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Studying the Single colony Penicillium, Aspergillus, and Fulvous fungi growth in different types of packed and unpacked Cacao

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ABSTRACT

Ochratoxin A is a species of toxic secondary metabolites or mycotoxins that have been responsible for many epidemics in the human communities and livestock, especially in recent times. Ochratoxin A is produced by toxic species, Aspergillus and Penicillium and the four main types of poisoning include acute toxicity, chronic toxicity, feature of mutagenic and malformations. Its major acute ill-effects are leaving on the liver and kidneys that can cause liver cancer, chronic hepatitis and cirrhosis of the liver and kidney. In this study, 70 samples of coffee and cocoa powder of Iranian and foreign companies, and 10 samples of Dutch opened cocoa powder, in general a total of 80 samples, were prepared from the market of Iran and cultured, in order to examine two species fungi, Aspergillus and Penicillium which produce toxigenic Akhratvksyn, and if growth of fungal colonies was positive, DNA would be extracted from fungi produced, and analyzed by PCR method to measuring toxin-producing level, then Ochratoxin A levels were tested by ELISA method and the data was analyzed by SPSS, Curve. Not growing fungi in 70 samples of Iranian and foreign companies, Penicillium and Aspergillus species were regarded growing in the Netherlands opened 10 Cocoa and DNA extraction from fungi was obtained properly. Measuring Ochratoxin in ELISA method, contamination of 80 samples would report much higher than standard. Mean concentration of Ochratoxin A in samples of coffee and cocoa on the market of Iran is ranged from120-10 ppb. By virtue of high contaminated products to Ochratoxin A toxin and also frequently consumption of Coffee and Cocoa powder in various food industries of Iran, and harmful effects cited on human health and prevailing dangerous diseases in adults and the impact of this on babies in utero, this information is given to health authorities, national institute standard and individuals in community to raise sensitivity toward and control for and standard level and quality of foods produced by these products, and one side, to prevent the build-up of toxins in the body of the different individuals of community, producing dangerous effects of cancer.

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INTRODUCTION

Ochratoxins are produced by three species of A.Ochraceus, A.niger, A.flavus, two species of Penicillium verrucosum, Penicillium Nordicum in coffee and cocoa. These two fungal species produce Ochratoxin A toxin in these products. This belongs to fungal secondary metabolites and contaminates living and dead plants' and animals' organs. So it is possibility of being infected by these fungi and their toxins in field crops. Study showed that after harvesting, the use of appropriate methods of processing, drying and preserving in storage required to reduce contamination and prevention of its spread. [12] Cereals and food packaging in good condition is important because the lack of ventilation and humidity and lack of proper treatment can be factors by which the food is infected by four species of fungi.

Ochratoxin A first in South Africa was isolated from Aspergillus Ochraceus [5]. Ochratoxin A is produced in food in tropical and subtropical areas such as cereals, grains, oats, wheat, coffee, cocoa, corn, grapes. The amount of toxins is reported in grains and cereals daily average between 50 to 80 percent and in coffee between 0.360-0.2 ug/kg. In East African countries, about 71% of cocoa products and its derivatives used in food and medicine are infected Ochratoxin A. Seasonal changes, farming and health conditions for plant and harvesting and transport coffee and cocoa would produce Ochratoxin A. In 2004, FAO/WHO has stated maximum permissible Ochratoxin A in Coffee and Cocoa to be 10-5 ug/kg. Maximum Limits of toxin in food, according

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manifesto of Europe Union, are allowed by a range between 0.05ug/kg and 5ug/kg in baby food, 5ug/kg in cereals, 3ug/kg in other products and 300-5 ug/kg in feedstuff [18]. Consuming 200 ug/kg toxins in food over the three months, it leads to brain, neurological, cardiovascular, kidney, liver and lung damage in humans. This toxin is a fat-soluble substance that accumulates in the fatty tissue of the body and is a strong Nefrotoxin [12]. The present study was designed to evaluate the quality of the coffee and cocoa powder in the Iranian market, by virtue of contamination to Ochratoxin A.

