International Journal of Food Microbiology 154 (2012) 79–84

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short Communication

Acid and alcohol tolerance of *Escherichia coli* O157:H7 in pulque, a typical Mexican beverage

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ARTICLE INFO

Article history:
Received 27 April 2011
Received in revised form 13 December 2011
Accepted 18 December 2011
Available online 23 December 2011

Keywords:
Pulque
Agave nectar
Escherichia coli O157:H7
Acid tolerance
Alcohol tolerance

ABSTRACT

Pulque is a traditional Mexican fermented alcoholic beverage produced from the nectar of maguey agave plants. No data exist on the behavior of *Escherichia coli* O157:H7 in agave nectar and pulque. An initial trial was done of the behavior of *E. coli* O157:H7 during fermentation of nectar from a single producer, a nectar mixture from different producers and "seed" pulque. A second trial simulating artisanal pulque production was done by contaminating fresh nectar with a cocktail of three *E. coli* O157:H7 strains, storing at 16° and 22 °C for 14 h, adding seed pulque and fermenting until pulque was formed. A third trial used pulque from the second trial stored at 22 °C as seed to ferment fresh nectar at 22 °C for 48 h (fermentation cycle). This procedure was repeated for an additional two fermentation cycles. During incubation at 16° or 22 °C in the first trial, the *E. coli* O157:H7 strains multiplied in both the single producer nectar and nectar mixture, reaching maximum concentration at 12 h. *E. coli* O157:H7 cell concentration then decreased slowly, although it survived at least 72 h in both fermented nectars. *E. coli* O157:H7 did not multiply in the seed pulque but did survive at least 72 h. In the second trial, the numbers of *E. coli* O157:H7 increased approximately 1.5 log CFU/ml at 22 °C and 1.2 log CFU/ml at 16 °C after 14 h. After seed pulque was added, *E. coli* O157:H7 concentration decreased to approximately 2 log CFU/ml, and then remained constant until pulque was produced. In the third trial, the *E. coli* O157:H7 cells multiplied and survived during at least three nectar fermentation cycles. The results suggest that *E. coli* O157:H7 can develop acid and alcohol tolerance in pulque, and constitutes a public health risk for pulque consumers.

1. Introduction

Pulque is a traditional fermented alcoholic beverage from central Mexico produced from *Agave* nectar, known as "aguamiel". In the early to middle 20th century, pulque represented a potential economic resource in central Mexico, and it is still consumed in the region. Most pulque production is done by artisans although some companies have industrialized the process. Pulque is a white, viscous liquid with an alcohol content of 5.4% and pH ranging from 3.5 to 4.2 (Ortiz, 1990; Ruvalcaba, 1983). Agave nectar is a crystalline liquid with a sugar content of 12° Brix and a pH of 7.5 (Nieto and Maecke, 1938; Ruvalcaba, 1983). Nectar is extracted from young (4–6 years of age) maguey agaves such as *Agave salmiana, A. mapisaga, A. feroz, A. atrovirens* and *A. americana* (Nieto and Maecke, 1938; Peña-Álvarez et al., 2004; Ruvalcaba, 1983; Sánchez-Marroquín, 1967; Sánchez-Marroquín et al., 1967). When an agave plant reaches the proper age its immature floral stem is removed to form a lesion in the form of a circular cavity in the central portion of the plant into which the plant excretes and stores nectar. Nectar collection is commonly done approximately every 12 h. After nectar collection, the cavity walls are scraped with a sharp tool to remove approximately 0.5-cm wall thickness and stimulate nectar secretion. Commonly, the cavity's external portion is covered with pieces of agave leaf or a large stone. Immediately after scraping, the plant begins excreting nectar and can produce up to 5 l in a twelve-hour period. Nectar fermentation begins at a very slow rate in the collection cavity where native microorganisms such as ethanol-producing bacteria, lactic acid bacteria, yeasts and exopolysaccharide-producing bacteria are present (Cervantes-Contreras and Pedroza, 2008; Escalante et al., 2004; Sánchez-Marroquín, 1967; Sánchez-Marroquín et al., 1967). Upon collection, nectar is placed in containers kept at 16–30 °C and inoculated with an amount of pulque known as a "seed". This seed pulque is selected by producers from pulque that generally has been fermented for 48–72 h and has very specific traits. It contains the main microorganisms required for nectar fermentation and pulque production. The

