Isolation and Identification of Fungi from Egyptian Ras Cheese Made with some Probiotic Lactobacillus spp. with Reference to Their Toxins and Enzymes

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Abstract

Isolation and identification of spoiled fungal species in Ras cheese made by adding probiotic *Lactobacillus* spp. as adjunct culture were investigated in fresh and 90 days stored samples. *Aspergillus flavus* and *Aspergillus niger* were the most predominant spices and have the highest percentages, while they showed lower percentages in the treated cheese samples. However cheese samples were completely free of mycotoxins, *A. flavus* spices had the ability to produce aflatoxin B_1 and G_1, and *A. niger* had the ability to produce Ochratoxin A. All tested *A. flavus* and *A. niger* isolates (28) had the ability to produce lipase and protease enzymes; from which 17 and 9 isolates possessed high activities, respectively. The results suggest that incorporation of probiotic bacteria in the manufacture of Ras cheese reduced the fungal growth in cheese, however, some isolated species considered dangerous because its production of toxins and enzymes in proportions affecting the product.

Introduction

Milk and dairy products are essential parts of a balanced diet. However, due to the possibility of contamination with pathogenic and/or toxinogenic microorganisms, particularly bacteria and fungi, they can pose a health risk (LeJeune and Rajala-Schultz 2009).

Ras cheese is Egypt's most popular traditional hard cheese. This type of cheese is made in large quantities under artisan conditions from raw cow's or buffalo's milk without the use of starter cultures and is sold when it has a strong flavour similar to kefalotyri cheese after 3 to 6 months. (Dabiza and El-Deib 2007). So, attention to the healthy production of Ras cheese is very important for consumers' health. Storage and ripening areas are the main source of contamination.

Part of the salting process of Ras cheese is done by sprinkling the salt superficially then salted water drips onto the wooden shelves where the cheese molds remain for months to develop its texture, leaving the wood to form a variety of microbial contamination including bacteria and fungi. The rotting fungi grow on the cheese blocks and begin to produce a variety of mycotoxins, including aflatoxins (Elramly et al. 2019).

Aflatoxins (AFs) are a major class of mycotoxins produced primarily by *Aspergillus* species including *A. flavus, A. parasiticus* and *A. nomius* (Creppy 2002) The presence of aflatoxin M_1 in milk and dairy products is an important issue, especially for developing countries (Prandini et al. 2009). AFs could be harmful if ingested in high quantities or over a long enough period. AFs B_1, B_2, G_1 and G_2 are the major classes of AFs (Sweeney and Dobson 1998). Aflatoxin B_1 is listed as a group I carcinogen by the International Agency for Research on Cancer (Iqbal et al. 2014).

The molds have more complex enzymatic systems than bacteria and their enzymes contribute to cheese, e.g., proteolysis and lipolysis enzymes, which are more extensive in these cheeses (Hayaloglu and Kirbag 2007). These enzymes could be responsible for several visible or non-visible defects, such as off-flavor, which leads to food waste, as well as large economic losses (Garnier et al. 2017).

Several investigations have shown that various Lactobacillus spp. can limit mycotoxinogenic mould growth by producing antifungal metabolites with low molecular weight (Lavermicocca et al. 2000; Dalié et al. 2010). In addition, El-Nezami et al. (1998) reported that some probiotic strains of *Lactobacillus* spp. had the ability to bind aflatoxins *in vitro*, such as *Lb. rhamnosus* GG and *Lb. rhamnosus* LC-705 which were very effective for removing aflatoxin B_1. Also, Piotrowska and Zakowska (2005) screened the ability of some *Lactobacillus* strains to reduce the concentration of ochratoxin A or remove it from liquid medium. Gomah and Zohri (2015) found that *Lactobacillus paracasie* subsp. *paracasie* inhibited the production of deoxynivalenol, zearalenone and fumonisin B_1 production to 56.8, 73.0 and 76.5%, respectively and also, *Lactobacillus rhamnosus* completely suppressed the mycelium growth of some *Fusarium* species and consequently, no toxin was produced in the presence of this bacterium.

