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MICROBIAL CHARACTERISTICS OF A TRADITIONALLY FERMENTED SPICE: THE CASE OF DATTA/QOCHQOCHA IN ETHIOPIA

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ABSTRACT

Introduction: This study assessed the presence of fungi, aflatoxin, pathogenic bacteria, and beneficial bacteria in Datta/Qochqocha, a spice traditionally fermented in Ethiopia, to ensure health and safety.
Methods: A sample of thoroughly homogenized Datta/Qochqocha was diluted in sterile water and subjected to serial dilutions. A 0.1 milliliters of each homogenate was spread to selective nutrient agar plates. The plates were incubated at varying temperatures and durations to isolate lactic acid bacteria, total aerobic mesophilic bacteria, coliforms, Enterobacteriaceae, staphylococci, yeasts, and molds. The microbial identifications were done based on morphological characteristics and biochemical tests. The mean colony-forming units (CFU) in the ideal dilution were measured using a colony counter. The concentration of aflatoxin antigen in Datta/Qochqocha was determined using high-performance liquid chromatography (HPLC), with results interpreted based on the area and height of the chromatographic peaks.
Result: The study found that Datta/Qochqocha contains 1.02 * 10^6 CFU/g of lactic acid bacteria, 8.5 * 10^4 CFU/g of aerobic mesophilic, 3.2 * 10^5 CFU/g of staphylococcus bacteria, and 1.3 * 10^6 CFU/g of the yeast of Datta/Qochqocha on average. A few samples of Datta/Qochqocha contain mold and lower pathogenic levels of aflatoxin with under-reportable colonies of salmonella, coliform, and Enterobacteriaceae. The pH level of Datta/Qochqocha was 3.82 ± 0.08 on its 7^{th} day of fermentation.
Conclusion: Datta/Qochqocha is a source of lactic acid bacteria and yeast, but it is free from pathogenic-level pathogenic bacteria, mold, and aflatoxins.

Keywords: Characteristics, Datta/Qochqocha, Ethiopian, Microbial, Traditionally Fermented

INTRODUCTION

Datta/Qochqocha is one of the traditionally fermented Ethiopian cultural spices. Its main components are chili pepper, green pepper with seeds, garlic, ginger, cardamom, fresh sweet basil leaf, coriander (fruit and leaf), and rue seeds **(Wedajo Lemi, 2020).** Fermentation is a biological process that naturally fortifies food with functional nutrients and maintains a healthy gut microbiota (**Knez, Kadac-Czapska, & Grembecka, 2023; Tasdemir & Sanlier, 2020**). Consuming fermented food is helpful for the gut microbiota's composition, as it contains probiotics and prebiotics that can prevent or alleviate digestive system issues **(Melini, Melini, Luziatelli, Ficca, & Ruzzi, 2019)**. Studies discovered that gut microbiota has multiple beneficial effects on its normal condition, including improving the effectiveness of medical care, preventing adverse effects of drugs, and boosting immunity in patients (**Knez *et al*., 2023; Lee *et al*., 2021; Sánchez-Alcoholado *et al*., 2020**). Probiotics influence the structure and function of the gut microbiota through interactions with commensal bacteria and the expression of microbial enzymes (**Melini *et al*., 2019**), which also improves the metabolic product (**Pellegrini *et al*., 2020).** Additionally, probiotics have anti-inflammatory and immune-boosting properties (**Malik *et al*., 2018; Melini *et al*., 2019**). However, it is unknown how human organisms interact with probiotics that can be given by adding them to foods (**Ciernikova *et al*., 2017**), or most fermented foods undergo steps that remove or hinder the probiotics' activity during preparation (**Nazir, Hussain, Abdul Hamid, & Song, 2018**). Unrefined, naturally fermented foods are a source of essential probiotics that help regulate metabolic processes and healthy physiological hemostasis (**Melini *et al*., 2019**). In Ethiopia, most of the traditional staple foods are fermented foods. However, the procedures (cooking) used to process these foods degrade the available essential microbiotas (**Asaithambi, Singh, & Singha, 2021; Carmody *et al*., 2019; Hassan, Elsabagh, Eleiwa, & Zohdy, 2018**) depending on the intensity of cooking and type of diet (**Nartey, Tei-Mensah, Adusei, Asante, & Abaati, 2021; Nova, Gómez-Martinez, & González-Soltero, 2022**). Generally, most fermented foods contain healthy microbial content, but some may contain aflatoxin and other unhealthy microbial content (**Skowron *et al*., 2022).** Nevertheless, the fungal, aflatoxin, pathogenic bacteria, and beneficiary bacteria characterization of Datta/Qochqocha have not been studied in Ethiopia. Therefore, this study has been conducted to evaluate the microbial contents of fermented Datta/Qochqocha for its health benefits in treating and preventing diseases.