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doi:10.1016/j.ijfoodmicro.2011.12.027

proportion of seed pulque to agave nectar can vary from 20:80 to 80:20, depending on type of producer. After inoculation with seed pulque, the nectar ferments into pulque in from 24 to 48 h. The principal variables determining nectar fermentation time are ambient temperature (Sánchez-Marroquín, 1967; Sánchez-Marroquín et al., 1967), microorganism type and the seed pulque concentration used to ferment the nectar (Cervantes-Contreras and Pedroza, 2008; Escalante et al., 2004; Nieto and Maecke, 1938, 1940; Sánchez-Marroquín et al., 1967). No standardized process exists for pulque production because producers do not control even basic variables such as temperature. Artisanal producers generally try to maintain constant seed pulque traits while industrial producers also control for initial nectar pH and ° Brix. Differences in fermentation process temperature among producers are due to factors such as changes in daytime room temperature, season and geographical region. Variation also exists between producers in terms of the initial nectar mixture:seed pulque proportion. Geographical region and season can also lead to different seed pulque microflora among producers (Cervantes-Contreras and Pedroza, 2008; Escalante et al., 2004; Sánchez-Marroquín et al., 1967). Finally, producers determine final fermentation time based on fermented product sensory characteristics (*e.g.,* viscosity, color, flavor and odor), adding yet another level of variability.

Very little is known about the microbial safety of pulque. It is highly probable that both nectar and/or pulque can be contaminated with pathogenic bacteria since they are exposed to contamination sources in the agave plant, during nectar collection, transport and fermentation. In addition, food safety practices are normally inadequate during the pulque production process. Although pulque is clearly important in Mexico, its production remains artisanal. This is largely due to the lack of or limited access to information on the frequency and behavior of pathogenic bacteria (e.g. *E. coli* O157:H7) in nectar and pulque. Thermal treatment equivalent to pasteurization eliminates any non-sporulated pathogenic bacteria possibly present in pulque, but no data is currently available on the time and temperature limits required to eliminate these pathogenic bacteria in this beverage. Therefore, research is needed on thermal death in non-sporulated bacteria in pulque to identify time and temperature limits and produce a safe beverage.

Pulque's alcohol content, low pH and the lactic bacteria activity during fermentation could inactivate human pathogenic microorganisms in the pulque. However, there are reports of bacteria such as *E. coli* O157:H7 developing tolerance to low pH levels (Conner and Kotrola, 1995; Leyer et al., 1995; Miller and Kaspar, 1994; Reinders et al., 2001), suggesting the possibility that these pathogens could survive in pulque.

E. coli O157:H7 was first recognized as a significant foodborne pathogen in the 1980s and early 1990s (Riley et al., 1983; Tuttle et al., 1999). It is known for its ability to cause severe disease and death. Hemorrhagic colitis caused by *E. coli* O157:H7 can lead to complications such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura, which may be fatal to young children or the elderly. Epidemiological evidence shows it to be virulent, requiring only a few cells to cause illness (Griffin and Tauxe, 1991).

Involvement of *E. coli* O157:H7 in foodborne illness outbreaks associated with consumption of acidic foods such as apple cider, fermented sausage, yogurt and mayonnaise (Besser et al., 1993; CDC, 1995; Morgan et al., 1993) has drawn attention to its acid resistance properties. Many subsequent studies have demonstrated that it can survive in a variety of acidic foods (Calicioglu et al., 1997; Glass et al., 1992; Massa et al., 1997; Miller and Kaspar, 1994; Semanchek and Golden, 1996; Zhao and Doyle, 1994). Other studies have shown that adaptation to acidic conditions can further improve survival of *E. coli* O157:H7 in foods preserved with low pH and acids (Leyer et al., 1995; Tsai and Ingham, 1997).

