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Evaluation of Probiotic Quality of Lactic Acid Bacteria Isolated from Locally Fermented Palm Wine and Kunu from Sellers in Oluku and Oba Markets in Benin City, Edo State, Nigeria

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ABSTRACT: Commonly used probiotics bacteria are the lactic acid bacteria (LAB) from the gastro intestinal tract. However, other LAB from exogenous origin having similar functional properties can also confer health benefit to the host. The objective of this work is to evaluate the probiotic quality of lactic acid bacteria isolated from locally fermented palm wine and kunu from sellers in Oluku and Oba markets in Benin City, Edo State, Nigeria using standard microbiological methods. *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus casei* were isolated from palm wine and kunu, and they were assessed for the following: resistance to simulated gastric and intestinal fluids, resistance to bile salt and low pH, haemolytic activity and their antimicrobial characteristic against some bacteria pathogens, mainly; *Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Escherichia coli*. Results showed resistance/tolerance and cell viability of all LAB isolates to simulated gastric and intestinal fluids, and low pH resistance of all isolates were negative at pH 2 but positive with viable cell growth at pH 3. The *L. fermentum, L. plantarum,* and *L. acidophilus* were not haemolytic while *L. casei* was haemolytic (alpha haemolysis). The LAB isolates were more resistance to bile salt (with viable cell growth) at 0.1% and 0.3% concentration compared to 0.5% concentration. Antibacterial activity testing showed that *L. acidophilus* and *L. casei* had antibacterial property against *Pseudomonas aeruginosa*, while *L. plantarum* and *L. fermentum* had antibacterial property against *Staphylococcus aureus* and *Klebsiella pneumonia* respectively. While all lactic acid bacteria (*Lactobacillus* species) isolated from the locally fermented food/product samples demonstrated strong probiotic characteristic properties, further *in-vivo* study to establish their probiotic potential is recommended.

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The use of probiotics, which, when given in sufficient proportions, benefit the host in terms of health (FAO/WHO, 2002), is one of the most promising areas for the development of functional food components. These probiotics, which are living microbial food additives, have an impact on the composition of the gut microbiota. The most prevalent non-pathogenic bacteria, lactic acid bacteria (LAB), are essential to our

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daily lives because they perform a variety of essential functions such as fermentation, food preservation, vitamin generation, and cancer prevention thanks to their probiotic qualities. These germs are one of the important bacterial groups that reside in the gastrointestinal system, and their significance has just come to light (Krishnendra *et al*., 2013). But certain lactobacilli have been found to provide health advantages, including enhanced lactose absorption, anti-cholesterol and anti-carcinogenic properties, and defence against various disorders (Krishnendra *et al*., 2013). In particular, Lactobacillus species are recognised as makers of antimicrobial substances, particularly bacteriocins, which have significant antibacterial activity (Aween *et al*., 2012). It is crucial to assess this characteristic in isolates that are candidates for probiotics since the generation of these chemicals by intestinal microflora is one of the most significant processes responsible for the antagonistic action against intestinal infections (Bilkova *et al*., 2011). Effective probiotics should have antibacterial properties, especially against gastrointestinal infections (Klayraung *et al*., 2008).

An alcoholic beverage known as "palm wine" is made from the sap of several types of palm trees. Particularly popular regions for the beverage include South India, the Philippines, and areas of Africa. Raffia, kithul, and Nipa palms, as well as oil palms like *Elaeis guineensis*, are where the sap is most frequently harvested in Africa (Ukhum *et al*., 2005). In addition to fermenting yeast from different genera, such as *Saccharomyces*, *Candida*, *Endomycopsis*, *Hausenula*, *Pichia*, *Saccharomycodes*, and *Schizosaccharomyces* (Ezeronye and Legras, 2009; Chandrasekhar *et al*., 2012), lactic acid bacteria strains of *Lactobacillus plantarum*, and *Leuconostoc mesenteriodes*. Because of the living bacteria and yeast that are present in palm wine as a consequence of spontaneous fermentation, it is milky white and bubbly (Ezeronye and Legras, 2009). It has been demonstrated that palm tree sap provides a rich medium that can sustain the growth of many different kinds of microorganisms. In general, the methods used to tap palm trees and collect palm sap, as well as the surrounding air and environment, have an impact on the sap's microbial composition (Amoa-Awwa *et al*., 2007; Naknean *et al*., 2010).In Nigeria, especially in the north, kunu (also known as kunu zaki) is a common fermented cereal-based food drink. The beverage kunu is a good source of vitamins, particularly B1 and B2, as well as important minerals. It can also be manufactured from maize, although it is often prepared from a grain like millet or sorghum. Sorghum-based beverages have a milky, light-brown hue, while millet- and maize-based beverages are whitish in colour. Few probiotic bacteria, such as those found in palm wine and kunu, are of exogenous origin; the majority of widely used probiotic bacteria were isolated from the gastro intestinal tract. Therefore, the objective of this work is to evaluate the probiotic quality of lactic acid bacteria isolated from locally fermented palm wine and kunu from sellers in Oluku and Oba markets in Benin City, Edo State, Nigeria

