ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH FERMENTED COW MILK SOLD IN SAMARU MARKET, KADUNA STATE, NIGERIA

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Abstract: This research was carried out for the isolation and identification of fungal spoilage organisms in fermented cow milk. Three samples were bought from Samaru market using sterile nylon bags for each samples and taken to the laboratory for analysis. One gram of each sample was put into nine (9) ml of sterile water. Five-fold dilution was carried out up to the fifth sterile test tube to reduce microbial load. Pourplate method was used by taking 0.1 ml from each dilution unto sterile prepared Solidified potato dextrose agar plates, the plates were incubated for 7 days for growth to occur. Representative growths from the plates were then sub cultured on agar plates to obtain pure cultures for identification. Slide culture technique was use for proper identification of the fungi and incubation was for 7 days. Lactophenol cotton blue was used to stain the fungi growth on the slide and the cover slip which was viewed under the light microscope. The fungi isolated were *Aspergillus flavus* (32.26%), *Aspergillus fumigates* (34.84%) and *Saccharomyces cerevisiae* (32.90%). The most abundant fungal specie was *Aspergillus fumigatus* (34.84). These food products can be contaminated due to daily exposure to air as fungal spores are known to be present in a large number in the air making it easy for them to invade exposed foods.

Keywords: Cow milk, isolation, subculture, inoculation, fungal spoilage

Introduction

Cow milk and dairy products due to their immense nutritive value form a major part of human food it play a prominent role in the diet. Cow Milk is unique in nutrient and a major source of protein and calcium (Pal *et al*., 2014). It is estimated that billions of people around the world consume cow milk and dairy products every day as they are the vital source of nutrition for human health. Approximately 50% of the cow milk produced is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing of many types of milk products (Pal and Jadhav, 2013). The dairy products such as cheese, butter, ice cream, milk powder and yoghurt are universally available. The microbial contamination of cow milk and cow milk products is largely due to unhygienic conditions and human factors. Pitt and Hocking (2007) mentioned that some factors that can contribute to the contamination of various cow milk products by fungi include; if the premises of milk processing plants are unsanitary, if the equipment and utensils are not properly sterilized, if the preservation is omitted, if the water is unwholesome, unsafe and non-potable, if the packaging material is not properly cleaned. Though the type of spoilage fungi differ widely among dairy products because of the effects of practices followed in the production, formulation, processing, packaging, storage, distribution and handling.

Warm climate and inadequate refrigeration are the principal causes of high level of contamination of fungi. Some physical defects such as off color, loss of firmness and loss of aroma can occur following the spoilage of cow milk products by fungi. Moulds and yeasts are recognized as an important cause of spoilage of various dairy products (Filtenborg *et al*., 1996; Fleet, 1999; Khalifa *et al*., 2013; Pal and Jadhav, 2013; Pal *et al*., 2014). The contamination of cow milk products with different types of fungi particularly of species of *Aspergillus*, *Fusarium* and *Penicillium* constitute a public health hazard as these fungi are known to produce mycotoxins that are injurious to human health (Pal, 2002; Sengum *et al*., 2008; Khalifa *et al*., 2013). The mouldiness can be considered as an indicator of deterioration in the milk products. The early detection of spoilage of cow milk products is necessary so that preventive measures can be applied.

Food products are a rich nutrient source that can attract both bacterial and fungal colonizers (Pitt *et al*., 2009). As such, the food product can be regarded as an ecological resource. Colonization with a number of food-borne microorganisms is beneficial with respect to nutritional value and prolonged storage of the food product, which is known as food fermentation in other case. After successful colonization of the product, its nutritional properties are altered (Samson *et al*., 2004). When the nutritional value, structure, and taste of the product are negatively influenced, this colonization is called food spoilage. It can be accompanied by the production of toxic secondary metabolites which may result in serious medical problems (Dijksterhuis *et al*., 2007). These two aspects of food colonization are two sides of the same coin. Food spoilage is a major threat for our food stock and is responsible for enormous losses (Pitt *et al*., 2009).

Fungi are the main degraders of the sturdy plant cell wall components that otherwise would accumulate within the ecosystems of the world. Prior to spoilage, the fungi can be present on or inside of the crop in low numbers, or as survival structures. Spoilage fungi can also be introduced to an empty habitat if the food is previously treated by pasteurization treatments. Food products include two main groups, which are living crops and processed food (Karlshøj *et al*., 2007).