METHODS AND MATERIALS

Study Design, Study Population, Sample Size, Sample Selection, and Data Source

A cross-sectional study design was employed from August 03 to 15, 2023. The snowball sampling method was used to collect 43 Datta/Qochqocha samples from producers in Hawassa and Nekemte cities. In addition four samples were prepared as standard samples. Totally 47 samples were included. The sample size was calculated using a 97% prevalence of lactic acid bacteria in selected vegetables at 95% certainty, a 4% margin of error, and a 5% nonresponse rate (**Ketema, 2014**) using the $n=z^2*p*(1-p)/d^2$ formula. Using a disinfected container, approximately 250 g of traditionally fermented Datta/Qochqocha samples were taken from each producer. Each sample was labeled with its production date, quantity, ingredients, area of production, and unique sample code. The sample was stored in the refrigerator at less than -80 degrees Celsius at the Holeta Agricultural Research Laboratory facility. The microbial composition of Datta/Qochqocha was assessed through conventional laboratory methods, including morphological identification and biochemical tests, to identify the intended bacteria.

Standard Datta/Qochqocha sample preparation procedures

The following ingredients had been prepared: each fresh fruit, one kilogram of red or green hot pepper, 100 grams of rue seeds, 125 grams of table salt, 350 grams of garlic, 125 grams of ginger, one coffee cup of fresh basil seeds, one coffee cup of fresh coriander seeds, and the seeds of three medium cardamoms. After donning the examination glove, fresh fruits and hot peppers were thoroughly washed under clean tap water. The hot peppers were cut into small

pieces using a washed and dried mortar and pestle after removing the tail/stem and leaves. The chopped hot pepper was combined with freshly peeled and sliced ginger and garlic, cardamom seeds, and fresh basil seeds with rue seeds and further chopped using a mortar. Then it was mixed with table salt and ground using a washed and dried traditional flat milling stone until a uniform composition of fine (avocado structure) greenish or red Datta/Qochqocha was produced. The sample was transferred to a container, tightly sealed, and stored in a cold environment for seven days before conducting a conventional laboratory analysis. The standard sample of Datta/Qochqocha was prepared based on the preliminary study findings that were done using in-depth interviews.

Laboratory procedure

A gram of thoroughly homogenized Datta/Qochqocha sample was diluted in 9 ml of sterile water (1 g: 10 ml), followed by a 10^{-1}-fold three-serial dilution. Nutrient agar culture media was prepared according to the manufacturer's recommendation. Approximately 20 ml of triplicate nutrient agar media was poured into each petri dish at 60°C and solidified overnight. Five percent of the batches of prepared media were placed overnight in the incubator at 37°C to check for sterility. 0.1 milliliter of homogenate of the three series (10^{-1}-10^{-3}) of dilution was pipetted, spread, plated, and incubated at different temperature levels and lengths of time based on the type of microbe intended to be identified. Subsequently, a strain of microbe found in a traditionally fermented Datta/Qochqocha was isolated based on its morphological presentation and biochemical tests accordingly. Colonies in an appropriate dilution (between 30 and 300) were counted using a colony counter machine (Scan® 300, India) and reported as a mean colony-forming unit per gram (CFU/g) of Datta/Qochqocha (**Ketema, 2014; Rahman *et al*., 2021**).

