract infection, endocarditis, respiratory syndromes, meningitis, etc. The common "yeast infection" is typically caused by Candida albicans. (Thapa et al., 2015) Nucleic acid detection methods by molecular biology such as PCR have become a common tool for identification and

characterization of microbial communities. (Madigan et al., 2000).

In the present study, conventional PCR system was used for the identification of some isolated yeast. It identified (7) strains were Candida parapsilosis ,Yarrowia lipolytica ,Rhodotrulla rubra ,Candida guiliermondii ,Candida albicanis ,Candida norvegenisis and Trichosporn beigelii broad range of clinically relevant yeasts. The primer used gives an amplicon (330-600 bp) from all isolates tested. That result similar to Dlauchy et al., (1999), Esteve-Zarzoso et al., (1999), Deak et al., (2000) , Akhtar et al., (2014) reported that PCR system was used for the identification of nine representative yeast isolates. Using the described primers, we could amplify DNA from all nine tested yeasts including Candida, Saccharomyces, Trichosporon, Cryptococus, Torulopsis and Rhodotorula representing a broad range of clinically relevant yeasts.

5. CONCULOSION

From the present study we concluded that there is contamination in yoghurt samples by a wide variety of yeast. Also, candida was the most common genera isolated from yoghurt. Phenotypic method not enough to identify most yeast species. Genotypic method is the most accepted method for identification of yeasts into species and varieties.