Materials and Methods

Study area

This research was carried out in the Department of Botany, Faculty of Life sciences, Ahmadu Bello University, Zaria. Zaria is located in the Northern Guinea Savannah zone of Nigeria and coordinates (11° 3'N, 7° 42' E).

Sample collection

Samples of fresh cow milk were obtained from three randomly selected places within Samaru Market, Kaduna state. The samples were collected in transparent nylons, the samples were labeled S1, S2, S3 and taken to the laboratory for analysis (S = Sample)

Preparation of culture media

Potato dextrose agar (PDA) was prepared according to manufacturer's instruction by suspending 39 g of the medium in 1000 ml of distilled water. The solution was placed in a water bath with frequent agitation and allowed to boil for one minute to completely dissolve the medium, chloramphenicol capsule (500 mg) was added to inhibit bacteria growth, the medium was autoclaved at 121°C for 15 min.

Preparation of sample

The collected samples were left for seven days to ferment; five-fold serial dilutions of each sample were prepared. Serial dilution was carried out where 1 ml of each sample was transferred into a test tube containing 9.0 ml of sterile distilled water and the test tube was shaken and labeled as 10^{-1}, from this tube 1.0 ml was transferred into another tube containing 9.0 ml of sterile distilled water and the test tube labeled as 10^{-2}. The procedure was repeated up to 10^{-5} using sterile syringes. The test tube 10^{-2}, 10^{-1}, and 10^{-5} was used for S1, S2 and S3, respectively.

Innoculation

1.0 ml from the distilled factors of the test tube was transferred into sterile Petri-dishes, containing solidified prepared potato dextrose Agar. The diluted samples were used to inoculate the prepared media using pour plate method. The inoculated plates were incubated at room temperature for 7 days.

Isolation of fungi

The samples of fermented cow milk were placed on potato dextrose agar and incubated at room temperature for 7 days. After incubation, colonies of different shapes and colors were observed on the plates (Cheesbrough, 2000). Pure culture of each colony type on each plate was obtained, this was done by sub-culturing each of the different colonies onto a freshly prepared PDA plates and incubated at room temperature again for 7 days and observed regularly for fungal growth.

Identification of fungi

The identification of fungi was based on macroscopic and microscopic examination. Macroscopic examination was based on color, texture, topography, and nature of hyphae. In microscopic examination, the technique of (James and Natalie, 2001) was adopted for identification of unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide, where a small portion of the mycelium was spread very well on the slide with the aid of a needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses. The species encountered was identified in accordance with (Cheesebrough, 2002).

Statistical analysis

Descriptive statistics was used to report percentage abundance of fungi in the form of a Bar chart using the formular:

% abundance $= \frac{No.\,of\ times\ a\ gungus\ is\ encountered}{Total\ No.of\ gungi\ isolated}\ x\ 100$ (Abu *et al.*, 2015)

Results and Discussion

Fungi isolated and identified from fermented cow milk

A total of three fungal species namely *Aspergillus flavus*, *Aspergillus fumigatus*, and *Saccharomyces cerevisiae* were isolated from three samples of fermented cow milk (Table 1). They were identified by studying their macroscopic and microscopic characters (Table 2) and compared with identification keys by Samson *et al*. (2004).

Table 1: Fungi isolated and identified from fermented cow milk

Species	Sample 1	Sample 2	Sample 3
Aspergillus flavus	+	+	+
Aspergillus fumigatus	+	+	+
Saccharomyces cerevisiae	+	+	+

+ = Present

Table 2: Macroscopy and microscopy of fungal species

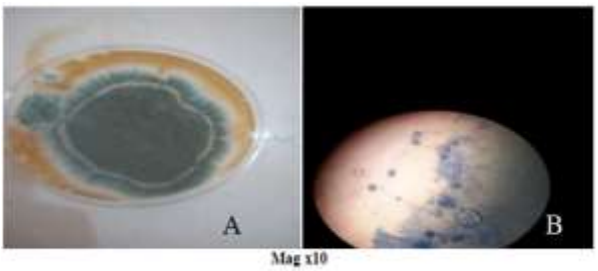
S/N	Fungi species	Macroscopy	Microscopy
1	*A.flavus*	The upper surface of the colony was yellow- green with edge, granular surface and green coloration on the reverse side.	The conidophores was thick walled, hyaline and slightly roughened, erect, long aseptate with a vesicle with short conidial chains.
2	*A. fumigates*	Gray- green with a slight yellow reverse.	Hyphae are septate and hyaline, conidiophores are smooth- walled and uncolored
3	*S.cerevisiae*	The colonies were creamy and yellowish-white in color	Cells are large, globose and also budding.