MATERIALS AND METHODS

Sample collection: Fresh quantities of palm wine and kunu samples were obtained in sterile plastic containers from local sellers in the Oluku and Oba markets in Benin City, Edo State, and were then immediately taken to the laboratory for microbiological analysis.

Isolation, characterization and identification of lactic acid bacteria (LAB): The LAB were isolated from the palm wine and kunu samples by pour plate method using De Man Rogosa and Sharpe (MRS) agar after serial dilution. Ten millilitres (10ml) of thawed samples were dispersed in 90ml of sterile saline to obtained 10^{-1} dilution. Further dilution was made until a 10^{-3} dilution was obtained. The Aliquot portion the dilutions were aseptically inoculated into sterile plates and 15ml of De Mann Rogosa Sharpe (MRS) agar was poured (using pour plate method) and allowed to solidify then the plates were incubated at recommended temperature 37^0c for 48 hours under anaerobic conditions (Cheesbrough, 2000). Upon incubation, the colony-forming units (cfu/ml) were counted, after which pure cultured under anerobic condition were obtained.

Bacteria isolates was identified based on colony morphological characteristics and standard biochemical tests as described by Cheesbrough (2000).

Isolation of bacterial pathogens: Bacteria pathogens (*Pseudomonas aeuroginosa, Escherichia coli, Klebsiella pneumonia, and Staphylococcus aureus*) needed for the antibacterial activity assay of the isolated LAB were obtained from stock cultures stored in nutrient agar slant.

Determination of total colony count of the Lactic Acid Bacteria (LAB): Total colony counts of LAB isolates were calculated using De Man Rogosa and Sharpe (MRS) agar and the pour plate technique. The samples were prepared as a threefold (10^{-3}) serial dilution in sterile normal saline. The number of colonies in cfu/ml was calculated after 48 hours of anaerobic incubation at 37 °C on triplicate MRS agar plates of each acceptable dilution (1ml each).

Determination of resistance to simulated gastric and intestinal fluids: The preparation of simulated gastric and intestinal fluids followed Fernández *et al*. (2003). Every day, fresh simulated gastric fluid was made by combining 0.35 g of pepsin with 100 ml of 0.2 % saline. Concentrated hydrochloric acid was used to bring the pH to 3.0, and a 0.22-µm filter was used to sterilise the fluid. 0.1 g of trypsin and 1.8 g of bile salts were suspended in 100 ml of a sterile solution containing 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride to produce simulated small intestine fluid. With 0.5 M sodium hydroxide, the pH was significantly reduced to 8.0. Through the use of a 0.45-µm filter, this solution was sterilized. The simulated stomach fluid was injected with the bacterial strains at a 10 % concentration and a pH of 2.0. The solutions were stirred for 10 seconds before being anaerobically incubated at 37 °C with agitation to mimic peristalsis. After three hours, aliquots of this solution were obtained, and the total number of bacteria that were still alive was calculated. The medium was then separated by centrifugation, replaced with simulated intestinal fluid, and incubated for a further three hours under anaerobic conditions at 37 °C to determine the total viable counts.