Lactic acid bacteria characterization procedure

The sample was aseptically applied to MRS (DeMan Rogosa and Sharpe) agar using the pour plate method. The plates were incubated using a GasPak system under an anaerobic jar at 37°C for 48 hours (**Imane & Amel, 2018**). If there was any growth of white, smooth, raised, and circular colonies (**Abdullah *et al*., 2021**), the colonies were tested for lactic acid bacteria using catalase and indole tests (**Hendrati, Kusharyati, Ryandini, & Oedjijono, 2017; Ketema, 2014**).

Total aerobic mesophilic characterization procedure

The sample was spread-plated using the standard plate count agar (PCA) medium and incubated at 37°C for 48 hours (**Degaga, Sebsibe, Belete, & Asmamaw, 2022; Ketema, 2014**).

Total coliform characterization procedure

The sample was spread on violet-red bile agar (VRBA) and incubated at 35°C for 24 hours. The rod-shaped, red, or pink colonies surrounded by precipitated bile were planned to be counted as coliforms (**Degaga *et al*., 2022; Rahman *et al*., 2021**).

Enterobacteriaceae characterization procedure

The samples were spread-plated on MacConkey agar and incubated at 32°C for 24 hours. The rod-shaped purple/pink colonies were identified as members of Enterobacteriaceae (**Degaga *et al*., 2022; Ketema, 2014**).

Staphylococci characterization procedure

The mannitol salt agar (MSA) was plated with a series of dilutions of the homogenized Datta/Qochqocha and incubated at 32°C for 36 hours. Golden yellow colonies were aseptically purified (**Degaga *et al*., 2022; Ketema, 2014**). Then, biochemical identifications were done using the catalase and indole tests (**Bano *et al*., 2020**).

Salmonella Species Characterization Procedure

After incubation of suspension (1 g in 10 ml of buffered peptone water) of Datta/Qochqocha for 24 hours at 37°C, 1 ml of the culture was transferred to separate tubes, each containing 10 ml of selenite cysteine broth in triplicate, and incubated for 24 hours at 37°C. Then, the samples were streaked onto xylose lysine desoxycholate agar medium and incubated at 37°C for 24 hours. Pink colonies with or without a black center from the selected medium were planned to be purified and tested biochemically (catalase and indole) (**Ketema, 2014; Kohli *et al*., 2018; Sohana *et al*., 2019**).

Yeast and mold characterization procedure

The quantification of yeasts and molds was performed via direct plate counting on potato dextrose agar (PDA). 0.1 ml of Datta/Qochqocha homogenate prepared in triplicate of 10^{-1} dilutions was spread plated on PDA and incubated at 28°C for 3-5 days. Smooth colonies without an extension at the margin were considered and counted as yeasts. Hairy colonies were classified as molds. Yeast and mold colonies in an appropriate dilution were counted using a colony counter machine. Then reported as a mean colony-forming unit per gram (CFU/g) (**Degaga *et al*., 2022**).

Sample preparation for mycotoxin analysis (total aflatoxin)

Twenty-five grams of Datta/Qochqocha and five grams of salt were dissolved in 250 ml of 60% HPLC standard MeOH. The dissolved sample was blended using a juice blender at medium revolution for 10 minutes and filtered using Whatman filter paper. Three milliliters of filtered fluid sample had been diluted in 6 ml of deionized water. Three milliliters of the diluted sample were re-filtered using glass fiber filter paper and passed to the VICAM water column at a flow rate of 2 ml/minute. Then 3 ml of air was injected into the column (**Hafez *et al*., 2021**).Then, one ml of MeOH was passed to the column with a flow rate of one drop per second. After 3 ml of air was passed to the column, 2 ml of deionized water was mixed well. The elution had been filtered using a 0.45 µm Whatman filter membrane. Then, 2 ml of elution had been collected using the elution collection flask for analysis via Agilent 1260 HPLC. To identify mycotoxin (aflatoxins: B1, B2, G1, and G2) antigen concentrations, 20 microliters of the elution were injected into HPLC, and then the result was interpreted using peak area and height. The HPLC data were analyzed in comparison to a standard curve (**Hafez *et al*., 2021**) .