For this reason, we conducted this research to evaluate Ras cheese made by adding some probiotic *Lactobacillus* spp. and to determine the natural occurrence of mycotoxins in the cheese samples; isolate and identify the spoilage fungal species contaminating the cheese samples; screen the potentiality of the isolated fungi for mycotoxins and hydrolytic enzymes production.

Materials And Methods

Materials

Milk used for the manufacture of Ras cheese was obtained from the experimental station's cow herd of the Faculty of Agriculture, Assiut University, Egypt. Pure cultures of *Streptococcus thermophilus* 14486, *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842, *Lactobacillus casei* subsp. *casei* 393, *Lactobacillus acidophilus* 4356 and *Lactobacillus helveticus* 15009 were supplied by the American Type Culture Collection (ATCC). The rennet, the commercial salt and cheese wax of fine grade were purchased from the local markets in Assiut city.

Ras cheese manufacture and treatments

The cow's milk was heated to 73°C for 15 sec. and then it was divided into 4 parts equally to make different treatments. Ras cheese was made as to the procedure of Hammam et al. (2020) which is explained in Fig. (1). Lactic acid bacteria as a starter (1%) was added at 32°C, mixed well and left for a half-hour for acid development then calcium chloride (0.02%) and rennet were added. After coagulation within 40 minutes, the curd was cut into small cubes and the temperature was raised to 45°C in 15 minutes. The curd was held at this temperature for 50 minutes. The whey was then drained down to the level of the curd (acidity 0.14 %). Then, the salt was added (2% of used milk) and mixed for 15 minutes then the curd was cooled, molded and pressed with 160

lbs. for the first 2 hours. Overnight pressing was done by increasing the weight up to 1000 lbs. Fig. 2 shows the form of Ras cheese after the pressing process is completed. The cheese wheel was then dried and salted on both sides with ten grams of dry salt day after day for 12 days. Cheese then was waxed and ripened for 3 months at 13± 2ºC and about 85% relative humidity. The experimental treatments varied according to the type of starters and are clearly explained in Fig 1.

Sample collection

Sixteen samples of Ras cheese were collected as 4 samples from each treatment individually and kept in a sterilized sealed container in the refrigerator for isolation of fungi and mycotoxins analysis.

Isolation of fungi

Czapek's Dextrose Agar (CzDA) and Potato Dextrose Agar (PDA) media were selected for isolation of fungi from different collected Ras cheese samples using the dilution plate method as recommended by Nyongesa et al. (2015); Pitt and Hocking (2009) Counts of fungi were carried out according to Wehr and Frank (2004). The pH of the two media was adjusted to 5.6 and sterilized in an autoclave at 121ºC for 20 min. After sterilization, the media were supplemented with chloramphenicol (250 mg/l) as bacteriostatic agents. Three replicate plates were prepared from each medium for each sample and incubated at 28±2°C for 7 days.

Identification of the isolated fungi

Identification of the isolated fungi was carried out based on their macro and microscopic characteristics using the taxonomic methods of Moubasher (1993); Raper and Fennel (1965); Rønhede et al. (2005); Samson and Pitt (1981).

Extraction of mycotoxins from cheese samples

Fifty grams of cheese samples were transferred into a blender jar, 100 ml of chloroform were added and the contents were homogenized for 5 minutes at low speed and 3 minutes at high speed. The extract was filtered through flouted Whatman No.1 filter paper. The extraction procedure was repeated twice. The combined chloroform extracts were washed with an equal volume of distilled water, dried over anhydrous sodium sulfate and then evaporated to near dryness on a steam bath and purified by column chromatography (AOAC 2012). The residue was transferred quantitatively to a small vial with 1 ml chloroform and kept in a refrigerator to the determination of mycotoxins occurrence.