In central Mexico, a lot of pulque is produced in farms and backyards; many of these pulque producers also raise cattle and sheep. The producers frequently graze cattle and sheep on pasture that grow in agave plantation. Cattle are a major reservoir for *E. coli* O157 (Hancock et al., 1998). In addition, *E. coli* O157 has been isolated from sheep and numerous other species (Chapman and Ackroyd, 1997; Chapman et al., 1997; Fischer et al., 2001; Cornick et al., 2000). However, ruminants appear to be more frequently colonized by *E. coli* O157 than are other animals. Agave plant could become contaminated with *E. coli* O157 by direct contact of contaminate manure or transmitted by other animal vectors (Hill et al., 2006; LeJeune et al., 2001; Sargeant et al., 2003; Thurston-Enriquez et al., 2005). *E. coli* O157 has been isolated from fecal feces of cattle and swine in central Mexico (Callaway et al., 2004). In addition, *E. coli* O157:H7 strains have been isolated from bovine and porcine carcasses (Varela-Hernández et al., 2007; Gallegos et al., 2009), as well as in our laboratory in 10% of ground beef samples from markets in Pachuca, Hidalgo state, Mexico (data not shown). It has also been reported in fecal samples from children with acute diarrhea (Flores-Abuxapqui et al., 1994; Paniagua-Contreras et al., 2007).

No reported data exist on the frequency and behavior of *E. coli* O157:H7 in agave nectar and pulque, neither the presence of coliforms bacteria in pulque. But, it is highly probable that both nectar and/or pulque could be contaminated with *E. coli* O157 since they are exposed to contamination sources in the agave plant, during nectar collection, transport and fermentation.

The objective of this study was to document the behavior of *E. coli* O157:H7 during nectar fermentation and its survival in finished pulque.

2. Materials and methods

2.1. Bacterial strains

Three *E. coli* O157:H7 strains were used in the trials: two isolated in our laboratory from ground beef (P1C6 and M5C8) and another isolated from meat (E09). The E09 strain was donated by E.F. Escartín of the Universidad Autónoma de Querétaro, Mexico. Rifampicin resistance (R+) was induced in all strains (Castro-Rosas et al., 2010). The R+ strains were kept in inclined trypticase soy agar (TSA; Bioxon, Becton Dickinson, México) tubes at 3 to 7 °C with monthly transfers over TSA. All strains maintained rifampicin resistance throughout the study.

2.2. Sample collection

Agave nectars and seed pulque were donated by a pulque producer which uses nectar from several nectar producers. Nectar from different producers is delivered to the company in the morning and afternoon, and mixed together in containers before inoculation with the seed pulque. The different nectars are mixed at a fixed ratio to formulate a nectar mixture with an initial pH between 7.0 and 7.5 and ° Brix of 9 to 10. Two nectar mixture (NM) samples, 5 l in the morning and 5 l in the afternoon, were taken from a container with nectar from ten producers. Fresh nectar was collected in the morning directly from 5 plants (2 l nectar each) randomly selected within an agave plantation; this fresh nectar was called single-producer nectar (Spn). One liter of seed pulque (SP) was provided by the producer. All samples were transported to the laboratory on ice and under aseptic conditions. In the laboratory, the 5 l NM samples were mixed together in a single disinfected stainless-steel container and the Spn samples stored in a separate stainless-steel container. Measurements of pH, soluble solids content (° Brix) and alcohol content were taken in triplicate from each NM, Spn and SP sample (AOAC, 1990). Finally, 50 ml subsamples of each sample were transferred to sterile tubes and stored at −18 °C until use.

2.3. Inoculum preparation

Tubes containing 3 ml trypticase soy broth (TSB, Bioxon) were inoculated with individual rifampicin-resistant strains and incubated at 35 °C for 18 h. The cultures were washed twice in sterile isotonic saline solution (ISS) by centrifuging at 3500 ×*g* for 20 min, and then the pellets were resuspended in ISS at about 10^9 CFU/ml. Samples (2 ml each) of the each strain suspension were combined to yield a cocktail containing approximately equal populations of each strain. Decimal dilutions of this cocktail were done with ISS to produce a final approximate concentration of 5 log CFU/ml.

2.4. First trial

Tubes of Spn, NM or SP were thawed by placing them in a 40 °C water bath for 10 min. The sample (50 ml) in each tube was inoculated with 100 μL of the R+ cocktail at a 2 log CFU/ml final concentration and homogenized for 10 s on a vortex. Samples were incubated at 16° ± 1 °C and 22° ± 1 °C, the temperature range used in pulque production in the central Hidalgo state region. Three samples were used for each studied temperature.