An overview of the research conducted:

To check toxin Ochratoxin A in coffee and cocoa products on the market in Iran by PCR method and test ELISA on the amount of toxin in the food, there have not been great records. In a study conducted in Tehran-Iran, it investigated Ochratoxin A levels in cereals by HPLC method. In this study, 18 samples were collected randomly in Tehran, and the results showed that the total amount of toxin was zero, given that the allowed limit to Ochratoxin A in cereals is 5 ppb and raisins 10 ppb. Also by regarding evaluation for cereals available in stores in Athens, the results indicated that HPLC test was conducted on 60% of the samples infected by Ochratoxin A. According to another study on breakfast cereals on the French chain-stories, in terms of Ochratoxin A by ELISA method, the results showed that Ochratoxin A in 60% of samples were found and 20% of them higher than allowed limit in Europe. According to the research in Turkey [Ankara] in 2005 on samples collected on coffee and cocoa, rice, spices and pulses, Ochratoxin A toxin have been tested by ELISA method. And, thus, the amount of toxin in the samples was above the 4.07-0.31ppb. In Brazil in March 2007 Ochratoxin A toxin measured by ELISA and HPLC methods and the amount of toxin in the coffee sample is reported 50pg/ml. According to another study in America in 2006 on the Columbia and Hawaii have some type of Arabica and Liberia coffee, after growth of fungi Aspergillus Ochraceus; they were able to isolate the Ochratoxin A toxin in these samples. The research on Albania in April 2011 to December 2011 on a sample of coffee and dried fruits, and beer was able to isolate Ochratoxin A from above samples, after growing the fungi Aspergillus Ochraceus A.

MATERIALS AND METHODS

1 - Culturing the samples:

In this experiment, 80 samples of Coffee and Cocoa were cultured. First, by means of the graduated cylinder, the amount of 5 g of physiology saline was measured and poured into a test tube. Sterile solution was prepared as many sample as cultured. Autoclave door was closed by a strong arm, and then turning on the device, the air valve was opened and the temperature was set to 95 ° C. After this temperature the device once again is closed and the air valve too, the device temperature until reaches 121 ° C. To prepare the culture medium, 65 g of Sobor powder and dextrose agar was measured by a digital scale and distilled or deionized water as much as one liter was measured by graduated cylinder and poured into an Erlenmeyer flask and agarose powder was added and mixed. The Erlenmeyer was flamed for 5 minutes, to dissolve the powder in water. When the temperature of sterilized culture medium was balanced and cooled, to pouring into the plates were prepared. Under the hood uv already cleaned thoroughly with cotton and alcohol and then the door of hood was closed and uv lights were turned on, and during 20 to 30 minutes, it got sterilized under a hood by uv light, any infection, bacterial or fungal spores were taken away. Uv lights turned off, after this time and the hood door was opened and hood vent turned on, to evacuating uv light to be gathered. After 10 minutes air evacuation, under the hood was entirely sterilized and provided for the preparation of culture plates. To culture coffee or cocoa powder for suspension on agarose medium, glass rod that was bent over by the flame was used and cultivation was carried on under the hood and side burner. Glass rod was placed inside the Beaker with alcohol and the lid of each plate took placed besides the flame, and the suspension of coffee or cocoa was taken as much as 500 ul or 100 ul by sampler and left on the culture medium. After the due time and fungal colony growth, fungal CFU was measured and fungus type grown can be determined.

2- DNA extraction method:

In this method, amount of the desired mycelium fungus of culture freshly grown was taken, therefore, a piece had been gotten as by one ounce as much as 0.5 cm of fungi, which were cultured, on the plate Sabordesstrozagar environment from where fresh mycelia grown and pigment not formed yet. Then smithereens of fungi were thrown by an ounce into micro-tubes and the extraction phases were performed.

3 - Method PCR:

In this study, 80 samples, including 10 Farmand Coffee samples and 10 Farmand cocoa samples, and 10 Khajik coffee samples and 10 Khajik cocoa samples packed and prepared by Iranian companies, 10 Starbucks Coffee samples, 10 Lavazza Coffee samples and Kruger Cocoa 10 samples produced and packed by foreign companies in Colombia, Italy and Germany and 10 open-coffee and open-cocoa samples collected randomly

from the Iranian market, after planting all 80 samples, growth of the fungus Penicillium and Aspergillus [flavus and niger] was observed only for 10 samples of cocoa collected in form of bulk from market of Iran, having extracted DNA of 10 fungi samples, it was examined whether fungal species were Toxigenic by PCR method.