Screening of the isolated fungi for mycotoxin production

a) Fungal isolates:

A total of 29 isolates recorded as common fungi from the different cheese samples belonging to *Aspergillus flavus* (18) and *A. niger* (11) were tested for their ability to produced mycotoxins.

b) Cultivation of fungal culture

Potato dextrose broth was used for mycotoxins screening. Erlenmeyer flasks (250 ml capacity) contained 50 ml of the medium were sterilized at 1.5 atmospheres for 20 min and inoculated after cooling with 3 discs taken from 7 day-old culture. Cultures were incubated at 28˚C as stationary cultivation for 10 days (Zohri et al. 2014).

c) Extraction of toxins from the fungal cultures

At the end of incubation, each culture was homogenized for five minutes in a high-speed blender (1600 rpm) with double volume of chloroform. The chloroform extracts were washed with an equal volume of distilled water, dried over anhydrous sodium sulfate, filtered then concentrated to near dryness and purified by column chromatography (AOAC 2012).

Determination of mycotoxins

The extracted mycotoxins were determined by thin layer chromatographic (TLC) technique on pre-coated silica gel plate 60 F254 (Merck) as described by El-Kady and Moubasher (1982). Mycotoxins were identified by comparison with appropriate reference standards (Scott et al. 1970; Schroeder and Kelton 1975; Gimeno 1979). Chemical confirmatory tests for positive samples were carried out using various treatments on TLC plates.

Screening of the isolated fungi for enzymes secretion

a) Protease secretion

It was tested using casein hydrolysis medium (Paterson and Bridge 1994). The medium was distributed into 20 ml test tubes (~10 ml/tube), sterilized by autoclaving at 121˚C for 20 minutes. Tubes containing medium were individually inoculated with the tested fungal isolates and incubated at 25˚C for 7 days. Complete degradation of milk protein was seen as clear depth in the tube. The clear depth below the colony was measured (in mm).

b) Lipase secretion

It was tested as the method of Mohamed and Hussein (2004). The medium was sterilized by autoclaving at 121˚C for 20 minutes. Tween 80 was autoclaved separately and added to the sterile and cooled basal medium and it was dispensed aseptically in 15 cm test tubes (~10 ml/tube). Tubes were incubated on the surface of agar by a plug of 3 mm diameter and were incubated at 25˚C for 7 days. The lipolytic ability by a fungus was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate (in mm) was measured.

Results And Discussion

Fungi isolated from Ras cheese samples

In this study, Ras cheese was prepared with different three treatments by using different probiotic bacterial strains as adjunct culture plus the control and it was stored for 90 days. A total of 15 species and one species variety belonging to 6 fungal genera were recovered from Ras cheese samples at 0 and 90 days using two isolation media at 28°C (Table, 1). On CzDA medium, 14 species and 1 variety belong to 6 genera. On the PDA medium, 12 species and 1 variety belong to 5 genera (Table 1). *Aspergillus* (represented by 6 species plus one variety on CzDA medium and 5 species plus one variety on PDA medium) and *Penicillium* (represented by 4 species on each of the two used media) were the most common fungal genera being isolated. Only one species of each *Cladosporium, Mucor* and *Rhizopus* were isolated on the two media used. On the other hand, one species of *Paecilomyces* was recorded on Czapek agar only.

Milk and its products provide a favorable environment for the growth of various microorganisms, whereas *Penicillium* and *Aspergillus* are the common fungal contaminants of cheese (Gandomi et al. 2009). Our results are in harmony with several other studies. El-Fadaly et al. (2015) isolated 66 fungal isolates from Ras cheese and classified them into13 species belonging to 6 genera of which, *Aspergillus* was the most predominant. These results are also confirmed by Elramly et al. (2019) who isolated *Aspergilus niger*, *A. flavus*, *A. fumigatus*, *A. ochraceus*, *A. candidus*, *A. terreus*, *Cladosporium* spp., *Mucor* spp., *Paeciliomyces* spp., *Penicillium* spp. and *Rhizopus* spp. from Egyptian Ras cheese.

On Czapek's medium

The results recorded in Table 2 clearly show that the total number of fungi in fresh Ras cheese in all three treatments (ranged from 277 to 348 CFU/g cheese) were less than those recorded in control samples (525 CFU/g cheese). Also, the number of total fungal colonies in storage control samples (334 CFU/g) was highly decreased than those recorded in the fresh control (525 CFU/g). At the end of the ripening period (90 days), the highest count of fungi was isolated from the sample of T_1 which was 344 CFU/g, while the least count was isolated from the sample of T_2 which was 302 CFU/g.