2.5. Second trial

Recently excreted nectar (REN) (collected immediately after scraping of agave plants) was collected under aseptic conditions and transported immediately to the laboratory under refrigeration. In the laboratory, 50 ml REN were placed in tubes and these inoculated with 100 μL R+ cocktail at a 2 log CFU/ml nectar final concentration. These inoculated tubes were incubated at 22 °C for 14 h and then SP added at a 20:80 (SP:inoculated REN) proportion. One series of tubes was incubated at 16° ± 1 °C for 48 h and another at 22° ± 1 °C for 48 h to produce pulque. Three replicates were done for each temperature.

2.6. Third trial

REN was mixed with pulque (Pu) from the second trial (at 22 °C/48 h) at a 80:20 (REN:Pu_1) proportion and incubated at 22° ± 1 °C for 24 h to produce new pulque (First fermentation cycle). The resulting pulque (Pu_1) was then mixed with new REN at a 80:20 (REN: Pu_1) proportion and incubated at 22° ± 1 °C for 24 h to produce a new batch of pulque (Pu_2; second fermentation cycle). Two further fermentation cycles were done following the above procedure. Three replicates were done for each fermentation cycle.

2.7. Microbiological counts

Duplicate microbial counts were done periodically by the pour-plate technique using TSA containing 100 mg/l rifampicin (Rif; Sigma-Aldrich St. Louis, MO, USA) and 50 mg/l natamycin (Sigma-Aldrich). Plates were incubated at 35 °C for 24 to 48 h. The presence of rifampicin-resistant *E. coli* O157:H7 strains in the plates was confirmed by transferring three to five colonies to plates of sorbitol MacConkey Agar (Bioxon) containing tellurite-cefixime [2.5 mg/L–0.05 mg/L (Sigma-Aldrich) (FDA, 2011)]. These were incubated at 35° ± 2 °C for 24 h. The isolated bacteria were then biochemically identified with a commercial biochemical kit (API-20E, bioMérieux, Mexico). Cultures identified as *E. coli* with the API-20E test were serologically identified using the *E. coli* O157:H7 latex agglutination test kit (RIM® *E. coli* O157:H7 Latex Test Kit, Remel, Lenexa, KS, USA).

3. Results and discussion

Temperature values correspond to samples at time of collection, whereas the pH, ° Brix and alcohol values were recorded after the samples were mixed in the laboratory (Table 1). The agave nectar results coincide with reported values (Cervantes-Contreras and Pedroza, 2008), and the pH and ° Brix values are considered adequate for pulque production.

3.1. First trial

The *E. coli* O157:H7 cocktail was slowly inactivated in the SP at 16° and 22 °C. This occurred more slowly at 16 °C than at 22 °C, but the strains in the cocktail survived at least 72 h in SP (Figs. 1 and 2). In the Spn and NM, the *E. coli* O157:H7 cocktail increased in concentration during the first 12 h at 16° and 22 °C, although at 16 °C growth was slower and therefore required more time to increase in concentration than at 22 °C. Concentration decreased slowly after the first 12 h of nectar fermentation (Spn and NM), but surviving cells persisted at least 72 h at 16° and 22 °C (Figs. 1 and 2). These results differ from our previous research (Gómez-Aldapa et al., 2011), in which the pathogens *Salmonella* Typhimurium, *Listeria monocytogenes* Scott A; *Staphylococcus aureus* and *Shigella flexneri* and *S. sonnei* were inactivated in the SP within the first 24 h at 16° and 22 °C. These same five pathogenic bacteria were also completely inactivated after the first 12 h of nectar (Spn and NM) fermentation and no pathogenic cells were isolated after 72 h fermentation at 16° and 22 °C (Gómez-Aldapa et al., 2011).

