IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 10, Issue 10 Ver. IV (October 2017), PP 31-38
www.iosrjournals.org

Microbiological Evaluation of Fruits of the Prickly Pear (*Opuntia Ficus Indica* L. Miller) Collected In Greece

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Abstract: *Cactus species have a worldwide expansion and are known since antiquity for a wide range of uses and properties. It has been used as a nutrient as well as a medicine in different cultures. In Greece Opuntia species grow almost everywhere in arid, semiarid and rocky areas following the pattern of wild plantation. The fruit from the species Opuntia ficus indica L. Miller is known as cactus pear and because of its delicious flavor is considered as a gift of nature. The aim of the present study is an attempt to assess the microbiology of the cactus pear fruit, collected from semi-arid and rocky areas in Greece. To this purpose 120 cactus pear fruits have been collected from the region of Epirus, in Northwestern Greece. The fruits were divided into two groups. The first group's fruits (n=60) were analyzed as whole entities (flesh - pulps and peel) while from the second group's fruits (n=60) only the flesh - pulps was analyzed after removal of the peel. All samples were analyzed for Total Aerobic Mesophilic Bacteria counts, S. aureus, coliforms, E. coli (with detection of E. coli O157:H7), Salmonella spp., Listeria monocytogenes, Clostridium perfringens and Lactobacillus spp.*
E. coli, Salmonella spp. and Listeria spp. have not been isolated. Lactobacilli were isolated from 29 samples of the whole fruit group (48.33%) and from all specimens from the only flesh group. These findings are of great interest to their potential probiotic action.

Keywords: *Opuntia ficus indica L. Miller, pathogenic bacteria, Lactobacillus*

Date of Submission: 28-09-2017 Date of acceptance: 06-10-2017

I. Introduction

Cactus pear or prickly pear (*Opuntia ficus indica* L. Miller) is a very common species in the dry and arid Mediterranean ecosystems. It has been observed and described since the era of classical antiquity. As well-known its origin and history are closely related to the ancient Central American civilizations and particularly to the Aztec culture. The confirmation of that comes by archaeological evidence. These plants were first cultivated by indigenous populations who settled in the semi-arid regions of Central America [1 – 5]. The plant has spread further – carried by people who traded and settled – to Africa, Asia, Europe and Australia, where the cultivated and wild plants continue to provide food and materials [6, 7].The prickly pear belongs to the Cactaceae family. Its taxonomy is highly complex; so many authors disagree with the classification of cactus pear in the Cactaceae family [5, 8, 9]. Following the classification proposed by the Germplasm Resources Information Network (GRIN) of the United States Department of Agriculture's Agricultural Research Service, the cactus pear belongs to the genus *Opuntia*. The scientific name *Opuntia* was coined by Tournefort in 1700, in view of their resemblance to spiny plants that grew in the town of Opus in Greece [8, 9]. Once introduced into Spain from Mexico, the species quickly spread throughout the Mediterranean basin. Today the plant, the cactus pear grow wild or are cultivated in southern Spain and around the Mediterranean in France, Greece, Israel, Italy and Turkey [10 – 12].

Opuntia plants are native to many environments, ranging from desert areas below sea level to high altitude areas and can grow to temperatures above 5 ºC or to -40 ºC in winter, meaning that the plant can be a viable option in regions where other plants will not survive (tropical regions of Mexico and areas in Canada additionally) [13]. While in most of the Countries the best plantations are located in coastal areas and more specifically in coastal zones with a width of more than 10 km wide undergoing maritime influence, in Greece the plants of *Opuntia* tend to grow in less fertile soils, non - mountainous and non - coastal areas [10, 14]. Quite literally, the plant is the basis for life for resource-poor people living in these marginal areas. One of the most striking characteristics of *Opuntia* is its anatomy and morphology, which have enabled it to adapt to many extremely stressful growth conditions, meaning that the plant can survive in regions where no other plants survive [15]. The plant has the appearance of cactus bush with green-grey flat rounded leaves (cladodes) and a yellow - orange fruit protected by numerous sharp spines (Picture 1) [16, 17]. Its fruit has a delicious characteristic flavor and is considered as a delicacy provided by nature. Fruits and vegetables are an