Aspergillus was represented by 6 species and one variety. *Penicillium* was represented by 4 species. The rest of the genera represent by one species. The percentages of *Aspergillus* species from the recorded total fungal counts in the fresh and stored samples were the highest compared to the other species (Table, 2). *Aspergillus flavus* and *Aspergillus niger* were the most common and have the highest percentages. However, the treated cheese samples showed lower percentages of these species compared to the control, which could reflect the positive effect of adding the *Lactobacillus* starters to cheese.

The number of species per sample ranged from 7-10 species/sample. The highest number was isolated from T_2 sample after 90 days of ripening (10 species).

On PDA medium

With reference to Table (3), the total number of fungal colonies of Ras cheese samples on PDA medium ranged from 243-363 CFU/g. The total number of fungi in control, T_1, T_2 and T_3 fresh cheese samples were 287, 363, 265 and 296 CFU/g, while were 301, 271, 267and 243 CFU/g after 90 days, respectively. The highest count was isolated from the sample of T_1 at fresh time (363 CFU/g), while the least count was isolated from the fresh sample of T_2 (265 CFU/g). At the end of ripening, the highest count was isolated from the control sample (301 CFU/g), while the least count was isolated from T_3 sample (243 CFU/g).

Aspergillus and *Penicillium* also were the most common isolated genera. *Aspergillus* percentage was higher in the fresh control cheese (87.8%) compared to the treated cheese (ranged from 78.49% to 84.45%), however, it ranged from 49.16% to 79.83% after 90 storage days (Table, 3).

The number of species ranged from 5 to 9 species/sample. The highest number was isolated from the fresh control sample (9 species). *Aspergillus* was represented by 5 species and one variety, while *Penicillium* was represented by 4 species. The other isolated fungal genera were represented by only one species for each (Table, 3). Also, it is noted that each of *A. flavus, A. fumigatus, A. niger, P. chrysogenum, P. corylophium, P. funiculosum* and *Rhizopus stolonifer* were isolated from control plus all the three treatments cheese, however each of *A. versicolor* and *P. duclauxii* appeared on control cheese samples at zero time only (Table, 3).

These results in Table 2 and 3 suggest that probiotic bacteria incorporated in the manufacture of Ras cheese prevented and reduced the fungal growth in cheese. This inhibition of growth could be due to the facultative anaerobic condition created by bacteria in the cheese (Batish et al. 1997), or due to their lactic and organic acids production, which decreases the pH of the growth environment (Bonestroo et al. 1993; Caplice and Fitzgerald 1999). Also, these bacteria have the potential to produce other components, e.g., reuterin, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds which have a negative effect on fungal growth (Dalié et al. 2010).

Occurrence of mycotoxins in cheese samples

The presence of mycotoxins were confirmed in different types of cheese by several studies (Siemens and Zawistowski 1993; Gandomi et al. 2009). Lieu and Bullerman (1977) showed that aflatoxins B_1 and G_1 were stable in cheese during storage at 5 °C. The high presence of *Aspergillus* species, especially *A. flavus*, in our studied cheese supported the hypothesis of the presence of mycotoxins in these cheeses. Therefore, Ras cheese samples were examined for the natural occurrence of mycotoxins. The results showed that the examined Ras cheese samples were completely free from the presence of mycotoxins. This result gives an assumption that the raw milk used in the preparation of Ras cheese was free from mycotoxins. Also, the use of probiotic bacteria as an adjunct starter as well as the production and storage conditions could be the reason to prevent the mycotoxins formation by the contaminated fungi.

In this respect, several bacterial species of *Bacillus*, *Lactobacilli*, *Pseudomonas* and *Ralstonia* have shown the ability to inhibit fungal growth and production of mycotoxins (Nesci et al. 2005; Palumbo et al. 2006; Zohri et al. 2018). El-Nezami et al. (1998); Wiseman and Marth (1981) found that the fungal growth and mycotoxins production are inhibited by antifungal metabolites produced by lactic acid bacteria which have the potential to bind aflatoxins. Gomah and Zohri (2015) found that *Lactobacillus rhamnosus* completely inhibited fungal growth as well as deoxynivalenol, zearalenone and fumonisin B_1 production by *Fusarium graminearum*, *F. culmorum* and *F. proliferation*, respectively.