Determination of resistance to bile salt: The approach outlined by Lin *et al*. (2007) was used to assess the impact of bile salts. Freshly prepared MRS broths were made with bile salt added at different concentrations of 0.1 %, 0.3 %, and 0.5 % (w/v) (Oxgall bile B8381, Sigma). The isolated lactic acid bacteria were suspended overnight and inoculated (1 %) into MRS broth. After 4 hours of incubation at 37°C, the optical density (OD) at 620 nm was read to determine the number of bacterial cells present in the culture broth. The OD values of the bacteria cultures in MRS broth with bile salt and those in MRS broth without bile salt were then compared to determine the bile tolerance.

$$Survival\ (\%) = \frac{Final\ OD\ value}{control\ OD} \times 100$$

Determination of haemolytic activity: The isolated LAB was then tested for haemolytic activity by streaking on blood agar plates made of sterile nutritional agar and human blood that was added aseptically, mixed, and distributed into sterile plates before setting. The haemolytic activity and zones were then monitored on the plates after a 24-hour anaerobic incubation period at 37 °C to look for evidence of alpha-haemolysis (green zones around colonies), beta-haemolysis (pale yellow/clear zones around colonies), or non-haemolysis (no clear zones around colonies) (Hawaz, 2014).

Determination of low pH resistance: LAB isolate overnight cultures were introduced to MRS broth that had been pH-adjusted to 2 and 3 using 1 M hydrogen chloride (HCl), respectively. The broths were incubated for 6 hours at 37 °C to represent the period food spends in the stomach, and repeated dilution and plating onto MRS agar were used to assess cell viability.

Determination of antibacterial activity: Using a slightly modified version of the agar-well diffusion experiment developed by Karaoglu *et al*. (2003), the antibacterial activity of the LAB isolates against four pathogenic bacteria strains (*Pseudomonas aeuroginosa*, *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus aureus*) was assessed. Using sterile cotton swabs, sterile, solidified nutrient agar plates were carefully streaked with each individual pathogenic bacteria strain's 24-hour-old broth culture. The plates were then rotated 360 degrees. Using a sterile cork borer, four wells were created in each of these plates, each of which was then injected with 0.1 ml of a 24-hour-old culture of lactic acid bacteria isolates. To examine the antibacterial activity of the LAB against the tested bacteria pathogens, plates were left on hold for 30 minutes and then incubated overnight at 37 °C for 24 hours. Results were shown by the presence of a free zone of inhibition close to each well; the diameter of these zones was measured in millimetres using a clear metre rule.

Statistical analysis: The data generated from the antimicrobial potential of the LAB isolates against bacterial pathogens were subjected to Two-way ANOVA to determine if there was a significant difference in the antimicrobial activity with respect to type of LAB isolates and pathogens used, using Microsoft Excel version 2007.

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) must have the ability to endure the stomach's (gastrointestinal tract's) low pH conditions, resist concentrations of bile salts, and possess an antibacterial action against pathogenic bacteria in order to be used as probiotics (Holzapfel, 1997). Based on their morphological and biochemical characterization, the four lactic acid bacteria isolated from palm wine and kunu samples in this study were identified as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus casei*. As recognised natural and spontaneous fermenters, these microbes have been regularly linked to samples of local food (Bertrand *et al*., 2015). Given that gastric acid in the stomach eliminates the majority of microorganisms (Chung *et al*., 1999), lactic acid bacteria as prospective probiotics

must show tolerance and resilience to such circumstance in order to survive. After being exposed to simulated gastric and intestinal fluids for 3 hours, the results showed that the LAB isolates were more resistant to or tolerated simulated gastric fluid with a pH of 3.0 compared to simulated intestinal fluid with a pH of 8.0, and that *L. fermentum* and *L. plantarum* had higher viable counts than *L. acidophilus* and *L. casei* at both times. According to Bertrand *et al*. (2015), LAB isolates' capacity to be more resistant to or tolerant of simulated gastric fluid than intestinal fluids may be connected to the lactic acid bacteria species' inherent adaptability to low pH environments. The findings of this study are consistent with the report of Muma *et al*. (2014), which reported that all lactic acid bacteria (*Lactobacillus* species) isolated from camel milk were more resistant to or tolerant of simulated gastric fluid at pH 3 than intestinal fluid at pH 8.