extraordinary dietary source of nutrients, micronutrients, vitamins and fiber for humans and are thus vital for health and well being. Usually the flesh of the prickly pear is consumed directly after the removal of the peel while its marmalade is also very popular. In the recent years cactus pear has been cultivated in a more systematic scale not only for the popularity of its fruit but also for the capacity of its leafs to produce after brewing an alcoholic beverage similar to the Mexican tequila. Prickly pear has high fiber content, it is rich in carotenoids and linoleic acid (an essential fatty acid) and contains minerals like calcium, potassium, magnesium and phosphorus, pectins, carotenes, betalains, ascorbic acid, quercetin and quercetin derivatives, the majority of which have antioxidant activity [18 – 20]. In Chinese medicine, cactus fruit is considered a weak poison and used as medicine for treatment of inflammation and pain [20, 21]. It has also been used as a detoxification agent for snake bite [20, 21]. Finally a study has showed that prickly pear cactus (type Arizona) effectively inhibited cell growth in several different immortalized and cancer cell cultures in vitro and suppressed tumor growth in a nude mouse of ovarian cancer model [22].

However, cactus pear fruit, as general all fruits and vegetables, are widely exposed to microbial contamination through contact with soil, dust and water and by handling at harvest or during postharvest processing. They therefore harbor a diverse range of microorganisms including plant and human pathogens [23, 24]. Thus despite their nutritional and health benefits, outbreaks of human infections associated with the consumption of fresh or minimally processed fruits and vegetables have increased in recent years [25, 26]. Pathogenic species such as, *Staphylococcus*, *Clostridium* and especially enteric pathogens such as *E. coli* and *Salmonella* are among the greatest concerns during food-related outbreaks [27, 28].

A few studies have been published concerning the phytochemical properties of the cactus pear fruit but the scientists ignored the microbiology issues. Yet the increased popularity of the fruit and its products (marmalades and juices) poses certain public health issues as well as an increased interest for these microbiological parameters of possible technological application (e.g. *Lactic Acid Bacteria*) The present study is the first attempt to evaluate the microbiology of prickly pear fruits collected from Greece and especially from semi – arid and rocky lands in the Epirus region in Northwestern Greece.

Picture 1: *Opuntia ficus-indica* L.Miller, Cladodes and mature fruits.

II. Material And Methods

IIa. Samples

One hundred and twenty (120) cactus pear fruits were collected from the region of Epirus in north western Greece. The fruits immediately packaged in sterile polythene bags that were sealed and transported to the laboratory in cooler boxes containing ice packs, where they were immediately stored in a refrigerator at 4^0C until analysis. Samples were divided into two groups. The first group's fruits (n=60) were analyzed as whole entities (flesh - pulps and peel) while from the second group's fruits (n=60) only the flesh - pulps was analyzed after removal of the peel (Picture 2).

IIb. Preparation for Microbial Analysis

Before samples were taken out of their transporting sterile bags, the possible contact surfaces were carefully sterilized using polyurethane sponges to prevent cross - contamination. Approximately 50 g from both fruit groups were removed aseptically. The sample was then homogenized in 450 ml peptone saline solution (8.5

g L^{-1} NaCl and 1 g L^{-1} peptone) for 2 min in a stomacher devise and right after was filtered through sterile Whatmann No.1 filter paper to remove the solid particles if any. Appropriate 1:10 dilutions of the resultant homogenate or the rinse fluid were prepared using Ringer's solution.

IIc. Microbiological evaluation of samples

After making serial dilutions, the samples were placed on different media as follows:

(1) Enumeration of Aerobic Total Mesophilic Plate Count.
Determination of the total microorganisms, mesophylic aerobic and facultative anaerobic microorganisms (bacteria) was performed in accordance with the ISO 4833-1:2003 standard [29].

(2) Enumeration of *Staphylococcus aureus.*
The APHA method was used for isolation of *Staphylococcus aureus* including enrichment of a 1 g sample in 10 ml cooked meat medium (Difco) plus 9% NaCl (w/v), streaking a loopful of the 24 h enrichment culture on Baird - Parker agar (Merck) containing egg yolk and potassium tellurite (Merck) and subsequent confirmatory coagulase test of lipase-positive jet-black colonies [30].