On the other hand, Aiad and Abo El-Makarem (2013) found that 56 % of examined Ras cheese samples were contaminated with aflatoxins with levels ranging from 7.40 to 111.50 ng/kg. Seddek et al. (2016) found that two out of three Ras cheese samples contaminated by aflatoxins B_1, B_2 and G_2. Also, Elramly et al. (2019) recorded the contamination of Ras cheese samples by aflatoxins and Ochratoxin A.

Mycotoxins production by fungi isolated from Ras cheese

A total of 29 fungal isolates from different Ras cheese samples were examined for their ability to mycotoxin production (Table 4). These isolates belong to *Aspergillus* (18 of *A. flavus* and 11 of *A. niger*). Seven out of the 18 tested *A. flavus* isolates had the ability to produce aflatoxin B_1 and two isolates had the ability to produce aflatoxin B_1 and G_1. Three isolates out of the 10 tested *A. niger* had the ability to produce Ochratoxin A (Table 5). This observation raises the possibility of contamination of cheese with the aflatoxins and ochratoxin, especially if it is stored for periods longer than 90 days.

In a study by Mohamed (2020), several isolates of *A. flavus* and *A. niger* groups showed positive production of aflatoxins B_1, B_2, G_1, G_2, and ochratoxin A from different dairy sources. Also, Varma and Verma (1987) found that three out of seven *A. flavus* isolates have the ability to produce aflatoxin B_1. Sánchez-Hervás et al. (2008) found that 49.2% of *A. niger* strains were able to produce ochratoxin A.

Extracellular lipases and proteases secreted by filamentous fungal isolates

Lipases and proteases enzymes are considered very important factors that can affect the sensory and the characteristics of Ras cheese during the ripening period (Seddek et al. 2016). Twenty-eight fungal isolates belonging to the species of *Aspergillus* (18 of *A. flavus* and 10 of *A. niger*) were collected from Ras cheese samples and were examined for their abilities to produce lipases and proteases (Table 5). All the tested fungal isolates showed lipase positive results. Seventeen possessed high lipase activities (activity depth more than 20 mm), while 3 and 8 had moderate and low ability to produce the enzyme, respectively. Two isolates of *Aspergillus flavus* showed the highest producers of lipase (24 mm as clear zone). This result is in agreement with Refaie (2013), who found that *Aspergillus flavus* was the highest producer of lipase enzyme with 30 mm clear zone as a depth of activity. Also, Seddek et al. (2016) recorded that *A. niger* was the highest lipase producer, followed by *A. ustus* and *A. flavus*.

Also, all the 28 fungal isolates tested in this study showed high and moderate positive results for their ability to proteases production. Nine of them possessed high production levels (more than 20 mm), where one of *Aspergillus flavus* isolates presented the highest producer with 31 mm clear zone.

Nineteen isolates had moderate production (10-20 mm). The lowest producer of proteases (16 mm) was *Aspergillus niger* (T1a / PDA / fresh) which was isolated from Ras cheese samples made with adding *Lb. acidophilus* as adjunct culture (Table 5).

The obtained results are in agreement with that reported by Seddek et al. (2016) who found that *A. flavus* and *A. niger* were the highest producers of casienase. In addition, El-Fadaly et al. (2015) found that the presence of casein in the fungal media caused a strong growth of the fungal strains compared with the growth on control medium without casein. Our results, in addition to the results found in previous studies, confirm the negative impact of the growth of filamentous fungi on Ras cheese and its expected defects on the sensory properties as a result of the secretion of lipolytic and protein degrading enzymes.