Sample preparation for pH level determination

Five grams of Datta/Qochqocha was blended with 20 ml of distilled water in a conical flask to create a homogenate and transferred to a beaker to make it ready for the test. The pH level of the Datta/Qochqocha was measured using a pH meter (pH-206043, Taiwan) calibration with pH4 and pH7 standard buffer solutions. Then the electrodes were washed with distilled water, dried, and placed in the homogenized Datta/Qochqocha (**Kitessa *et al*., 2022; Medalcho, Nigusse, & Banerjee, 2021**). The pH measurement result was reported on average.

Data quality control

A standard Datta/Qochqocha sample had been prepared using a clean glove. A calibrated digital weight scale was used to measure each nutrient agar and Datta/Qochqocha sample. The materials were cleaned, dried, and autoclaved. The culture media were prepared aseptically in triplicate. Five percent of each batch of the nutrient agar media was incubated at 37°C overnight to verify sterility before use. Plate pouring and inoculation were done in level II biological safety cabinets to prevent contamination. The incubation period and temperature were followed strictly using a temperature recording logbook.

Data analysis

Conventional laboratory analyses were done to ascertain the microbial composition of Datta/Qochqocha from their point of view. A descriptive analysis was performed using Excel software to quantify the means and standard deviations of the microbial load of Datta/Qochqocha.

Ethical consideration

The Addis Ababa University's College of Natural and Computational Science Institutional Ethical Review Board granted the necessary ethical approval (Ref. No: CNCSDO/514/15/2023). The participants were then told of the study's objective, the significance of their involvement, no known potential hazards, complete confidentiality, the right to deny selling the Datta/Qochqocha sample, and the need for their verbal informed consent before sample collection. The participants were asked if they had any questions before obtaining consent and then confirmed their agreement by stating they agreed to participate in the study.

RESULT

Microbial characteristics of traditionally fermented Datta/Qochqocha

The study revealed that lactic acid bacteria, total aerobic bacteria, and yeast grew in all 47 (100%) samples of a traditionally fermented Datta/Qochqocha. In contrast, mold and aflatoxin were present in 10.64% and 8.51% of the samples, respectively. Staphylococci, Enterobacteriaceae, total coliform, mold, and aflatoxin did not grow on the standard sample plated plate. Salmonella didn't grow in all Datta/Qochqocha sample plates "Table 1". The mean pH level of Datta/Qochqocha was 3.82 ± 0.08. *No significant variation of values observed between the study areas.*

Table 1 Microbial characteristics of traditionally fermented Datta/Qochqocha in Ethiopia, 2023 (n=47).

Identified Microorganisms in Datta/Qochqocha	N (%)	CFU/g of Datta/Qochqocha in Mean ± Standard Deviation
Lactic acid bacteria	47 (100)	$1.02*10^6 \pm 0.12*10^6$
Total aerobic mesophilic	47 (100)	$8.5 \times 10^4 \pm 0.51*10^4$
Salmonella	Not detected	Not detected
Total coliform	43 (91.49)	Under reportable
Enterobacteriaceae	43 (91.49)	Under reportable
Staphylococci	43 (91.49)	$3.2*10^5 \pm 0.08*10^5$
Yeast	47 (100)	$1.3*10^6 \pm 0.19*10^6$
Mold	5 (10.64)	$6.0*10^5 \pm 0.22*10^5$

*Under reportable: less than 30 colonies of bacteria grown per petri dish

Lactic acid bacteria characterization

White, smooth, and raised colonies were grown on DeMan Rogosa and Sharpe (MRS) agar medium. The catalase test showed weak bubble formation, while the indole test resulted in a yellowish-colored ring formation.

Total aerobic mesophilic count

Cloudy colonies exhibiting diverse morphologies were cultivated on standard and wild sample plates using plate count agar (PCA) medium.

Total coliform count

The study identified under-reportable red colonies on violet red bile agar (VRBA); however, colonies didn't grow on the standard sample spread plates.

Enterobacteriaceae count

The study identified under-reportable pink colonies on MacConkey agar plates; however, these colonies did not grow on the standard sample spread plates.

Staphylococci count

Golden yellow color colonies with golden and pink mediums have been identified using mannitol salt agar plates of homogenized Datta/Qochqocha samples. The biochemical tests were positive for catalase (bubble formation) and negative for the indole test (cloudy ring formed). But it wasn't found in the standard samples plated.