Table 1: Determination of LAB resistance to simulated gastric fluid

Isolates	Gastric fluid resistance pattern
Lactobacillus fermentum	++
Lactobacillus plantarum	++
Lactobacillus acidophilus	++
Lactobacillus casei	++

Key: *++ indicates resistance / tolerant*

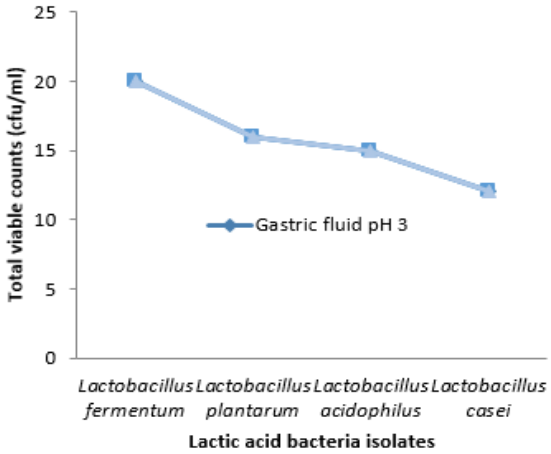

Fig 1: Total viable counts of LAB isolates in simulated gastric fluid

Table 2: Determination of LAB resistance to simulated intestinal fluid

Isolates	Intestinal fluid resistance pattern
Lactobacillus fermentum	++
Lactobacillus plantarum	++
Lactobacillus acidophilus	++
Lactobacillus casei	++

Key: *++ indicates resistance/tolerant*

However, it has been demonstrated that low pH resistance or tolerance is a critical requirement for the probiotic characterization of *Lactobacillus* species, and a pH value of 3.0 has been deemed typical for such a probiotic strain examination (Sahadeva *et al*., 2013). Although pH in the stomach can be as low as 1.0, pH 3 has traditionally been recommended in most *in-vitro* experiments since strain survival is frequently significantly reduced at pH 2 and lower (Prasad *et al*., 1998). Following a 24-hour incubation period, the results of the LAB isolates' resistance to low pH showed that all of the isolates were both resistant and tolerant to pH 3; however, *Lactobacillus fermentum* and *L. plantarum* were more resistant in terms of total viable counts than *L. acidophilus* and *L. casei*. However, none of the LAB isolates had any viable counts as they could not tolerate the pH 2 environment. This was consistent with the earlier study by Argyri *et al*. (2013), which found that nine strains of *Lactobacillus* were more resistant and viable in a pH 3 environment than they were in a pH 2 or lower environment. Additionally, according to Shokryazdan *et al*. (2014), all examined *Lactobacillus* isolates were resistant to or tolerant to low pH 3. When exposed to acidic environments, *L. plantarum* strains were more viable at pH 3 than pH 2. Additionally, in earlier studies by Liu *et al*. (2013) and Jose *et al*. (2015), it was found that *Lactobacilli* tolerated and survived in MRS broth at pH 3, while viability was reduced at pH 2. This capacity of LAB to survive low pH conditions is attributed to their capacity to ferment lactose and produce lactic acid at pH ranges of 2.5–3.0 (Jacobsen *et al*., 1999).

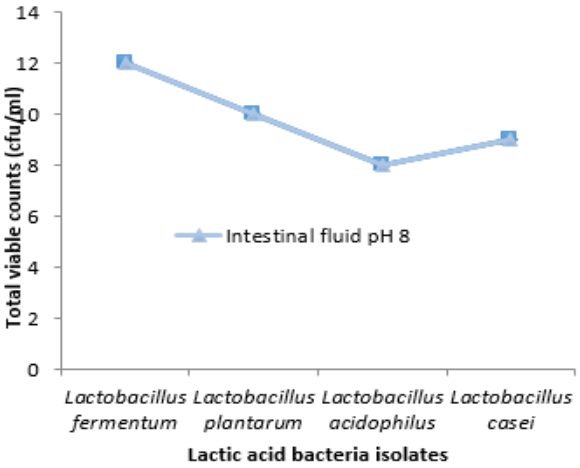

Fig 2: Total viable counts of LAB isolates in simulated intestinal fluid

Table 3: Determination of low pH resistance of LAB isolates

Isolates	pH 2	pH 3
Lactobacillus fermentum	_	++
Lactobacillus plantarum	_	++
Lactobacillus acidophilus	_	++
Lactobacillus casei	_	++