The *E. coli* O157:H7 cocktail did not grow in SP at either temperature (Figs. 1 and 2), probably due to its initially lower pH and higher alcohol content (Table 1). At inoculation, the SP had a pH near 4 and a 6.0% alcohol content, while the Spn and NM had an average 7.3 pH and 0.4% alcohol content (Table 1). Low pH levels and higher alcohol percentages like the initial SP values do not promote microbial survival. Very little change in pH was observed in the SP during incubation, whereas in the Spn and NM the pH decreased notably during fermentation at 16° and 22 °C (Table 2).

In the first trial, agave nectar was fermented using its own microbial flora, but this is not the way pulque is prepared. In central Mexico, pulque is produced by inoculating nectar with seed pulque and then storing at 16°–30 °C for 24–48 h. In addition, the nectar used in the first trial (Figs. 1 and 2) had been previously exposed at ambient temperature (16°–22 °C) for an average of 14 h, that is, the temperature and time between scraping the plant to stimulate nectar release, its collection and transport to the processing plant. *E. coli* O157:H7 may not behave in the same way if it enters the nectar at the moment of excretion instead of being inoculated 14 h later. Initial nectar excretions could be contaminated by *E. coli* O157:H7 present in the cavity which therefore remain in contact with the nectar for up to 14 h. Under these circumstances, microorganisms native to the nectar may begin to multiply and inhibit pathogenic bacteria growth, or vice versa; or the native microorganisms may begin to ferment the nectar creating acidic and alcoholic conditions that could induce pathogens to develop greater acid or alcohol tolerance rather than killing them (as occurs in some acid foods) (Leyer et al., 1995; Menz et al., 2010). The second and third trials were designed to closely simulate common pulque production conditions.

Table 1
Values of temperature, pH, ° Brix and alcohol in seed pulque and nectar samples collected from a processing plant or in agave plants.

Sample	Temperature (°C)	pH	° Brix	Alcohol (%)
Spn	11 ± 0.3	7.2 ± 0.2	12 ± 0.2	0.3 ± 0.02
NM	10 ± 0.5	7.4 ± 0.2	10 ± 0.2	0.5 ± 0.02
REN	12 ± 0.5	7.3 ± 0.1	13 ± 0.2	nd
SP	22 ± 0.2	4.1 ± 0.2	4.2 ± 0.2	6.1 ± 0.05

Spn: single-producer nectar, NM: nectar mixture, REN: recently excreted nectar, SP: seed pulque, nd: not detected.

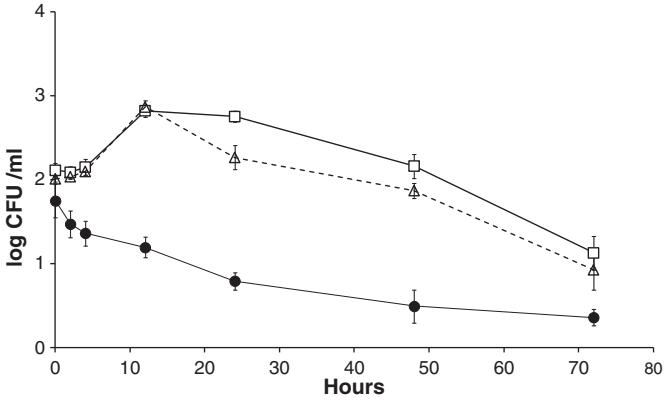

Fig. 1. Behavior of *E. coli* O157:H7 in nectar mixture (Δ), single-producer nectar (□) and seed pulque (●) at 16 °C.

3.2. Second trial

In the second trial, agave nectar was collected in sterile glass bottles immediately after the plant was scraped (REN), placed in glass tubes in the laboratory and these inoculated separately with *E. coli* O157:H7 cocktail and allowed to incubate at 22° or 16 °C for 14 h. Seed pulque was then added and the mixtures left to ferment an additional 48 h at 22° or 16 °C to produce finished pulque. During the first 14 h at 22° or 16 °C, the *E. coli* O157:H7 cocktail multiplied in the REN, exhibiting an approximately 1.5 log increase at 22 °C and a 1.2 log increase at 16 °C (Fig. 3). Introduction or addition of the SP into the contaminated REN reduced count by approximately 1.7 log CFU/ml at 22 °C and 1.2 log CFU/ml at 16 °C. After this reduction, cell numbers remained relatively stable. *E. coli* O157:H7 survived well during the following 43 h of fermentation. During this time, pulque pH was 4 and alcohol content was 6%.