Yeast counts

White smooth colonies (yeasts) were grown in all plates of potato dextrose agar, including the standard sample spread plates.

Mold counts

It was identified that hairy colonies (mold) with extension at the periphery had been identified to grow among 5 (10.64%) samples on plates of potato dextrose agar (PDA). However, it was not grown in standard sample spread plates.

Mycotoxin species (total aflatoxin)

The study found that both traditionally fermented Datta/Qochqocha were free from pathogenic levels of aflatoxins (B1, B2, G1, and G2). The probability of aflatoxin presence in the samples was 4 out of 47 (8.5%). The aflatoxin concentration level was below the normal cutoff for aflatoxins in food, with a total peak area of 2.640215 and a total height of 1.96807e-1 on average, corresponding to a concentration of 1.345 micrograms per kilogram (µg/kg).

DISCUSSION

The study characterized different microbial types in traditionally fermented Datta/Qochqocha through culturing and biochemical tests to ensure its health and safety. A traditionally fermented Datta/Qochqocha contains lactic acid ($1.02 * 10^6$ CFU/g), total aerobic mesophilic ($8.5 * 10^4$ CFU/g), staphylococcus bacteria ($3.2 * 10^5$ CFU/g), yeast ($1.3 * 10^6$ CFU/g), and mold ($6.0* 10^5$ CFU/g). The prevalence of mold and the lower pathogenic level of aflatoxin (1.345 micrograms/kg) in Datta/Qochqocha were 5 (10.64%) and 4 (8.5%), respectively. The pH level of Datta/Qochqocha was 3.82 ± 0.08 on its 7th day of fermentation.

The occurrence of lactic acid bacteria and yeast in Datta/Qochqocha can be attributed to the effects of natural fermentation (**Mora-Villalobos *et al*., 2020**). This finding is supported by a study that identified lactic acid bacteria and yeasts as the predominant microorganisms involved in the fermentation of traditional foods (**Fikadu**, **2021**). This could be due to an association of fermentation processes with either yeast, lactic acid bacteria, or a combination of both (**Faria-Oliveira *et al*., 2015**).

Staphylococcus bacteria were successfully cultivated in fermented Datta/Qochqoch samples but not in standard samples. This observation aligns with research suggesting that fermented foods can harbor Staphylococcus (**Skowron *et al*., 2022**). The presence of these bacteria may be attributed to skin contact and inadequate hygiene practices (**Oyedeji *et al*., 2023**).

Under reportable numbers of colonies of Coliform and Enterobacteriaceae, and no Salmonella were observed to grow on fermented Datta/Qochqocha. This could be due to the antimicrobial effect of lactic acid bacteria and yeast on pathogenic and spoilage microorganisms (**Amenu & Bacha, 2024; Ibrahim *et al*., 2021; Ma, Wu, Qin, Dong, & Li, 2023; Mgomi, Yang, Cheng, & Yang, 2023; Siddiqui *et al*., 2023**).

The study revealed the presence of mold in a few samples of Datta/Qochqoch, while the standard sample showed no signs of mold growth. This might be due to skin contact and poor hygiene (**Oyedeji *et al*., 2023**).

Datta/Qochqocha contains minimal aflatoxin levels, which are below the limits set by the European Union (**Regulation No. 165/2010 of 26 February 2010**) and Ethiopian standards (**ES 6687:2021**). Furthermore, existing literature suggests that fermentation processes involving lactic acid bacteria can prevent or significantly lower the toxicity of bacterial, fungal, and mycotoxin contaminants (**Es, Raeisi, & Fakhri, 2018; Opoku, Hudu, & K. Mahunu, 2021; Wacoo *et al*., 2019**). It also might be because there is little chance that fresh fruit and vegetables can contain aflatoxin (**Oyedeji *et al*., 2023**). The aflatoxin content of Datta/Qochqocha is lower than the content of maize, which was 2.79 ± 0.17 ng/g. This might be because there is less probability that fresh fruit and vegetables can contain aflatoxin (**Oyedeji *et al*., 2023**).