3-1- Identifying Primer:

To examine and design, in this study, front primers [Forward] and back ones [Reverse] for using PCR method, mentioned articles were applied, and to create these primers, Sina Gen Company was helped.

To design the primers Penicillium verrucosum and Nordicum, study conducted in 2005 by Caroline Bogs *et al*. was helped [8].

To design the primers Aspergillus flavus, study conducted in 2003by Maria Helena Fungaro *et al* was helped [30].

To design the primers Aspergillus niger, study conducted in 2005 by Amaia Gonzalez-Salgado *et al*. was helped [30-42].

Table 1: Sequences of primers used in this study.

Fungus	Primer	Sequence
Penicillium Verrucosum	Otanps-for	5'GTCTTCGCTGGGTGCTTCC3'
	Otanps-rev	5'CAGCACTTTTCCCTCCATCTATCC3'
Penicillium Nordicum	Otapks-for	5'TACGGCCATCTTGAGCAACGGCACTGCC3'
	Otapks-rev	5'ATGCCTTTCTGGGTCCAGTA3'
Aspergillus Flavus	OPX7F 809	5'GGCTAATGTTGATAACGGATGAT3'
	OPX7R 809	5'GCTGTCAGTATTGGACCTTAGAG3'
Aspergillus niger	ITS-1	5'TCCGTAGGTGAACCTGCGG3'
	NIG	5'CCGGAGAGAGGGGACGGC3'

3-2- Dilution of the primers:

To dilute primer Liufelizeh, according to the following instructions for the preparation of the parent stock, it was added to each vial certain amount of distilled, deionized water.

Table 2: volume of primers dilution.

Oligo Name	Ml for 100 ml
Otanps-for	397 ml
Otanps-rev	379 ml
Otapks-for	310 ml
Otapks-rev	353 ml
OPX7F 809	322 ml
OPX7r 809	402 ml
ITS-1	206 ml
NIG	245 ml

So, to prepare working stoke, we reached 10ul of mother stoke with distilled deionized water to volume 100 ul.

3-3 - Polymerase chain reaction PCR:

For the PCR method, a proprietary district of single-stranded DNA [ss DNA] is propagated, using Neokleotidtery phosphates, DNA-Polymerase enzymes called Taq polymerase.

The method in this study [2x] PCR Master Mix was used which is produced Sina Gen Company, including for the following components:

Table 3: Components [2x] PCR Master Mix.

Taq DNA Polymerase	0/08 Unit/ml
Mgclz	3 mM
dATP	0/4 mM
dCTP	0/4 mM
dGTP	0/4 mM
dTTP	0/4 mM

To perform the reaction PCR, the total volume of reactants was considered 25 ul, shown in a table.

Table 4: volume of reactants PCR.

PCR Master Mix[2x]	12/5ul
Forward Primer[10 Mm]	1 ul
Reverse Primer[10 ml]	1 ul
Template [DNA]	4 ul
d H2o	6/5 ul

Thermal Program in Thermo-cycle device was as follows, to reproduce specific sequences in this study:

Table 5: Thermal Program in Thermo-cycle device in the reaction PCR.

Step	Temperature	Time	Number of cycle
First denaturation	95 ℃	5 min	1
Denaturation	95 ℃	30 sec	35
Annealing	60-65 ℃	50 sec	
Extension	72 ℃	1 min	1
Final extension	72 ℃	10 min	1

4- Enzyme Linked Immunosorbant Assay:

In this method, antigen is pasted and fixed along with antibody on the solid surfaces such as poly Stern of plastic verities or microniter plates. Firstly, the antigen often is bound to the solid phase and then the antibody is added which is studied. After washing antibody binding to the antigen, adding the marked antibody, directly against the primary immunoglobulin, will be clear. The mark may be a radioisotope or an enzyme. Today, due to financial problems and limitations, enzyme marks have been replaced radioisotopes. To perform this test, 80 Coffee and Cocoa samples were evaluated.