Key: ++ indicates resistance / tolerant: -- indicates sensitive/non-tolerant

Lactobacilli probiotic ability to survive in the presence of bile salt is physiologically significant in terms of bile salt resistance or tolerance (Sjovall, 1959).When the LAB isolates were tested for their capacity to tolerate and resist bile salt at concentrations of 0.1%, 0.3%, and 0.5% (w/v), respectively, and without bile salt (0.0%) as control, it was revealed that at a concentration of 0.1% bile salt, the percentage survival rates of the LAB isolates were: 88% for *L. fermentum*, 81% for *L. plantarum*, 90% for *L. acidophilus* and 88% for *L. casei*. However, the percentage survival rates for *L. fermentum*, *L. plantarum*, *L. acidophilus*, and *L. casei* were 74%, 61%, 76%, and 85% respectively at 0.3% bile salt concentration, and at 0.5% bile salt concentration, the percentage survival rates were 67%, 50%, 57%, and 41% respectively; indicating that the LAB isolates were more resistant/tolerant to 0.1% and 0.3% bile salt concentration than to 0.5%. This result is consistent with a related study by Abriomiel *et al*. (2012), who reported that all LAB isolated from fermented olives were able to grow and survive at a 0.3% bile salt concentration as opposed to a 0.5% concentration. Similar to this, according to Koll *et al*. (2008), test strains of *Lactobacillus* were tolerant to bile concentrations of 0.3%. At a bile salt content of 0.3% - 0.5%, Rahman (2015) showed the growth and viability of *L. fermentum* and *L. acidophilus* isolates from buffalo milk. Additionally, it has been reported that *Lactobacillus* was seen to develop luxuriously and to live well in a 0.3% bile salt supplement, in contrast to a 0.5% bile condition, where poor tolerance was recorded (Jose *et al*., 2015). Due to the presence of bile salt hydrolase (BSH), an enzyme that mitigates harmful effects by conjugating bile, most *Lactobacillus* species exhibit a wide range of resistance (DeRoos *et al*., 2000).

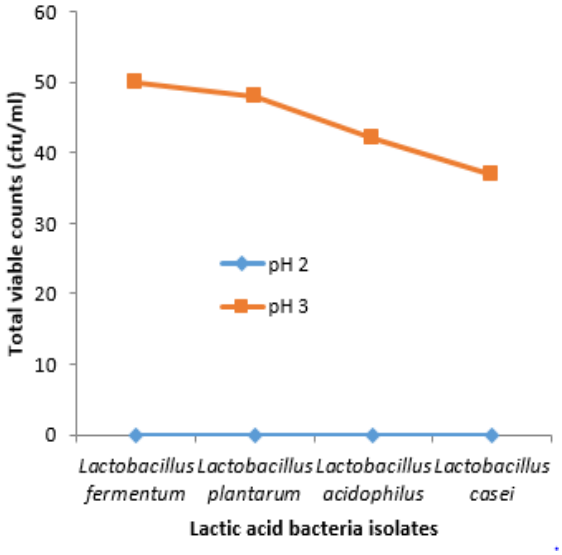

Fig 3: Total viable count of LAB isolates at pH 2 and pH 3

Table 4: Optical density readings at 620nm for resistance to bile salt of LAB isolates

Isolates	Concentration			
	0.0%	**0.1%**	**0.3%**	**0.5%**
Lactobacillus fermentum	0.097	0.085	0.072	0.065
Lactobacillus plantarum	0.110	0.089	0.068	0.054
Lactobacillus acidophilus	0.102	0.092	0.077	0.057
Lactobacillus casei	0.112	0.095	0.058	0.046