Datta/Qochqocha contains a lower lactic acid bacterial load compared to milk and milk products, which range from 1.12×10^7 to 2.75×10^9 CFU/g (**Taye, Degu, Fesseha, & Mathewos, 2021**). The lactic acid bacteria load of Datta/Qochqocha is consistent with the lactic acid bacteria load of yogurt and milk, ranging from 6.1×10^5 to 5.4×10^8 CFU/mL (**M'hamed *et al*., 2022**).

The yeast load of Datta/Qochqocha is in line with the yeast load of yogurt and cheese, which ranges from 10^6 to 10^7. It was also similar to the yeast load of indigenous fermented foods and beverages of Ethiopia, which ranges from 1.4×10^2 to 3.74×10^6 CFU/g or milliliter (**Dabassa, Han, Bacha, & Bai, 2020**). However, the yeast load of Datta/Qochqocha is higher than that of Awaze, which was 2.11×10^2 CFU/g (**Dabassa *et al*., 2020**).

The total aerobic mesophilic load of Datta/Qochqocha is similar to a study's finding, which ranges between $1.63x10^1$ to $3.6x10^1$ (CFU/g) in different types of food (vegetables, meat, and others) (**Al-Busaidi, Al-Bulushi, & Al Subhi, 2023**). The total aerobic mesophilic load of Datta/Qochqocha is within the lower border average standard of aerobic mesophilic load of foods, which is less than 10^4 CFU/g (**Degaga *et al*., 2022**).

A total staphylococci load of Datta/Qochqocha is similar to a study's findings, which ranged between $5.48x10^1$ to $6.72x10^1$ CFU/g in different types of food (vegetables, meat, and others) (**Al-Busaidi *et al*., 2023**). The staphylococci load of Datta/Qochqocha is within the safe range of the staphylococci load of food, which is less than 6 log CFU/g (**Degaga *et al*., 2022**).

A salmonella, coliform and Enterobacteriaceae load of Datta/Qochqocha's aligns with the microbial safety standard recommendation, which indicate no Salmonella, less than 10^4 CFU/g for total coliform, and Enterobacteriaceae (**Firehiwot *et al*., 2024**).

The pH level of Datta/Qochqocha is similar to the pH level of fermented red cabbage (3.76 ± 11) (**Vatansever, Vegi, Garden-Robinson, & Hall, 2017**). However, it is lower than the pH level of fermented red tamarillo (3.94 ± 0.06) (Cao *et al*., 2024), carrot (3.99 ± 0.04), and daikon radish (4.17 ± 0.05) (**Vatansever *et al*., 2017**). These might be due to temperature and ingredient's pH level differences.

Datta/Qochqocha could help improve gut health, absorb nutrients better, boost the immune system against diseases, reduce harmful microbes, control blood sugar, and support mental health because due to it's contain of Lactic acid bacteria and Yeast. This idea is supported by studies' findings (**Agarbati *et al*., 2024; El-Raghi, El-Mezayen, & Areda, 2024; Rehamnia, Lee, Kuktaite, & Kacem Chaouche, 2022**).

The fermented Datta/Qochqocha may not be perished easily because of its lactic acid bacteria and yeast effect. This finding is supported by various studies findings (**He, Degraeve, & Oulahal, 2024; Kruk, Lalowski, Hoffmann, Trząskowska,**

& Jaworska, 2024).

STRENGTHS AND LIMITATIONS

It would have been better if the microbial characterization of traditionally fermented Datta/Qochqocha had been done using genomic analysis. This is the first study to characterize the microbial content of traditionally fermented Datta/Qochqocha.

CONCLUSION

Datta/Qochqocha is a source of lactic acid bacteria and yeast. It is free from pathogenic levels of pathogenic bacteria, mold, and aflatoxins. Datta/Qochqocha has a good effect on nutrient absorption, boosting immunity, increasing nutrient content, infection prevention, gut health, and antioxidant effect because of its lactic acid content (**Ayivi *et al*., 2020; Coelho, Malcata, & Silva, 2022; Mathur, Beresford, & Cotter, 2020**).
Strengthen adherence to maintaining hygiene on Datta/Qochqocha preparation. Community health education to strengthen Datta/Qochqocha use for its lactic acid bacteria, which has disease prevention and treatment-enhancing effects (**Mathur *et al*., 2020**).

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