(3) Enumeration of *Coliforms* and *Escherichia coli.*
For each selected dilution, 0.1 ml of sample was spread-plated into brilliance *E. coli*/*coliform* agar (Oxoid, Cambridge, UK) and Violet red bile agar (VRBA, Merck). The plates were incubated at 37 °C for 24 h, following which, the number of pink (*coliform*) and purple (presumptive *E. coli*) colonies was counted. Identification of *E. coli* was carried out with IMVIC tests [31].

Picture 2: Removal of the peel from the prickly pear

IId. Isolation of *E. coli* O157:H7

Samples were prepared as described above. Homogenate or the rinse fluid was prepared using Buffered Peptone Water (BPW) (Oxoid, Cambridge, UK). Laboratory analyses to detect of *E. coli* O157:H7 were performed in accordance with the ISO 16654:2001 standard [32].

IIe. Isolation of *Salmonella* spp.

Samples were prepared as described above. Laboratory analyses for the detection of *Salmonella spp*. were performed in accordance with the ISO 6579:2002 standard [33]. 25 g of sample and 225 ml buffered peptone water were homogenized using the Stomacher devise. The analysis depends on the principles of preculturing for 18 - 24 h in Buffered Peptone - Water at 35 - 37^{0}C, then the selective culturing for 24 h in Rappaport–Vassiliadis Soya Reptone Broth at 41.5 ^{0}C for 24 h. The broths were streaked onto Brilliant Lactose Green (BLG) Agar and Xylose Lysine Desoxycholate Agar (XLD agar) incubated at 37^{0}C for 24 h.
Suspected transparent colonies with or without black center on XLD agar and red colonies on BLG agar were subjected to biochemical analyses and serotyped using commercial Kits API20E [34, 35].

IIf. Isolation of *Listeria* spp and *Listeria monocytogenes*

Samples were prepared as described above. Laboratory analyses for the detection of *L. monocytogenes* were performed in accordance with the ISO 11290 method [36]. For lecture, 5 g of sample were homogenized in 225 ml of Half Fraser Broth (Oxoid) and incubated at 37^{0}C for 24 h.

Aliquots of 100 μl of primary enrichments were transferred to 10 ml of Fraser Broth with Ferric Ammonium Citrate (FAC) and incubated again at 37^0C for 24 - 48 h. Aliquots of 0.1 ml of secondary enrichments were plated in duplicate in PALCAM Listeria selective agar (Merck) supplemented with PALCAM Listeria selective supplement (Merck). Suspected colonies were confirmed by Gram staining, motility test (hanging drop), catalase and b-haemolysis tests and sugar fermentation tests for rhamnose, xylose and mannitol [30, 37]. Serotypes were determined using Bacto-Listeria-O polyvalent.

IIg. Isolation of *Clotriduim perfringens*

In the present study the L.S. (Lactose-Sulfite) broth was used [38, 39]. The composition of the L.S. broth is as follows: 5 g tryptic digest of casein; 2.5 g yeast extract (Difco); 2.5 g sodium chloride; 2.5 g lactose; 0.3 g L^{-1} cysteine hydrochloride; 1 L distilled water. The pH was adjusted to 7.1 ±0.1 and 9 ml of the medium was dispensed into tubes. Sterilization was done by autoclaving at 115^0C for 20 min. Before use, the medium was boiled for 20 min to reduce the oxygen content and 0.5 ml of a 1.2% solution of anhydrous sodium metabisulfite ($Na_2S_2O_5$) and 0.2 ml of a 1% solution of ferric ammonium citrate, were added to each tube. The above solutions were prepared and sterilized by filtration (0.45 mm) just prior to use. Incubation was performed aerobically in a water bath at 46^0C for 24 h. Additionally, an aliquot of each sample was heated for 20 min at 80^0C for detection of germinated spore forms and L.S. broth was seeded to each sample.

IIh. Detection of *Lactobacillus* spp

Blood Agar enriched with nalidic acid (0.01%) was used incubated at 37^0C and 45 ^{0}C for 48 h, under aerobic and anaerobic conditions [40].