These results differ from our previous research (Gómez-Aldapa et al., 2011), in which *L. monocytogenes*, *S. aureus* and *Salmonella* were completely inactivated within 8 h after SP addition at both temperatures, while *Shigella* species survived for approximately 24 h at both temperatures. The *E. coli* O157:H7 behavior observed here in pulque coincides, at least in part, with that reported by Menz et al. (2010). In their study, *E. coli* O157:H7 was capable of growth during the initial fermentation stages in hopped wort, but were quickly inactivated when added during the later fermentation stages. Pathogen growth and survival was enhanced as pH was increased, and as both ethanol percentage and original gravity decreased. In contrast, in pulque *E. coli* O157:H7 survived well during the following 43 h after addition of seed pulque.

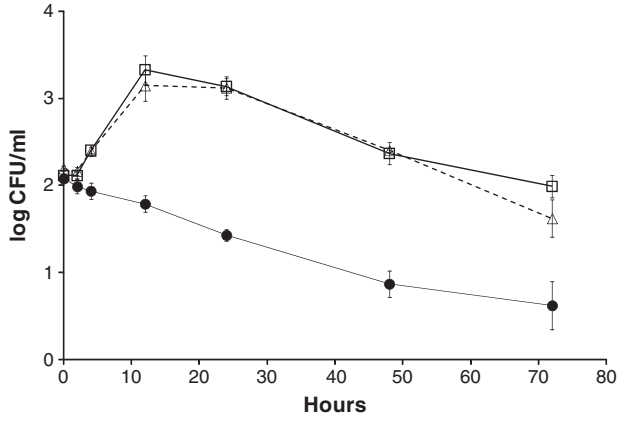

Fig. 2. Behavior of *E. coli* O157:H7 in nectar mixture (Δ), single-producer nectar (□) and seed pulque (●) at 22 °C.

Table 2
Changes in pH values during incubation of seed pulque (SP), nectar mixture (NM) and single-producer nectar (Spn) at 16° and 22 °C.

Hours	SP		NM		Spn	
	16 °C	22 °C	16 °C	22 °C	16 °C	22 °C
0	4.1[a] ± 0.2[b]	4.1 ± 0.1	7.4 ± 0.2	7.4 ± 0.2	7.3 ± 0.2	7.3 ± 0.1
2	4.1 ± 0.2	4.1 ± 0.2	7.4 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	7.3 ± 0.2
4	4.1 ± 0.3	4.0 ± 0.3	7.3 ± 0.1	7.1 ± 0.05	7.2 ± 0.1	7.0 ± 0.1
8	4.0 ± 0.2	3.8 ± 0.2	7.0 ± 0.1	6.4 ± 0.2	7.1 ± 0.2	6.5 ± 0.1
24	3.9 ± 0.2	3.6 ± 0.1	5.0 ± 0.2	4.3 ± 0.2	5.2 ± 0.3	4.5 ± 0.2
48	3.7 ± 0.1	3.4 ± 0.2	4.3 ± 0.1	4.0 ± 0.1	4.3 ± 0.1	4.1 ± 0.05

[a] pH value.
[b] Standard deviation.

E. coli O157:H7 clearly has the ability to tolerate acid and alcoholic conditions in pulque. This leads to questions of how *E. coli* O157:H7 would behave if the contaminated pulque produced in the second trial was used as seed pulque to ferment nectar, and if it remains viable throughout a succession of fermentations.

3.3. Third trial

Pulque (Pu; fermented for 48 h at 22 °C) from the second trial was used to seed recently excreted nectar (REN) and allowed to ferment (24 h/22 °C); this was treated as one fermentation cycle. Pulque (Pu_1) was taken from this first cycle and used in a second cycle, and then a third cycle run, resulting in a total of four cycles (including that in the second trial). Throughout the four cycles, *E. coli* O157:H7 multiplied to 0.5 log CFU/ml during the first 8 h and then remained constant (Fig. 4). The decrease in *E. coli* O157:H7 concentration when the pulque was mixed with the REN was caused by dilution rather than cell inactivation or death due to mixing. Values for pH and alcohol content achieved were rather similar for each fermentation cycle (pH 4.3 ± 0.2, 4.5 ± 0.2, 4.4 ± 0.1, 4.5 ± 0.2 for cycle 1, 2,3, 4, respectively, and alcohol 5.0 ± 0.05, 5.2 ± 0.1, 4.2 ± 0.2, 4.7 ± 0.2).