Conclusion

Making of Ras cheese with probiotic *Lactobacillus* sp. as adjunct cultures showed a marked decrease in the total number of fungi. However, the samples of Ras cheese were completely free of mycotoxins, they were contaminated with mycotoxin-producing fungi such as *A. flavus* and *A. niger* which are considered very dangerous because of its production of B_1, G_1 and Ochratoxin A. Also, the isolated fungi were positive to the production of the lipolytic and proteolytic enzymes and thus could affect the sensory properties and the nutritional value of Ras cheese. Therefore, we can recommend that we do not rely on the standard specifications on the number of fungi only because the numbers may be within the permissible limits, but some species can produce the toxins in proportions affecting the product.

Declarations

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table (1)

Distribution of fungal genera and species isolated from Ras cheese made with adding some probiotic *Lactobacillus* spp. using two isolation medium*.

Isolated media	Czapek agar		Potato dextrose agar	
Fungal genera	No. of species	No. of varieties	No. of species	No. of varieties
Aspergillus	6	1	5	1
Cladosporium	1	0	1	0
Mucor	1	0	1	0
Paecilomyces	1	0	0	0
Penicillium	4	0	4	0
Rhizopus	1	0	1	0
Number of genera	6		5	
Number of species and variety	14+1		12+1	

* The figures of the fungal species isolated from Ras cheese samples are given in Online Resource

Table (2)

Average counts (CFU /g cheese) of fungal genera and species isolated form cheese samples made with adding some probiotic *Lactobacillus* spp. when fresh and after storage for 90 days on Czapek medium at 28±2°C

Fungal species	C				T1				T2				T3			
	fresh		90 days		fresh		90 days		fresh		90 days		fresh		90 days	
	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC
Aspergillus	443	84.38	198	59.28	236	81.94	215	62.5	296	85.05	173	57.28	226	81.58	226	69.7
Aspergillus flavus	63	12	24	7.18	80	27.7	43	12.5	83	23.85	51	16.88	40	14.44	53	16.40
Aspergillus flavus. var. *columnaris*	6	1.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus fumigatus	2	0.38	35	10.47	30	10.41	16	4.65	22	6.32	13	4.30	32	11.55	33	10.21
Aspergillus niger	366	69.71	134	40.11	120	41.6	150	43.60	190	54.59	103	34.10	147	53.06	129	39.93
Aspergillus sydowii	2	0.38	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus terreus	2	0.38	5	1.49	6	2.08	6	1.74	1	0.28	6	0	7	2.52	11	3.40
Aspergillus ustus	2	0.38	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.30
Mucor himealis	0	0	0	0	10	3.47	0	0	0	0	0	0	18	6.49	0	0
Paecilomyces varioti	0	0	0	0	0	0	0	0	0	0	23	7.61	0	0	0	0
Penicillium	2	0.38	98	29.33	16	3.67	99	28.77	34	9.77	73	24.15	33	11.90	48	14.85
Penicillium chrysogenum	0	0	0	0	10	3.47	0	0	0	0	33	10.92	0	0	9	2.78
Penicillium corylophium	2	0.38	85	25.44	3	0.10	96	27.90	15	4.31	15	4.96	23	8.30	18	5.57
Penicillium duclaucii	0	0	0	0	3	0.10	3	0.87	0	0	14	4.63	3	1.08	0	0
Penicillium funiculosum	0	0	13	3.89	0	0	0	0	19	5.45	11	3.64	7	2.52	21	6.50
Rhizopus stolonifer	80	15.23	38	11.37	26	9.02	30	8.72	18	5.17	33	10.92	0	0	48	14.86
Total count	525		334		288		344		348		302		277		323	
Number of genera	3		3		4		3		3		4		3		4	
Number of species + variety	8 + 1		7		9		7		7		10		8		9	

C: (Control) (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* (1:1)). T1: (*Lb. acidophilus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T2: (*Lb. helveticus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T3: (*Lb. casei* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). Lb. = Lactobacillus.