Percentage abundance of fungi associated with fermented cow milk

A total of 155 colonies occurred on the fermented cow milk *Aspergillus flavus* occurred 50 times with percentage of abundance of 32.26, *Aspergillus fumigatus* occurred 54 times with percentage abundance of 34.84 and *Saccharomyces cerevisiae* occurred 51 times with percentage abundance of 32.90 (Table 3).

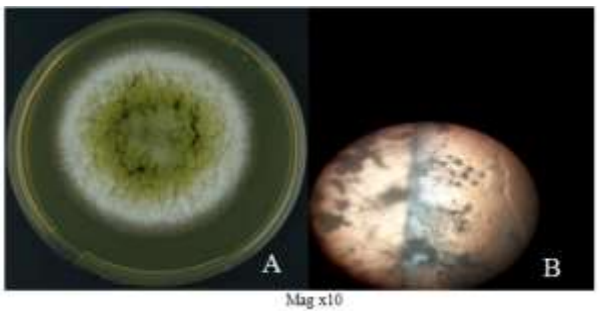
Table 3: Percentage abundance of fungi associated with fermented cow milk

S/N	Fungi species	No. of colonies	% abundance
1	*A. flavus*	50	32.26
2	*A. fumigatus*	54	34.84
3	*S. cerevisiae*	51	32.90



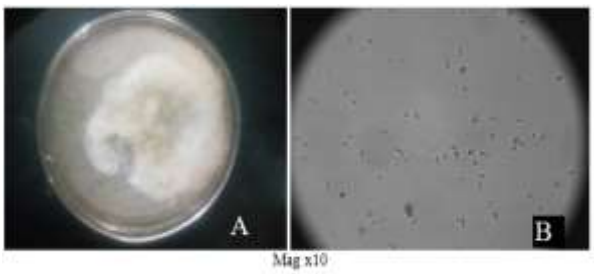
A= Macroscopic features B= Microscopic features

Plate 1: Pure culture of *Aspergillus fumigatus*



A= Macroscopic features B= Microscopic features

Plate 2: Pure culture of *Aspergillus flavus*



A= Macroscopic features B= Microscopic features

Plate 3: Pure culture of *Saccharomyces cerevisiae*

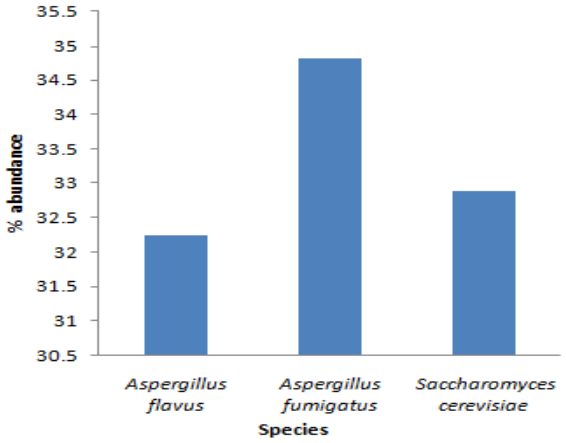


Fig. 1: Percentage abundance of fungi species isolated from fermented cow mil

All the three samples of fermented cow milk were found to be contaminated with the same fungal species; the species of fungi identified were *Aspergillus flavus*, *Aspergillus fumigatus*, and *Saccharomyces cerevisiae* (Fig. 1). However, all the isolated fungi when compared with already described species by Samson *et al*. (2004) were found to be similar.