Results:

1 - Results of culturing the samples: in culture phase, 80 Iranian and foreign Coffee and Cocoa powders was carried out in two parts as a suspension culture 100λ for each sample in two plates, and a suspension culture 500λ for each sample in two another plates and by putting plates within incubator for 3 to 5 days at [room] temperature 30^{o}C. Single-colony fungal growth was observed only for 10 Dutch Cocoa samples purchased from the market openly, that the results are presented for CFU of fungi and mean values in Table [1]. Table [1] distribution of CFU values in cultured fungi.

Table 6: research Result.

Number / Fungus	The number of cut fungi	Mean	S.D	Minimum number	Maximum number
Penicillium	7	18.57	9	10	30
Aspergillus Flavus	2	15	7.1	10	20
Aspergillus niger	1	20	0	0	20

CFU of fungi is obtained as calculating the number of fungi in each colony by a formula One gram of powder was mixed in saline solution at a concentration 0.5 cc of cultured suspension that how fungi are grown that in any single colonies, there have been 10 fungal fruits.

There have existed, for Cocoa powder sample in number 71, two Penicillium colonies equivalent to 20 fungi in Cocoa powder sample, in number 72, one Penicillium colony equivalent to 10 fungi, in number 73, three Penicillium colonies equivalent to 30 fungi, in number 74, 2 Penicillium colonies equivalent to 20 fungi, in number 75, one Penicillium colony equivalent to 10 fungi, in number 76, one Aspergillus flavus colony equivalent to 20 fungi, in number 77, one Aspergillus niger colony equivalent to 10 fungi, in number 78, 2 Penicillium colonies equivalent to 20 fungi.

1-2- Results of PCR:

1-2-1- The results of fungal DNA extraction: DNA extraction of fungi was successful and every 10 fungi with DNA extracted, formed 10 visible bands on SDS gels. DNA was maintained inside the freezer at a temperature of - 21 ° C. 1-2-2- Performing PCR: having performed PCR on DNA, there had been 2 Aspergillus niger fungi and one Aspergillus flavus and 7 Penicillium species and the answer was negative in all 10 cases that there was not found any bands based on being positive due to existence of Ochraceus A gene, in ranges under investigation for each fungus species.

3-1- ELISA results:

In examining amount of Ochraceus A concentration in 80 coffee and cocoa powder samples ELISA kit and then reading an OD of each sample by reader- spectrophotometry device and comparing the OD of the samples with zero-standard OD ppb, 2ppb, 5ppb, 20ppb and 40ppb, the results were analyzed by the CURVE software. And a chart was plotted and analyzed using SPSS software and another chart and table was plotted. Table 6 shows optical or OD of reading standards and 80 samples.

Table 6: OD of reading standards and 80 of coffee and cocoa powder samples.

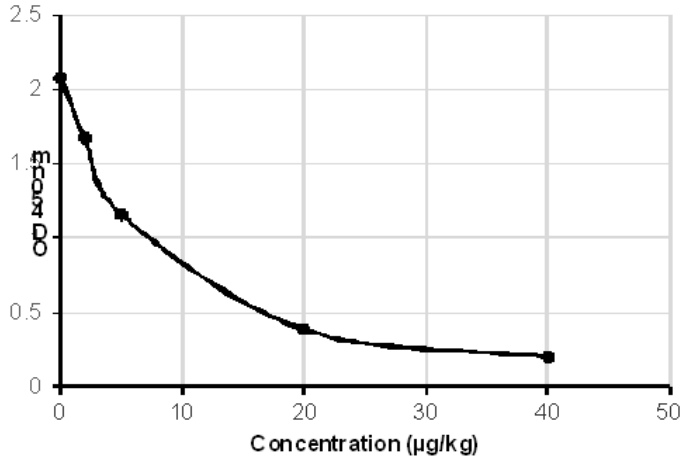
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.702	0.797	2.141	0.755	1.236	2.067	0.932	0.689	2.063	0.297	1.512	0.739
B	1.584	0.965	1.29	0.782	1.167	1.667	0.742	0.968	1.689	0.255	1.427	0.787
C	0.965	0.914	1.602	1.088	1.188	1.145	0.73	0.524	1.175	0.4	1.439	0.357
D	0.303	0.716	1.451	1.551	1.166	0.398	0.508	0.478	0.377	0.544	1.386	1.24
E	0.13	0.585	1.482	1.244	0.843	0.206	0.308	0.728	0.197	1.551	1.403	1.051
F	0.848	0.728	1.507	1.236	0.843	0.843	0.627	0.242	0.313	1.487	1.449	0.952
G	1.114	0.347	1.427	1.231	0.867	0.841	0.646	0.447	0.26	1.384	1.167	1.029
H	0.738	0.819	1.385	1.244	0.941	0.861	1.006	0.456	0.456	1.483	0.71	1.065

By CURVE software, existing Ochraceutoxin A concentration is obtained in each sample by placing the OD which readings as the Y-axis.

