Antibiotic resistance and tolerance to simulated gastrointestinal conditions of eight hemolytic *Bacillus pumilus* isolated from pulque, a traditional Mexican beverage

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Abstract Traditional fermented sap beverages from two provinces of Mexico were studied for the isolation of pathogenic bacteria from these beverages. Eight strains of hemolytic Gram positive bacteria, identified as *Bacillus pumilus* (*B. pumilus*), were isolated. They showed different antibiotic resistance profiles and tolerances to the simulated gastrointestinal conditions. All the *B. pumilus* isolated were resistant to at least one antibiotic tested. Regarding the simulated gastrointestinal conditions, strains S1 and S3 were able to tolerate all the conditions. Hemolytic activity has been associated with a health risk and is often considered as a virulence determinant associated with pathogenicity. Therefore, the hemolytic activity along with the ability to tolerate gastrointestinal conditions and the resistance to antibiotics exhibited by *B. pumilus* strains S1 and S3 could be associated with a health risk for pulque consumers.

Keywords: pulque, *Bacillus pumilus*, hemolytic activity, antibiotic resistance, simulated gastrointestinal stress

Introduction

Pulque (fermented agave sap) is one of the most important traditional non-distilled alcoholic beverages produced and consumed in the rural areas of the central states of Mexico and remains popular in many communities, dating back to the Prehispanic era; it is consumed daily in these rural areas during meals by men, women, children, and the elderly and is often a central feature of festive occasions. Its consumption is because of the popular belief that it provides curative and health-promoting properties, exhibits hydration qualities, replenishes lost carbohydrates, enhances milk production; hence, consumed postpartum, and proves to be a nutritional beverage as a source of important nutrients such as ascorbic acid, iron, riboflavin, and likely folic acid (1–3). However, it has been suggested that pulque has adverse effects because of its ethanol content (2). From a microbiological point of view, there is little information about the safety from and behavior of indigenous pathogens in pulque. This is of importance since the production systems are largely artisanal, with inadequate hygiene conditions and exposure to multiple contamination sources (4). Some pathogenic bacteria such as *Salmonella* Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes*,

Shigella flexneri, and *Shigella sonnei* when inoculated into pulque have shown the ability to survive under acidic and alcoholic conditions of this beverage. It is highly probable that pulque can contain indigenous pathogenic bacteria since they are exposed to contamination sources in the agave plant during nectar collection, transport, and fermentation (4).

Bacillus strains are widely distributed in different environments; they have been isolated from sources such as food, soil, air, and faeces (5,6). Different strains of *Bacillus* have been identified in pulque (7) and in other traditional fermented food such as *Bacillus pumilus* (8). *Bacillus licheniformis*, *B. subtilis*, *B. pumilus*, and *B. cereus* among others have been involved in food poisoning incidents (9,10) because of the production of enterotoxins and hemolytic activity such as in *B. thuringiensis* (11), *B. cereus*, and *B. subtilis* (12). It has been reported that *B. pumilus* induce the proinflammatory cytokine interleukin 6 in culture macrophage cell line and *in vivo* induce proinflammatory cytokine tumor necrosis factor alpha and Th1 cytokine gamma interferon (13).

In a previous study, under conditions of isolation of microorganisms capable of surviving under simulated gastrointestinal (GI) stress conditions (14), the majority of our isolates were bacilli Gram-

positive, which interestingly showed hemolytic activity (data not previously reported). This activity has been associated with a health risk since hemolytic activity is often a virulence determinant and is associated with pathogenicity (1). Therefore, the aim of this study was to identify the bacilli isolates and to individually test the ability of each of the isolate to survive in simulated GI stress conditions and to resist certain antibiotics.

Material and Methods

Sample collection and pulque production Newly collected agave sap (AS) and overnight fermented (FAS) samples were obtained from the following two regions of México: Huitzilac in the Province of Morelos and Singuilucan in the Province of Hidalgo, with a distance of 150 km between them. All the samples were placed in sterile tubes and transported at 4°C. Each sample was fermented in the laboratory by the addition of FAS and AS in a ratio of 1:3 v/v (pulque) at 36°C (14).

Control strains The methodology reported by González-Vázquez *et al.* (14) was used to isolate, grow, produce and conserve a pure culture of *Lactobacillus casei* Shirota from Yakult® and the strain *E. coli* ATCC 160211. The control strain used for biochemical, antibiotic, acid, simulated gastric juice, bile salt tests, and genotypic characterization was *L. casei* Shirota.