All the above isolates were further characterized by the sugar fermentation pattern, which was determined by using API microsystem, as specified by the manufacturer (API® - BioMerieux, Marcy-l'Etoile, France). Moreover, the VITEK 2 (BioMerieux, Marcy-l'Etoile, France), an automated system for identification, was applied.

IIi. Statistical Analysis

Colony counts were converted into log10 CFU/g. The mean values obtained from the microbiological evaluation of prickly pears fruits were analyzed by independent samples t-test and by one-way analysis of variance (ANOVA) to determine any statistically significant difference ($P<0.05$) among the all commodities means.

III. Results And Discussion

In the present work a systematic study on the microbiology of prickly pear was attempted for the first time and thus it is not possible to discuss its findings by comparing them with the findings of other researchers. Table 1 shows the isolated bacteria from Greek prickly pears. The "whole fruits" group showed statistically significant higher Total Plate Counts (TPC) by a factor of 4 than the "flesh only" group ($Z= 45.82$, $P<0.001$, Figure 1). The variance of the former group was 11% while the variance of the second one was 34.69%. These results indicate that the main bacterial load is due to the peel. The relatively small variance suggests a balanced ecosystem. The increased variance of the TPC of the flesh of the fruit suggests that various factors such as the location, the soil, the degree of maturity of the fruit etc. can affect this parameter on an individual basis. Another reason for the decreased bacterial load of the flesh is its acidic pH (<5.2). In fresh fruits and vegetables the aerobic colony court does not correlate with food poisoning incidences [41], yet it reflects their quality as an indicator [42]. In our research the fresh pulp was characterized as "very good".

Coliforms were present in moderate TP counts in 18 specimens of whole fruit (30%) but were absent in the flesh. The fact that most of the whole fruit specimens (70%) were negative to *coliforms* suggests that the presence of *coliforms* - always indicating fecal contamination - depends on the location of the cactus plant (perhaps near a passage of animals).The relatively low variance (14.85%) indicates that *coliforms* are balanced by the ecosystem.

E. coli, *Salmonella* spp. and *Listeria* spp. have not been isolated. Also in the present study *E. coli* 0157:H7 was not detected. It has been reported that *E. coli* O157:H7 is found sporadically in fruits and vegetables at very low levels together with very high levels of competitor organisms and is therefore very difficult to detect [43]. *S. aureus* was isolated from 8 specimens (13.34%) of the whole fruit group. This bacterium is known to be well adopted in low humidity environments such as the arid ecosystems that *Opuntia ficus indica* L. Miller thrives. The low incidence of isolation and the large variance (76.76%) indicate a rather circumstantial contamination. *Coliforms* and *S. aureus* were not isolated from the flesh of the fruit and this finding indicates the protective role of the peel. *Lactobacilli* were isolated from 29 specimens of the "whole fruit" group (48.33%) and from all specimens (100 %) from the "only flesh" group. Furthermore, *Lactobacillus spp*. was isolated in statistically significant higher counts from the "only flesh" group by a factor of 1.8 ($Z= 8.76$, $P<0.05$) with respect to the "whole fruit" group (Figure 2). Obviously *lactobacilli* are a significant part of the micro flora of the flesh of the fruit. These microorganisms consist a barrier against other bacteria (pathogens

or not) through mechanisms of bacterial competition. Other lactic acid bacteria were also isolated from 18 specimens (30%) of flesh, which is to be expected, given that the flesh has a pH of 5.7 or less and that the flesh is rich in nutrients, particularly carbohydrates. These findings are of great importance not only due to the technological significance of these bacteria but also due to their potential probiotic action. The local populations have always considered the prickly pear as a "healthy food" especially for the diseases of the alimentary tract.
C. perfringens' vegetative forms were isolated in low incidences in both groups (5 specimens and 4 specimens, 8.33% and 6.67%). The incidence of isolation of the spore forms was slightly increased in the "only flesh" group (9 specimens, 15.00%) but showed a spectacular increase in the "whole fruit" group (58 specimens, 96.67%) (Figure 3). This finding is justified by the abundance of microorganisms in the natural environments and indicates – again - the protective role of the peel.