Clearly, *E. coli* O157:H7 was able to adapt and develop tolerance to the acid–alcohol conditions of the pulque and survive for at least four fermentation cycles. In addition this conclusion is supported by results from second (Fig. 3) and third trials (Fig. 4): when *E. coli* O157:H7 and SP were added at the same time into the fresh REN (Fig. 4), pathogenic bacteria survived better during REN fermentation and in final pulque that when fresh REN was initially contaminated only with *E. coli* O157:H7 and then SP was added (Fig. 3). The results suggest that *E. coli* O157:H7 tolerance to the acid–alcohol condition is developed during agave fermentation in presence of SP.

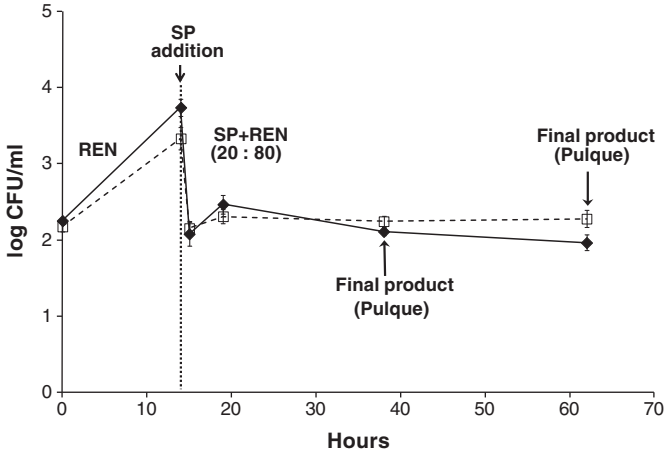

Fig. 3. Behavior of *E. coli* O157:H7 in recently excreted nectar (REN) at 16° (□) or 22 °C (♦) for 14 h and during fermenting of REN after adding seed pulque (SP). SP + REN = mixture of seed pulque and recently excreted nectar at a 20:80 proportion.

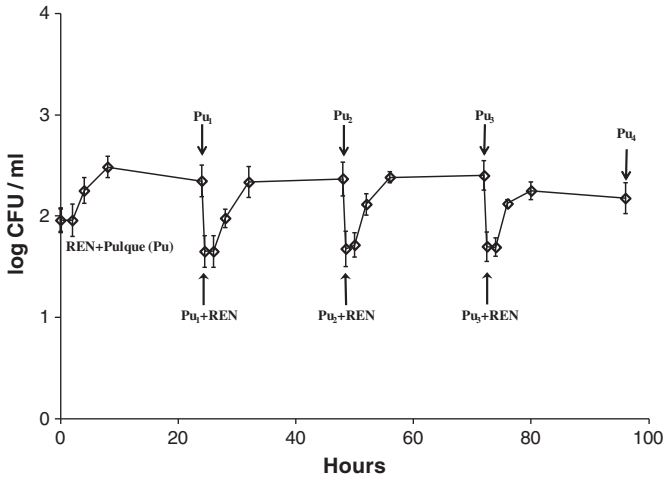

Fig. 4. Behavior of *E. coli* O157:H7 during four fermentation cycles of recently excreted nectar (REN) using in each cycle a contaminated pulque with pathogenic bacteria to ferment REN at 22° ± 1 °C. REN + Pulque (Pu) = mixture of recently excreted nectar and contaminated pulque at a 20:80 proportion; Pu_1 = final product (pulque) of first fermentation cycle; Pu_1 + REN = mixture of Pu_1 and REN at a 20:80 proportion; Pu_2 = final product of second fermentation cycle; Pu_2 + REN = mixture of Pu_2 and REN at a 20:80 proportion; Pu_3 = final product of third fermentation cycle; Pu_3 + REN = mixture of Pu_3 and REN at a 20:80 proportion; Pu_4 = final product of fourth fermentation cycle.