Table 7: Ochraceutoxin A concentration present in the 80 cocoa and coffee powder samples.

company / The number of samples	Iranian Farmand Coffee	Iranian Farmand cocoa	Iranian Khajic coffee	Iranian Khajic cocoa	Columbia starbucks coffee	Italy Lavazza coffee	Germany Kruger cocoa	Dutch open cocoa
1	16.1	10.9	30.3	26	72.8	48	42	107.55
2	17.25	14.25	28.2	22.5	75.2	46.3	34.5	153.5
3	17.25	13.55	30.45	22.3	82.7	48.4	32.55	130.1
4	15.9	12.3	28.8	34.5	52.1	49	42	150.2
5	17.2	14.1	30.75	22.65	58.1	50.55	40.84	123.1
6	16.65	14.25	31.15	23.3	60.05	42	36.8	162.5
7	21.9	13.4	29.66	24.9	70	42.15	36.5	125.2
8	22.15	15.25	29	27.7	78.85	45.94	35.4	84
9	22.15	15.2	31.35	25.15	80.05	43.5	40.4	95.85
10	20.45	15.55	28.2	25.15	53.95	49.66	36	84

Also a chart was plotted by CURVE software that standard concentration on the x-axis and OD on y-axis.



80 samples were analyzed and the results were drawn in tables and charts. Table 8 shows pairwise comparisons between samples and the results.

Table 8: pairwise comparison of the samples and representation of the mean and standard deviation and P_v

Comparison of two groups	Type of samples	Mean	S.D	T-test
Comparison between 40 foreign and Iranian cocoa and coffee	Total coffee	40.85	19.84	- $P_v > 0.05$
	Total cocoa	49.40	45.23	
Comparison between 40 foreign companies and 40 Iranian companies	All Iranian	21.7	6.3	$P_v < 0.05$
	All foreigners	68.55	36.21	
Comparison between 10 Iranian Farmand coffee samples and 10 Iranian Farmand cocoa samples	Farmand coffee	18.7	2.36	$P_v > 0.05$
	Farmand cocoa	13.88	1.43	
Comparison between 10 Iranian Khajic coffee samples and 10 Iranian Khajic cocoa samples	Khajic coffee	29.79	1.18	$P_v > 0.05$
	Khajic	24.42	1.74	
Comparison between 10 foreign Starbucks coffee samples and 10 foreign Kruger cocoa samples	Columbia Starbucks coffee	68.38	11.39	$P_v < 0.05$
	Germany Kruger cocoa	37.7	3.36	

Chart 1 was plotted based on the average of 10 Iranian cocoa and coffee samples and 10 foreign cocoa and coffee samples and in general, comparing the average of 80 samples.

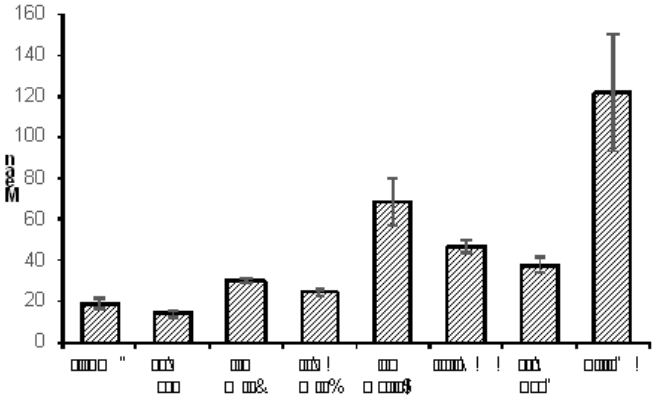


Chart 1: Comparison between Iranian mean samples and foreign ones.