Bacterial isolation One milliliter of pulque was added to a stress-inducing medium, consisting of MRS Broth (Difco, Sparks, MD, USA) at pH 1.5 with HCl (0.1 N) and oxgall (0.5% w/v) (Difco) and incubated for 2 h at 200 rpm, 37°C; then 10 µL of the mixture were plated onto an MRS Agar (Difco) and incubated for 12 h at 37°C. Rod-shaped Gram-positive microorganisms (bacilli) were selected (14).

Genotypic identification DNA extraction was conducted using an Easy-DNA kit (Invitrogen, Carlsbad, CA, USA) and as per to the supplier's instructions. Polymerase chain reaction (PCR) amplification was conducted using primers LAC1 (5'-AGCAGTAGGGAATCTTCCA-3') and LAC2 (5'-ATTTCACCGCTACACATG-3') (15). To genotype the strains, amplifications, comparisons, and sequence alignments were performed using the methodology reported by Gonzalez-Vazquez *et al.* (14).

Phenotypic identification

Catalase: The test was conducted by placing a drop of hydrogen peroxide (J.T. Baker, Phillipsburg, NJ, USA) over a colony of microorganisms in triplicate. If bubbles or froth were formed, the test was considered as positive (16). *E. coli* ATCC 160211 was used as a positive control and *L. casei* Shirota as a negative one (14).

Blood agar screening: A modified version of the method reported by Ruiz-Moyano *et al.* (17) was used. In brief, a colony of each strain was

plated onto blood agar and incubated for 48 h at 37°C in triplicate. The control strains were the same as those in the catalase test.

Antibiotic resistance: BIO-RAD multidisc (Gram-positive II bacteria) containing the antibiotics shown in Table 1 were used. The multidisc was placed onto Mueller Hinton (Difco) agar plates at pH 7, previously inoculated with the isolates or *L. casei* Shirota. Plates were incubated for 72 h at 37°C in triplicate (14). The results were expressed according to the instructions given by the supplier of the test kit. Before the test, we used *E. coli* ATCC 160211 to measure the reproducibility of the technique (data not shown).

Tolerance to acid, simulated gastric juice and bile salts Tolerance to simulated GI stress conditions: acidity, gastric juice, and bile salts was measured at different times (0, 1, 2, and 4 h) by following the same methodology as that of González-Vázquez *et al.* (14). Bile salt tolerance was tested using bovine bile (Difco), primary bile acids conjugated either taurine (taurocholic acid) or glycine (glycocholic acid) (Sigma-Aldrich, St. Louis, MO, USA), and a secondary bile acid (deoxycholic acid; Sigma-Aldrich). Each test was conducted in triplicate.

Statistical analysis The results of tolerance to the simulated GI stressing conditions among the isolates were statistically analyzed by ANOVA ($p \leq 0.05$) using an R free software (<http://www.R-project.org>, 2010) The data used were generated from triplicates examined in three independent experiments.

Results and Discussion

Isolation, identification and partial characterization of *B. pumilus*

It has been suggested that different geographical regions and seasons can result in different pulque microbiota (4). Despite this, after 3 h of fermentation, eight Gram-positive rod shape catalase positive bacteria were isolated, they showed hemolytic activity and high identity (97-99%) with *Bacillus pumilus*. These results were corroborated with those of the phylogenetic analyses between the isolates and their respective *Bacillus* relatives (Fig. 1). The isolates from the region of Singuilucan were classified as S1, S2, S3, and S4. The ones from Huitzilac were H1, H2, H3, and H4.

Cervantes and Pedroza (18) reported that during the production of pulque using agave sap from Tlaxcala state, the Gram-positive bacilli CFU increased from 1.7×10^2 to 3.4×10^9 per mL after 24 h of fermentation. In this study, we isolated eight *Bacillus* strains using a methodology to isolate lactobacilli that can tolerate simulated GI stress conditions. The MRS broth has been used by other authors to isolate and grow bacillus strains (19) and because it is an enriched medium, some other acid-resistant bacteria can grow in it (20). Some authors have identified *Bacillus* in pulque (8) but its toxicity or role in its fermentation has not been studied. Our results are the first report about the isolation of *Bacillus* with hemolytic activity from pulque of

Fig. 1. Phylogenetic tree based on 16S ribosomal gene sequences showing the relationship between the isolated *Bacillus* and *Bacillus pumilus*. The phylogenetic tree was constructed using the Neighbor Joining algorithm. Bootstrap analysis of 1,000 replications was performed and is expressed as percentage.

two different Mexican regions and suggest that not all Gram-positive *Bacillus* from pulque are safe, since the isolates showed hemolytic activity, resistance to some antibiotics (Table 1), and different grades of tolerance to simulated GI conditions ($p \leq 0.05$) (Fig. 2).