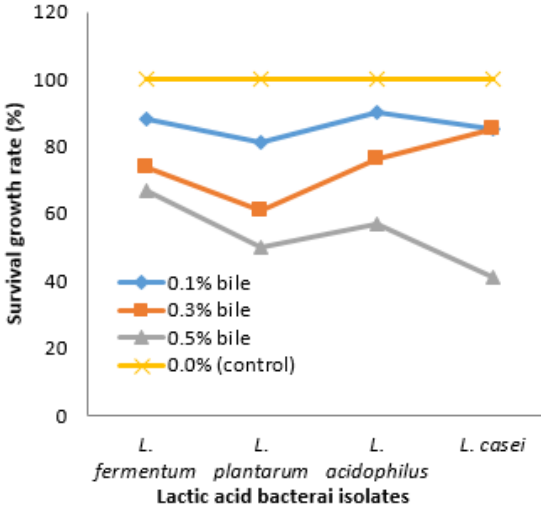

Fig 4: Percentage survival growth rate of LAB isolates at different bile salt concentrations

Table 5: Haemolytic activity assay of LAB isolates on blood agar

Isolates	Haemolytic activity
Lactobacillus fermentum	Non- haemolysis
Lactobacillus plantarum	Non- haemolysis
Lactobacillus acidophilus	Non- haemolysis
Lactobacillus casei	Alpha haemolysis

In order to be evaluated and used as probiotics, lactic acid bacteria strains must not be haemolytic. From this study, *L. acidophilus*, *L. plantarum*, and *L. fermentum* are not haemolytic (no clear zones around colonies indicating non-haemolysis of red blood cells on blood agar), whereas *L. casei* demonstrated alpha haemolysis (green zones around colonies indicating partial or poor haemolysis of red blood cells on blood agar). This finding is in consistent with those of Hawaz (2014), who found that *Lactobacillus plantarum* and *Lactobacillus casei* isolated from curds did not haemolyze when cultured on blood agar, and Halder *et al*. (2017), who found that all *Lactobacilli* isolated from curds did not exhibit haemolysis. Similarly, Amengialue *et al*., 2023 reported an increased haemoglobin level and packed cell volume in *Lactobacillus* species treated rats. The fact that lactic acid bacteria often exhibit either non-haemolysis or just weak haemolytic activity indicates that they are used safely in food. Exotoxins released by microbial strains with beta-haemolytic activity on blood agar plates lyse blood cells, clearing the areas around bacterial colonies. The isolated LABs that were

evaluated for antibacterial activity against some selected pathogenic bacterial strains revealed that all of the isolates had varying degrees of antibacterial activity. The study by Bassyouni *et al*. (2012), who tested *Lactobacillus* species against *Salmonella thyphimurium*, *Escherichia coli*, and *Staphylococcus aureus*, found that the pathogenic bacteria were resistant to a broad spectrum of antimicrobial effects. Additionally, according to Halder *et al*. (2017), all *Lactobacillus* isolates from curd samples exhibited antibiotic activity against *Proteus vulgaris* and *Escherichia coli*. According to some sources, lactic acid bacteria have the potential to create peptides and antimicrobial chemicals such as organic acids, short-chain fatty acids, and bacterocins (antibacterial proteins) that prevent the growth of pathogenic bacteria when present in the same environment (Salminen *et al*., 1998).

Table 6: Antibacterial activity of LAB isolates against bacteria pathogens

LAB isolates	**Zone of inhibition (mm)**			
	S. aureus	*K. pneumonia*	*P. aeruginosa*	*E. coli*
Lactobacillus fermentum	10.00	13.00	10.00	11.00
Lactobacillus plantarum	15.00	10.00	09.00	10.00
Lactobacillus acidophilus	10.00	13.00	15.00	12.00
Lactobacillus casei	11.00	13.00	15.00	12.00

Conclusion: Probiotics serves as dietary supplements with immune system modulation potential. In many instances, dietary supplements containing probiotics provide many of the advantages of immune system strengthening and can also improve defence against infectious diseases. The probiotics that have been and frequently chosen for usage have human origin, either through food or food items. This work have shown that the isolated lactic acid bacteria (*Lactobacillus* species) possessed probiotic-potential traits. To further establish their utility as probiotics, more *in-vivo* research is advised.

Conflict Of Interest: The authors declare that they have no competing/conflicting interests

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