Table (3)

Average counts (CFU, colony forming unit/g cheese) of fungal genera and species isolated form cheese samples treated by different bacterial strains during their preparation when fresh and after storage for 90 days on PDA medium at $28\pm2°C$

Fungal species	C				T1				T2				T3			
	fresh		90 days		fresh		90 days		fresh		90 days		fresh		90 days	
	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC	TC	%TC
Aspergillus	252	87.80	148	49.16	295	81.26	209	77.12	208	78.49	178	66.66	250	84.45	194	79.83
Aspergillus flavus	58	20.20	8	2.65	30	8.26	36	13.28	25	9.43	20	7.49	40	13.51	23	9.64
Aspergillus flavus. var. *columnaris*	0	0	0	0	66	18.18	0	0	0	0	0	0	0	0	0	0
Aspergillus fumigatus	26	9.05	8	2.65	33	9.09	0	0	25	9.43	16	5.99	10	3.37	0	0
Aspergillus niger	164	57.14	129	42.85	166	45.73	173	63.83	158	59.62	141	52.80	198	66.89	168	69.13
Aspergillus terreus	2	0.69	3	0.99	0	0	0	0	0	0	1	0.37	2	0.67	3	1.23
Aspergillus versicolor	2	0.69	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	0	0	0	0	0	0	0	0	1	0.37	0	0	0	0	0	0
Mucor himealis	0	0	0	0	0	0	0	0	12	4.52	0	0	18	6.08	1	0.44
Penicillium	23	6.43	62	20.59	52	14.31	46	16.97	36	13.58	66	24.71	28	9.45	35	14.39
Penicillium chrysogenum	0	0	27	8.97	6	1.65	0	0	0	0	18	6.74	0	0	4	1.64
Penicillium corylophium	5	0.17	33	10.96	26	7.16	26	9.59	18	6.79	29	10.86	20	6.75	23	9.64
Penicillium duclaucii	2	0.69	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium funiculosum	16	5.57	2	0.66	20	5.50	20	7.38	18	6.79	19	7.11	8	2.70	8	3.29
Rhizopus stolonifer	12	4.18	91	30.23	16	4.40	16	5.90	8	3.01	24	8.98	0	0	13	5.34
Total count	287		301		363		271		265		267		296		243	
Number of genera	3		3		3		3		5		3		3		3	
Number of species + variety	9		8		7 + 1		5		8		7		7		7	

C: (Control) (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* (1:1)). T1: (*Lb. acidophilus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T2: (*Lb. helveticus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T3: (*Lb. casei* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). Lb. = Lactobacillus.

Table (4)

Mycotoxins produced by some fungal isolates from Ras cheese made with adding some probiotic *Lactobacillus spp.* (grown on Potato dextrose broth at 28±2°C for 10 days.

Fungal isolates	Source	Mycotoxins
Aspergillus flavus	Ca/ PDA / fresh	Aflatoxin B_1
	Cb/ PDA / fresh	-
	Cc/ PDA / fresh	Aflatoxin B_1
	Ca/ Cz / 90 days	-
	Cb/ Cz / 90 days	-
	T1 / PDA / fresh	-
	T1 / Cz / fresh	-
	T1 / Cz / 90 days	-
	T1a / PDA / 90 days	Aflatoxin B_1 and G_1
	T1b / PDA / 90 days	Aflatoxin B_1
	T2a / Cz / fresh	Aflatoxin B_1
	T2b / Cz / fresh	Aflatoxin B_1
	T2 / Cz / 90 days	-
	T2 / PDA / 90 days	-
	T3 / PDA / fresh	Aflatoxin B_1
	T3 / Cz / fresh	Aflatoxin B_1 and G_1
	T3a / Cz / 90 days	Aflatoxin B_1
	T3b/ Cz / 90 days	-
Aspergillus niger	Ca/ Cz / 90 days	-
	Cb/ Cz / 90 days	Ochratoxin A
	T1 / PDA / fresh	Ochratoxin A
	T1 / PDA / 90 days	-
	T1 / Cz / 90 days	-
	T2a / PDA / 90 days	-
	T2b / PDA / 90 days	-
	T3 / PDA / fresh	-
	T3 / Cz / fresh	-
	T3a/ PDA / 90 days	-
	T3b / PDA / 90 days	Ochratoxin A

C: (Control) (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* (1:1)). T1: (*Lb. acidophilus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T2: (*Lb. helveticus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T3: (*Lb. casei* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). Lb= Lactobacillus.