The results of third trial differ from those reported by Menz et al. (2010), they reported that *E. coli* O157:H7 grew rapidly in unpitched sweet wort at 0 to 2% (vol/vol) ethanol, but that growth rate decreased as ethanol concentration neared 4% (vol/vol). Previous studies suggest that *E. coli* O157:H7 exhibits some acid resistance or tolerance in acidic foods (Conner and Kotrola, 1995; Glass et al., 1992; Leyer et al., 1995; Miller and Kaspar, 1994; Reinders et al., 2001). *E. coli* O157:H7 has developed multiple mechanisms to survive under low-pH conditions (Lin et al., 1996), and acid tolerance plays a vital role in the survival and virulence of diarrhea-causing *E. coli* strains (Castanie-Cornet et al., 1999; Price et al., 2004; Slonczewski and Foster, 1996). However, this is the first report of *E. coli* O157:H7 strain alcohol tolerance in fermented beverages.

Ethanol accumulation produced by fermentation represents an adverse environmental change to which organisms must evolve an adaptive response if they are to survive (Ingram and Vreeland, 1980). Ethanol changes the cell environment's physical characteristics (Franks and Ives, 1966) and may alter the way in which a cell interacts with its environment. The primary site through which cells maintain contact with the environment is the plasma membrane, suggesting that the plasma membrane may be a vital site for expression of adaptive responses to ethanol.

E. coli synthesizes ethanol as a product of glucose metabolism (Ingram, 1986), suggesting it may have developed an adaptive response to this compound (Ingram and Vreeland, 1980). Indeed, *E. coli* is known to tolerate ethanol concentrations as high as 5% (Ingram, 1986). At a pH of about 4.5, *E. coli* can survive a 5% ethanol concentration in McIlvaine's medium. However, a pH below 4.5 accentuates the effect of the alcohol, meaning at pH 3 and a 5% ethanol concentration no viable cells can be isolated from some *E. coli* O157:H7 strains (Jordan et al., 1999). The effect of the pH/alcohol combination depends, therefore, on the susceptibility of individual *E. coli* O157:H7 strains. In the present study, pH was 4.0 and alcohol concentration > 6%, conditions generally comparable to those in Jordan et al. (1999), but the *E. coli* O157:H7 remained viable, making this the first report of *E. coli* O157:H7 tolerance to alcohol and acid pH in a fermented beverage.

The high pathogenicity and low doses required for *E. coli* O157:H7 infection, perhaps less than 50 cells (Mackey and Gibson, 1997), make contamination with *E. coli* O157:H7 and its behavior in foods of particular concern to the food industry, public health authorities and consumers. Determining the behavior of pathogenic microorganisms in agave nectar and pulque is important given the nectar's natural source, handling conditions, pulque fermentation methods and the way it is consumed in Mexico. Agave nectar is an agricultural product and, like fruits and vegetables, is exposed to multiple contamination sources (*e.g.,* dust, rain, fauna, utensils and human contact). The personal hygiene and utensil disinfection conditions prevailing during nectar collection strongly favor nectar contamination with pathogens. Once collected, nectar is exposed to additional sources of contamination (*e.g.* machinery, containers, domestic and wild animals, workers, dust and vehicles) each of which can expose nectar to different pathogenic microorganisms.

Much of the *E. coli* O157:H7 contamination risk in agave pulque could be mitigated by using proper handling and correct food safety practices, such as thorough washing and disinfection of utensils and containers used in all harvest and production stages, and use of basic personal hygiene practices by those harvesting and processing the raw material. Implementing these measures could greatly reduce and perhaps prevent contamination of nectar in the field and during harvest, and of pulque during fermentation and storage. They would also allow for production and use of *E. coli* O157:H7-free seed pulque.

Under the present study conditions, the *E. coli* O157:H7 cocktail survived during the pulque fermentation process, suggesting that this pathogen poses a high potential health risk to pulque consumers as long as the pulque production practices common in Hidalgo state, Mexico, remain in use. However, further research is required using other pulque production conditions (*e.g.* different seed pulque: agave nectar proportions) and different inoculums and more strains of *E. coli* O157:H7 to better understand the real microbial risk to consumers from this traditional Mexican fermented beverage.

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