The study indicates that sample 2 had the higher fungal population, and *Aspergillus fumigatus* with the highest percentage abundance this is because they are the dominant in tropics. These samples were possibly contaminated by either the biotic or the abiotic factors which appeared to be one of the major factors that support fungal growth in food products (Hill and Waller, 1999). Moreover, food products can encounter fungal infestation by influences from the outside environment, such as insect's infestation, wound and presence of foreign matter such as sand, dust and debris among others (Djerbi, 1983). Thus some of the identified fungal species could have come from any of these sources. Similarly, the constant exposure of food products to the outside environment at the time of sales could have aided in deposition of the fungal spores on them. Therefore, spores can germinate on the food products when temperature and humidity triggers the growth processes. Damage by insects has also been known to provide entry points for fungal infection (Dennis, 2002) and aid in their rapid spread. Hence, presence of insects may under certain critical circumstances be quite essential for establishment of infection. While several fungal species cause spoilage of food products worldwide,the presence of this known organisms isolated from these samples, are known to produce different mycotoxins like the ochratoxin, neurotoxin and aflatoxin. Thesemycotoxinsmay cause serious mycotoxicoses in man and in animals (Williams *et al*., 2004). The extent to which neurotoxin affects nerve function depends on the toxicity of the substance either by ingestion or by inhalation depending on the individual age and immune status. This toxin can have long lasting effect by causing neurons to malfunction or by disrupting interneuron communication which may eventually lead to paralysis or death (Ram *et al*., 2012). The ochratoxin is a naturally occurring foodborne mycotoxin found in a wide variety of agricultural products that can be produced by several fungal specie and genera like *Aspergillus* specie. Ochratoxin (OTA) causes nephrotoxicity and renal tumors in different animal species (Hagelberg *et al*., 1989). However health effect has linked OTA exposure with human disease known as Balkan endemic nephropathy (BEN) and chronic intestinal nephropathy (CIN) as well as other renal diseases (Duarte *et al*., 2011). The generally most common and deadly mycotoxin produce is the aflatoxin known to be produce by *Aspergillus* species. *Aspergillus* infections have grown in importance in the last years. However, most of the studies have focused on *Aspergillusfumigatus*, the most prevalent species in the genus. In certain locales and hospitals, *Aspergillusflavus* is more common in air than *A. fumigatus*, for unclear reasons (Guinea *et al*., 2005). After *A. fumigatus*, *A. flavus* is the second leading cause of invasive aspergillosis and it is the most common cause of superficial and systemic infection. Common clinical syndromes associated with *A. flavus* include chronic granulomatous sinusitis, keratitis, and cutaneous aspergillosis, wound infections, osteomyelitis following trauma, liver disease and even deep or systemic aspergillosis (Van Burik *et al*., 1998). Outbreaks associated with *A. flavus* appear to be associated with single or closely related strains, in contrast to those associated with *A. fumigatus*. In addition, *A. flavus* produces aflatoxins, the most toxic and potent hepatocarcinogenic natural compounds ever characterized which is very deadly because it is a byproduct that can cause serious damage to the DNA. Prolong exposure to aflatoxin, cell accumulate DNA mutations and thus are at risk of developing into cancer cells (Morgan *et al.*, 2005). Aflatoxins are known to be heat stable therefore heating of food products contaminated with aflatoxin cannot be destroyed by heat (Payne and Brown *et al*., 1998). This known mycotoxins that can be produce by this fungi species gets into the body either by inhalation of the spores, ingestion or cuts from the skin and this mycoses are more severe in immunocompromised individual. The spores of this fungi are in the air making it very easy for them to invade exposed food products or during the administration of immunosuppressive drugs use during organs transplanting thereby giving them easy access to cause infection (Chu, 1998).

Some species of *Saccharomyces cerevisiae* are opportunistic pathogens that can cause infection in people with compromised immune systems (Cogliati, 2013).

Conclusion

In conclusion, *Aspergillus flavus* (32.26), *Aspergillus fumigates* (34.84) and *Saccharomyces cerevisiae* (32.90) were found associated with cow milk sold in samara market, zaria , Kaduna state.

.Recommendations

This research showsthat there is fear of consumption of mycotoxins because of their serious health implication, as they can be highly toxic and carcinogenic (AOAC, 2002; Shenasi *et al*., 2002); thus rendering the food products unfit for human and animal consumption. Therefore, creation of awareness on proper hygiene in the production, packaging, distribution of cow milk, and proper sterilization on the equipment been used. Contaminated food products should be sorted and eliminated to avoid re-infection. This will help to reduce the rate of mycoses. Further studies should be carried out using other growth media.

Conflict of Interest

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

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