B. pumilus is a spore-forming bacteria commonly resistant to extreme conditions; possibly by air flow, the agave sap incidentally gets the spore which is coming from the soil. There are various issues that strongly influence the contamination of agave sap with pathogens, such as the agave collected from plants that grow in pastures can be easily contaminated by animal feces. The agave's cavity is opened to induce secretion of the sap by using no aseptic tools and is then sealed with rocks and agave leaves to keep the pests away with varying degrees of efficiency, which in turn can affect the cavity's temperature, thereby influencing the sporulation of bacteria; the sap is collected manually through a hollow plant stalk using a hand-held cup or through other regional methods (1); conditions of poor

personal hygiene and utensils prevail during the collection (4). Therefore, the safety of this beverage has to be assured by diminishing the sources of contamination and improving the inadequate artisanal practices.

Genotypic identification In this study, all *Bacillus* isolates were identified as *B. pumilus* using primers LAC1 and LAC 2, which are usually used to identify lactobacilli. Furthermore, these primers would not bind exclusively to the 16S rRNA gene of lactobacilli but would also anneal with other species (15).

Phenotypic identification Regarding the resistance to antibiotics, strains S1, S2, and H3 showed resistance to the β -lactamic antibiotics group. This group is more prescribed in primary care and hospitals (21). Strain S3 showed resistance to pefloxacin and erythromycin. The first one is a quinolone commonly used to treat lower urinary

Table 1. Resistance to antibiotics shown by the isolated *Bacillus pumilus* from Singuilcan and Huitzilac regions

Strain	Antibiotic										
	AM	CF	CTX	PEF	CXM	DC	E	GE	LEV	PE	SXT
<i>L. casei</i> Shirota (control)						+		+			
<i>B. pumilus</i> S1	+						+			+	
<i>B. pumilus</i> S2	+						+			+	
<i>B. pumilus</i> S3				+			+				
<i>B. pumilus</i> S4				+							
<i>B. pumilus</i> H1				+							
<i>B. pumilus</i> H2				+							
<i>B. pumilus</i> H3	+									+	
<i>B. pumilus</i> H4				+							

Ampicillin (AM, 10 μ g), cephalothin (CF, 30 μ g), cephalexin (CTX, 30 μ g), pefloxacin (PEF, 5 μ g), cefuroxime (CXM, 30 μ g), dicloxacillin (DC, 1 μ g), erythromycin (E, 15 μ g), gentamicin (GE, 10 μ g), levofloxacin (LEV, 5 μ g), penicillin (PE, 30 μ g), tetracycline (TE, 30 μ g), and trimethoprim-sulfamethoxazole (SXT, 25 μ g). Resistance is shown by + sign.

Fig. 2. Tolerances to (A) acidity, (B) gastric juices, (C) taurocholic acid, (D) glycocholic acid, (E) deoxycholic acid, and (F) oxgall of *L. casei* Shirota (Yakult®) (●), S1 (○), S2 (▼), S3 (△), S4 (■), H1 (□), H2 (◆), H3 (◇), H4 (▲). Values are means of triplicates from three separate runs.

tract infections. In spite of being from different regions, strains S4, H1, H2, and H4 were also resistant to pefloxacin (Table 1). These results suggest that the *B. pumilus* isolates could be different strains but their resistance to this and other antibiotics is of high relevance since it has been reported that *B. pumilus* contains in its genome a putative integrative and conjugative element associated with the spreading of antibiotic resistance in bacterial communities (22). It has been suggested that although the soil resistome is the most important reservoir of resistance from an evolutionary perspective, the human commensal microbiota incorporates the resistome that is most accessible to human pathogens (23); therefore, owing to the possibility of genetic information interchange between *B. pumilus* and the intestinal microbiota, there are strong reasons why this kind of a beverage must be drunk with caution. This evidence also suggests that the production of pulque must be improved to avoid the presence of such microorganisms in the beverage. In addition, *B. pumilus* can produce toxins (24) and is associated with bacteremia in patients with cancer (25). It also has toxic properties; cytopathic effects in Vero cells, lecithinase production, proteolytic action in casein (26), and β - or γ -hemolytic effect in blood agar (27). In fact, all *B. pumilus* isolates were hemolytic, indicating a reduction in the hemoglobin or red blood cells in the medium to methemoglobin. The reduction of hemoglobin could produce a health condition known as methemoglobinemia in which the iron within the hemoglobin molecule is oxidized from the ferrous to the ferric state. This oxidized hemoglobin has an impaired ability to transport oxygen and carbon dioxide (28). This activity is not desirable in bacteria involved in fermented foods because of their pro-oxidative effects (29). Hemolytic activity has been associated with pathogens such as *Rickettsia*, *Shigella*, *Trypanosoma cruzi*, and *L. monocytogenes* since they use it to enter to the host cytoplasm (30). In patients with