Table (5)

Lipases and Proteases secretion by some filamentous fungi isolated from Ras cheese and grown at 282°C for 10 days.

Fungal isolates	Source	Lipases		Proteases	
		Depth of activity (mm)	Levels	Depth of activity (mm)	Levels
Aspergillus flavus	Ca/ PDA / fresh	23	H	20.5	H
	Ca/ Cz/ fresh	24	H	19	M
	Cb/ PDA / fresh	22	H	18.5	M
	Ca/ Cz/ 90 days	22	H	20.5	H
	Cb/ Cz / 90 days	24	H	20.5	H
	T1a / Cz / fresh	20.5	H	20.5	H
	T1a / PDA/ fresh	22.5	H	20.5	H
	T1a / PDA / 90 days	21	H	19.5	M
	T1a / Cz / 90 days	22	H	20	M
	T1b / PDA / 90 days	21	H	20	M
	T2a / Cz / fresh	21	H	20	M
	T2b / Cz / fresh	21	H	19	M
	T2a / Cz / 90 days	21.5	H	24.5	H
	T2a / PDA / 90 days	23	H	20	M
	T3a / Cz / fresh	22.5	H	29	H
	T3b / Cz / fresh	22	H	19.5	M
	T3c / Cz / fresh	10	M	31	H
	T3a / Cz / 90 days	22	H	21.5	H
Aspergillus niger	Ca/ PDA / fresh	3	L	19	M
	Ca/ Cz / 90 days	8.5	L	20	M
	T1a / PDA / fresh	1	L	16	M
	T1a / PDA / 90 days	3.5	L	17	M
	T1a / Cz / 90 days	8.5	L	19	M
	T2a/ PDA / fresh	11	M	16.5	M
	T2a/ PDA / 90 days	10.5	M	19	M
	T3a / Cz / fresh	9	L	18	M
	T3a / PDA / fresh	2	L	17	M
	T3a / PDA / 90 days	9.5	L	20	M

L- Low level = less than 10mm, H- Moderate level = 10-20mm, H- High level = more than 20mm

C: (Control) (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* (1:1)). T1: (*Lb. acidophilus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T2: (*Lb. helveticus* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). T3: (*Lb. casei* + (*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*) (1:1). Lb= Lactobacillus.

Figures

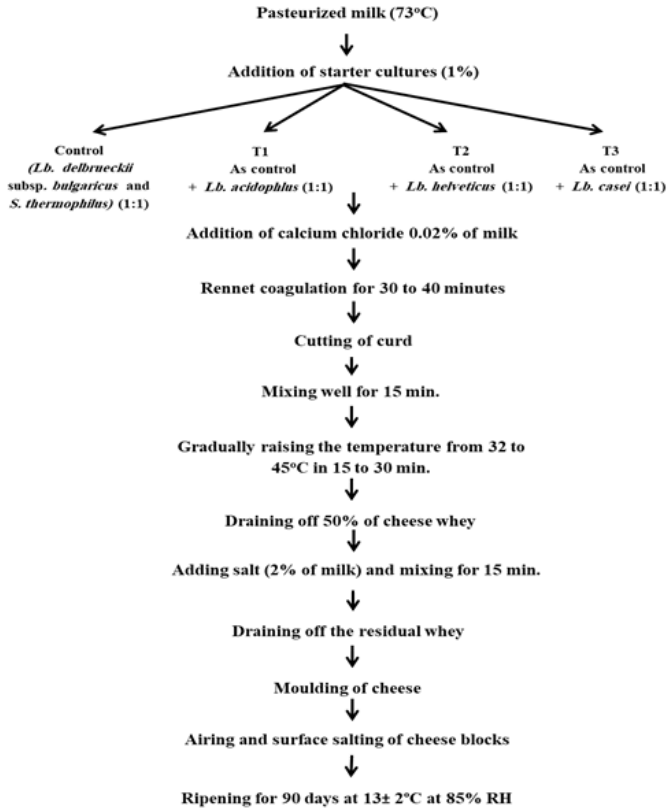

Figure 1

Diagram showing the steps of Ras cheese making

Figure 2

The form of Ras cheese after the pressing process

Supplementary Files

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