REFERENCES

1. A.P.H.A (2002): American Public Health Association. Standard Methods for the Examination of Dairy Products, F.P. Downes and K. Ito (editors), American Public Health Association, 16th Edition, Washington DC.
2. Akhter, N., Javed, M.M., Zahoor, S., Babar, M.E. and Haq, I.U., (2014): Rapid isolation/selection of bestyeast culture and its metabolic control for the biotransformation of benzaldehyde to 1-hydroxy- 1-phenyl-2-propanone. Pakistan J. Zool., 46: 783- 787.
3. Aly, M.M.; Al-Seeni , M. N.; Qustib, S.Y. and El-Sawi, N.M. (2010): Mineral content and microbiological examination of some white cheese in Jeddah, Saudi Arabia during summer 2008.food and chemical toxicology, 48(11): 3031-3034.
4. Büchl, R., and Seiler, H., (2011): YEASTS AND MOLDS. Yeasts in Milk and Dairy Products, 4: 2761–2769.
5. Deak, T.; Chen, J. and Beuchat, L.R. (2000): Molecular characterization of Yarrowia lipolytica and Candida zeylanoides isolated from poultry applied and environmental. Microbiology, 66(10) 4340–4344
6. Dhama, K., Chakraborty, S., Verma, A.K., Tiwari, R.,Barathidasan, R., Kumar, A. and Singh, S.D. (2013): Fungal/mycotic diseases of poultry-diagnosis,treatment and control: A review. Pakistan J. biol. Sci., 16: 1626-1640.
7. Dlauchy, D.;Tornay-Lehoczki, J. and Péter, G. (1999): Restriction enzyme analysis of PCR amplified rDNA as a taxonomic tool in yeast identification. Systematic and Applied Microbiology, 22: 445-45
8. El-Bakri, J.M. and EL-Zubeir, I. E. M. (2009): Chemical and microbiological evaluation of plain and fruit yoghurt in Khartoum state, Sudan. International journal of dairy science, 4(1): 1-7
9. El-Diasty, E.M. and Salem, R.M. (2009): Incidence of lipolytic and proteolytic fungi in some milk products and their public health significance. Arab J. Biotech, 12(1): 4956.
10. El-Diasty, E.M. and El- Kaseh, R.M. (2008): Microbiological monitoring of raw milk and yoghurt samples collected from El-Beida city. Arab journal of biotechnology, 12 (1) 57-64
11. El-Fadaly, H.A.; El-Kadi, S.M.; Hamad, M.N. and Habib, A.A (2015): Role of Fungal Enzymes in the Biochemistry of Egyptian Ras Cheese during Ripening Period. Open Access Library Journal, 2: e1819
12. Espinel-Ingroff, A.; Stockman, L.;Roberts, G.;Pincus, D.; Pollack, J. and Marler, J. (1998) :Comparison of RapID Yeast Plus system with the API20C system for identification of common, new, and emerging yeast pathogens. J. Clin. Microbiol., 36:883–886
13. Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. (1999): Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol., 49:329–337.
14. Finegold, S. M. and Martin, W. J. (1982): Bailey and Scott's diagnostic microbiology, 6th Ed. The C.V. Mosby Company, St. Louis.
15. Flee, G.H. (1990). A review, Yeasts in dairy products. J. Appl. Bacteriol.68: 199-211
16. Garnier, L.; Valence, J.and Mounier, J. (2017): Diversity and Control of Spoilage Fungi in Dairy Products: An Update, microorganisms.5 (42): 3-33
17. Hube, B. and Naglik, J. (2001): Candida albicans proteinases. Resolving the mystery of a gene family. Microbiology, 147:1997–2005.
18. Khan, K. R.; Khan, S. U.; Anwar, M. A. and Bhadar, F. S. (2008): Physical and chemical quality appraisal of commercial yoghurt brands sold at Lahore. ARPN. Journal of Agricultural and Biological Science, 3 (3):14-21
19. Koneman, E.W.; Allen, S.D.; Janda, W.M.; Scheckenberger, P.C and Winn, W.C. (1992): Color Atlas and textbook of Diagnostic Microbiology. 4th Ed. J.B. Lippincott Company. Philadelphia.
20. Lodder, J. and Krger-Van Rij, N.J.W. (1970): The Yeasts: A taxonomic Amestrdam. North Holland Publishing.
21. Madigan, M. T.; Martinko, J.M. and Parker, J. (2000): Biology of microorganisms. 9th ed. by T.D. Brock, Prentice Hall Upper Saddle River, NJ 07458.
22. Mayoral, M.B.; Martin, R.; Sanz, A.; Hernandez, P.E.; Gonzalez, I. and Garcia, T. (2005): Detection of Kluyveromyces marxiamus and other spoilage yeasts in yoghurt using a PCR-culture technique. International Journal of Food Microbiology, 105(1): 2733
23. Neppelenbroek, K.H.; Campanha, N.H.; Spolidorio, D.M.; Spolidorio, L.C.; Seo, R.S. and Pavarina, A.C. (2006): Molecular fingerprinting methods for the discrimination between C. albicans and C. dubliniensis. Oral Dis., 12: 242–253.
24. Olugbuyiro, J.A. (2011): Physico-chemical and sensory evaluation of market yoghurt in Nigeria. Pakistan Journal of Nutrition, 10(10): 914-918.
25. Rohm, H.; Eliskases-Lechner, F. and Brauer, M. (1992): Diversity of Diversity of yeasts in selected dairy products. Journal of Applied Bacteriology, 72: 370- 376
26. Saint-Eve, A.; Lévy, C.; Martin, N. and Souchon, I. (2006): Influence of proteins on the perception of flavored stirred yogurts. Journal of dairy science, 89(3): 922-33.
27. Šalomskienė, J. and mačionienė, I. (2009): the influence of contamination yoghurt, quark and semi-hard Cheese by yeasts on their sensory properties.veterinarija ir zootechnika (vet med zoot), 48 (70)
28. Sharma, S.D.; Gill, J.P.; Joshi, D.V. and Kwatra, M.S. (1993): Mycoflora of Indian fermented milk product. Indian J. Dairy Sci., 46 :(2) 85- 87
29. Soliman, N. S.M. and Aly, S.A. (2011): Occurrence and identification of yeast species isolated from Egyptian Karish cheese. Journal of Yeast and Fungal Research, 2(4): 5964.
30. Spreer, E. and Mixa, A. (1998): Milk and dairy product technology. CRC Press, 339-62.

31. Thapa, S.; Shrestha, R.; Tibrewal, A.; sharma, A. and Yuvraj, k.c (2015): isolation of yeast from soil and different food samples and its characterization based on fermentation. Nepal journal of biotechnology, 3 (1) 29-34.

32. Yeo, S.F. and Wong, B. (2002): Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev., 15: 465－484.