debilitated immune states, *B. subtilis*, *B. licheniformis*, and *B. pumilus* have been associated with a range of clinical conditions, food spoilage, and incidents of food-borne gastroenteritis (31). In this study, the amount of *B. pumilus* in the samples of the pulque studied was not determined; however, the fact is that these microorganisms were present in this beverage and could be dangerous to the vulnerable population.

Performance in simulated GI conditions In contrast, *B. pumilus* is salt tolerant (32) and this is associated with the tolerance of the GI tract stress by the mechanism of cross resistance in bacteria (33), which is not desirable in pathogens because it could lead to the colonization of GI (22,33). Therefore, we decided to evaluate the ability of the *B. pumilus* isolates to tolerate this stress. During the process, some tests were not done on some *Bacillus* because they lacked the ability to tolerate GI stress. The results indicate that the use of a stress-inducing medium allowed the isolation of the bacteria that could tolerate these conditions. It also allowed the isolation of hemolytic *B. pumilus*. During the acid tolerance test, *L. casei* Shirota survived for 1 h. A similar survival profile was exhibited by *B. pumilus* strains S4 and H2. Strain H3 exhibited a lower resistance compared with *L. casei* Shirota. The other *B. pumilus* strains (S1, S2, S3, H1, and H4) survived over 4 h under the experimental conditions (Fig. 2A).

Regarding the tolerance to gastric juice, strains S4, H1, H2, and H3 could not tolerate this condition for 1 h (Fig. 2B); therefore, it could not come up to the duodenum, where bile salts are released. Regarding strain S2, it survived only for 3 h, whereas strains S1, S3, and H4 survived for 4 h (Fig. 2B); therefore, these strains could likely stay alive until they reach the duodenum. During bile salt tolerance test, when the strains were exposed to taurocholic acid, only strains S4 and H3 were not able to survive during the 4 h of exposure (Fig.

2C). Two isolates (S2 and H2) were not able to survive when exposed to glycocholic acid (Fig. 2D). In contrast, only the strain S4 did not survive when exposed to the secondary bile salt deoxycholic acid (Fig. 2E). Only three strains (S1, S3, and H2) survived during 4 h of the exposure under a mixture of bile salts (oxgall), whereas the other five strains did not survive (S2, S4, H1, H3, and H4). Strains S1 and S3 showed tolerance to low pH (Fig. 2A and 2B) and bile salts (Fig. 2C-2F). Ridlon *et al.* (34) suggested that glycine conjugates are higher in proportion (3:1) than taurine conjugates in human bile; therefore, this strain may die on interaction with this salt in the intestine. Oxgall had a larger effect over the viability of all the strains than the other salts tested, suggesting a toxic synergistic effect since oxgall contains fatty acids, inorganic salts, sulphates, bile pigments, cholesterol, mucin, lecithin, glucuronic acids, porphyrins, and urea (14). Strain S2 and H4 did not tolerate oxgall for more than 1 h. Although strains S1, S3, and H2 did tolerate this condition, only strains S1 and S3 survived the other stress conditions; therefore, they could exert negative effects on the host depending on their immunity conditions.

Some work related to pulque in the presence of *Bacillus* has been published; however, it has not been isolated and little attention has been focused on these microorganisms in terms of their participation in the process of pulque elaboration and their impact on health. In the present study, the topic regarding their health effects has been studied since the results showed that some of the isolated hemolytic *Bacillus* could tolerate the GI tract stress and could lead to a negative health effect. Therefore, the study of bacteria other than probiotics, present in this beverage and that prove to be non-beneficial to human consumption, is necessary. These results suggest that *B. pumilus* could be non-beneficial to pulque consumers and also there is a high risk of pulque contamination with other pathogenic bacteria because of their production system, nevertheless further information about certain toxins such as the gene responsible for possible toxin production by *B. pumilus* and the ability of this microorganism to show pathogenicity is needed.

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Disclosure The authors declare no conflict of interest.

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