A study on the isolation and selection of potential probiotic lactic acid bacteria from *Opuntia ficus-indica* L. fruits that grow in Northwestern Argentina was carried out [44]. According to that study 17 strains of *lactic acid bacteria* were isolated from *Opuntia ficus-indica* L. fruits that grow in arid regions from Argentina. Isolates were screened for probiotic traits such as gastrointestinal stress tolerance, cell surface properties and antimicrobial activity, and also for their effects on functional properties of fermented juices. Among these 17 isolates, 4 showed unique properties suitable for starters in prickly pear juice fermentation. Our results on LAB are similar to these findings. This point is of considerable commercial and scientific interest because it can "deliver" LAB isolates with promising characteristics for further studies on fermentation and technological applications, on survival in different food matrices as well as on their impact on their quality characteristics. Last but not least are the potential health benefits of these strains and particularly their therapeutic value in chronic diseases [45].

Table 1. Bacteria isolated in prickly pears collected in the region of Epirus in Northwestern Greece.

	Whole fruit (cortex and flesh)			Flesh only		
Species	Number of positive samples	CFU/g range (positive samples)	Mean and SD (positive samples)	Number of positive samples	CFU/g range (positive samples)	Mean and SD (positive samples)
TPC	60	5.11-8.97	6.74 ± 0.73	60	0,5-2,49	1,47 ± 0,51
Coliforms	18	1.28-2.34	1,82 ± 0,27	-	-	-
E. coli	0	-	-	-	-	-
S. aureus	8	<1-1.96	0.99 ± 0.76	-	-	-
Salmonella spp.	0	-	-	-	-	-
Listeria spp	0	-	-	-	-	-
Lactobacillus spp.	29	1.51-2.48	1,77 ± 0.57	60	<2-3.91	2,73 ± 0.23
Other *Lactic acid bacteria*	0	-	-	18	-	-
C. perfringens (vegetative)	5	<1	<1	4	<1	<1
C. perfringens (spores)	58	<1	<1	9	<1	<1

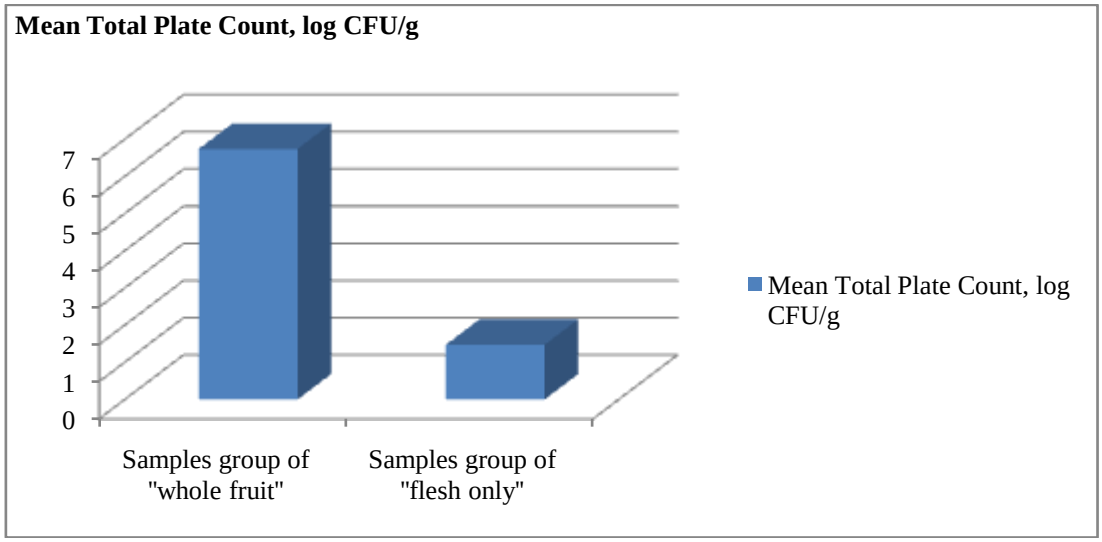

Figure 1. Mean Total Plate Count (TPC) from the "whole fruit" group and from the "flesh only" group.