Discussion:

Considering the results in the cultivation section of 80 Iranian and foreign coffee and cocoa in accordance with table 1 , since the fungal species of Penicillium and Aspergillus have fast growing within 3 to 5 days of incubation temperature and formed fungal colonies, and in outcomes of cultivating the 10 Farmand coffee samples, 10 Farmand cocoa samples, 10 Khajic coffee samples and 10 Khajic cocoa samples, the productions of 40 Iranian companies, and cultivating 10 Starbucks Colombia coffee samples, 10 Lavazza Italian coffee samples, 10 Kruger German cocoa samples and 10 Dutch open-cocoa, the productions of 40 foreign companies, it observed that single-colony fungi of Penicillium and Aspergillus and flavus were grown just in 10 Dutch open-cocoa and in 70 rest samples, fungal growth was not observed. This is my conclusion that the lack of stability of two fungal species at temperature higher than 85^{o}C and since, at least, a temperature is necessary higher than 100^{o}C to extract and evaporate water into the intra-tissues of green coffee beans and cocoa beans, during roasting and cooking them for curing, so in this temperature, definitely, cellular components and textures of fungi growing inside the green beans of them are removed from the products, then it is justifiable and accords with the results, not being any fungus colonies in 70 packing samples of Iranian and foreign; and since fungal growth was observed in 10 samples of Dutch open-cocoa, I have concluded that there are a lot of spores of the two fungal species in air and water, and in all areas, including in vitro, so mold growth of 10 samples can be explained by saying that this species belongs certainly to the peripheral and secondary fungal species that have been prepared as an open space around the cocoa powder in market of Iran. Harvested green coffee beans from plantations, due to the humidity and high temperatures, may be contaminated with above fungi were Toxinzay and when making hot 200^{o}C to cook, toxin does not remove but toxin-producing fungi are destroyed.

Given that in the PCR, the DNA of 10 Dutch open cocoa samples were extracted, but during there were not found any bands in performing PCR process, based on recording gene-specific-producing toxin, Ochraceutoxin A, in two species of Penicillium and Aspergillus in specific range of each them, it can verify the results of a samples' culture which in 70 samples of Iranian and foreign, primary fungi producing Ochraceutoxin A toxin have destroyed and removed, in virtue of high temperature, existing fungi of 10 open samples belong to environmental secondary fungi samples, they are not agents producing the toxin.

In the ELISA, in terms of table, based on high contaminant to Ochraceutoxin A toxin for 80 the cocoa powder and coffee samples of Iran and foreign, it is over than standard and normal is determined 5 µg/kg. I concluded that with respect to the spatial and chemical structure of the toxin and Chloride ion make toxin resistant against heat even higher than 200^{o}C. Obtained results determined from measuring Ochraceutoxin A toxin in 10 Farmand coffee samples with mean 18.7 and in 10 samples of Farmand cocoa with 13.8 µg/kg, 10 Khajic coffee samples with 29.79 µg/kg, 10 Khajic cocoa with 24.4^{2} µg/kg, 10 Starbucks coffee with 68.38 µg/kg an 10 Dutch open cocoa samples with 121.6. This type of toxin is resisted to temperature higher than 100^{o} C during roasting and even higher than 200^{o}C, the high toxicity of the samples by Ochraceutoxin A can be explained.

Suggestions:

According to the results of high contamination of samples by Ochraceutoxin A in study conducted, thus it must inform the public individuals and Health Organization and National Standard Organization towards the

widespread use of these products as a powder in the food and pharmaceutical and industrial use for companies and the domestic use and in producing various types of chocolate and sweets and cakes and also the use coffee and cocoa powder for drinking, that has a lot of popularity in recent years, and also referring to toxic impacts and dangerous side effects of the toxin in the body and causing a variety of cardiovascular and renal and hepatic, finally, in the long-term use can lead to cancer, in order to make high sensitivity for controlling and monitoring existence of the Ochraceutoxin A toxin within these industrial and food products, which mostly are foreign and imported from foreign countries into Iran and Iranian and foreign packaging and delivery of bulk and open them wide and frequent use by the general public, because the organizations monitor further and people need to be aware and avoid the widespread use of these toxic products purchased and their physical health not to endangered by consumption of these foods.

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