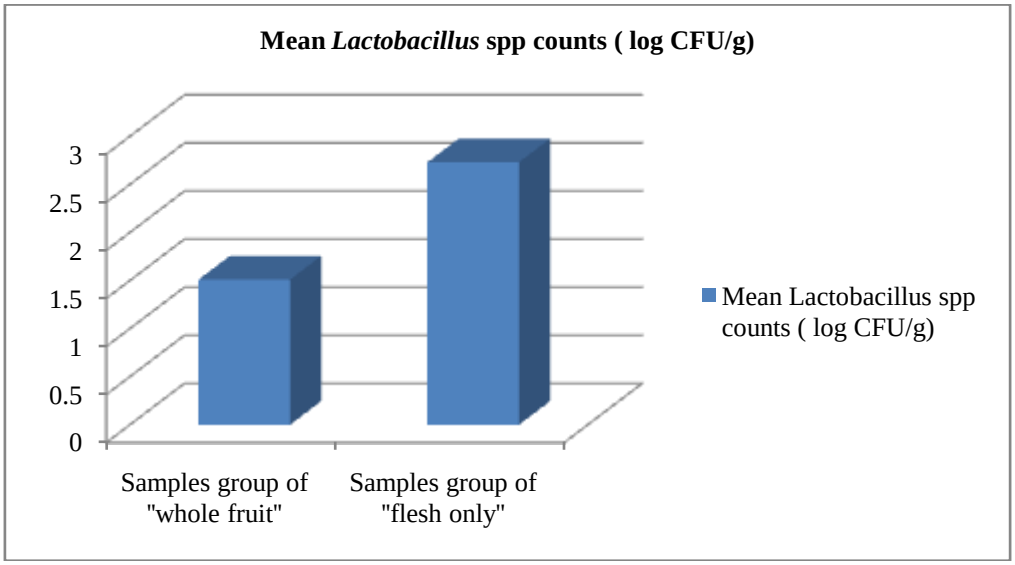

Figure 2. Mean *Lactobacillus spp.* counts from the "whole fruit" group and from the "flesh only" group.

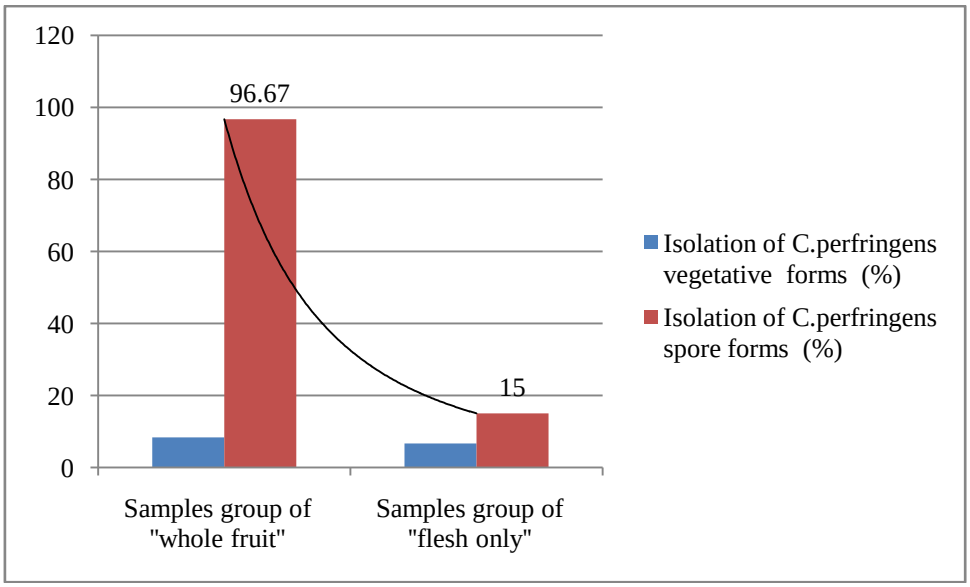

Figure 3. Isolation of *C. perfringens* spore forms from the "whole fruit" group and from the "flesh only" group.

IV. Conclusion

The absence of pathogenic bacteria, such as *E. coli*, *Salmonella* spp. and *Listeria monocytogenes*, strengthens the view that prickly pear can be considered as a safe food for human consumption. Moreover, the presence of *Lactobacilli* especially in the flesh of the fruit is very important with regard to its potential probiotic action.

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