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Original Research Article

Quantitative assessment of available probiotic products in community pharmacies in Benin City, Nigeria

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Abstract

Purpose: *To assess four probiotic products available in community pharmacies in Benin City, Nigeria for accuracy of information on product labels with regard to the quantity and type of microorganisms, pH and bile tolerance, and antimicrobial activity.*
Methods: *Percent label compliance of products was determined, in addition to isolation, identification and enumeration of microorganisms. Determination of pH and bile tolerance was conducted using turbidity studies in MRS broth. Antimicrobial activity against Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Klebsiella pneumonia, Staphylococcus aureus and Candida albicans pathogens was investigated using agar overlay technique.*
Results: *In each product, there was 100 % label compliance with regard to name of probiotic organism, storage condition, dose, expiration date, contact details and batch number. Three-quarters (75 %) of the probiotic products indicated product net quantity, National Agency for Food and Drug Administration and Control (NAFDAC) number, and microbial count; 50 % of products indicated the excipients used, while only 25 % of the products showed their indications. None of the products indicated strain designation. In species identification, Enterococcus faecium was absent in a multi-species product PB1, while PB3 contained Saccharomyces cerevisiae instead of Saccharomyces boulardii. Enumeration showed comparatively low quantities of probiotic organisms. Tolerance to pH 3 and pH 7, and bile levels of 0.3 and 2 % were within acceptable range. The probiotic organisms demonstrated antimicrobial effect specifically against P. aeruginosa, E. coli, B. subtilis, K. pneumonia, S. aureus and C. albicans.*
Conclusion: *Antimicrobial effect and tolerance to pH and bile salts were consistent with acceptable properties of probiotics. However, there is need for total compliance with the indications, strain designation, excipients, and actual quantity of the individual probiotic organisms in the formulations.*

Keywords: *Probiotics, Strain designation, Agar overlay technique, Saccharomyces cerevisiae, Saccharomyces boulardii*

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INTRODUCTION

Probiotics are active microorganisms which, when prescribed in sufficient quantity, provide specific health benefit to the end users [1]. Some microorganisms such as *Sporolactobacillus, Lactococcus, Propionibacterium, Escherichia coli* and *Leuconostoc mesenteroides* [2,3] exhibit similar probiotic properties. These organisms have gained recognition because of their various

health benefits such as prevention of diarrhea in children, modulation of immunity, alleviation of lactose intolerance, prevention of some forms of cancers, and lowering of serum cholesterol [4]. Moreover, it has been reported that some of these organisms prevent the occurrence of infectious diseases [5].

The nomenclature "probiotic" refers to supplements, foods and drugs for human and veterinary use [4]. Regulatory authorities require that the storage condition, expiration date, names of species, strain designations and their corresponding quantities be indicated on the label of each probiotic formulation [6]. It has been suggested that approximately 10^8 to 10^9 probiotic organisms should be prescribed daily for achievement of required health benefits [4].

Healthcare professionals in Nigeria seem to have limited awareness and knowledge of the use of probiotic products, notwithstanding their beneficial effects, which with time can be improved upon by pharmacists [7]. In specific terms, the pharmaceutical microbiologist has a crucial role of ensuring that the emerging awareness and knowledge of probiotic effects are used to match label claims with actual contents so as to ensure the achievement of effective pharmaceutical care in Nigeria.

The present study assessed four probiotic products available in community pharmacies in Benin City, Nigeria, with respect to the accuracy of information on product labels regarding the quantity and types of microorganisms, pH and bile tolerance, and antimicrobial effects.

EXPERIMENTAL

Collection of samples of probiotic products

Four available probiotic products (designated PB1, PB2, PB3 and PB4) were purchased from community pharmacies in Benin City, Edo state, Nigeria. The products were kept appropriately in a cool, dry environment, away from light for two days before the commencement of the study. Information such as name of probiotic organism (genus and species), strain designation, indication, batch number, product net quantity, dose and contact details were recorded. Then, the percentage compliance of the information on each product label was determined.

Isolation and identification of probiotic microorganisms

The content of a capsule or sachet of each product was dispersed in 9 mL of sterile phosphate buffered saline (PBS), pH 7.4 and mixed thoroughly. Then, ten-fold serial dilutions were made in PBS, and appropriate dilutions of PB1-PB4 were plated onto appropriate agar media, and incubated under specific conditions. Dilutions of PB1 were plated on MRS agar (De Man Rogosa and Sharpe) adjusted with 0.05 % (w/v) L-cysteine hydrochloride under anerophilic condition. The PB1 dilutions plated on MRS agar were also incubated under microaerophilic condition. Moreover, PB1 dilutions were plated on nutrient agar incubated under aerobic condition. The PB2 and PB3 dilutions were plated on Sabouraud Dextrose Agar (SDA) and incubated aerobically, while PB4 dilutions were plated on MRS agar and incubated under microaerophilic condition. The seeded agar plates were incubated at 37 ºC for 48 h. Discrete colonies were isolated and labelled. Gram staining status, catalase status [8] and sugar fermentation tests were carried out using ribose, galactose, maltose, mannitol, sucrose, D-fructose, D-glucose and D-lactose. The probiotic microorganisms were identified based on bacterial sugar utilization as measured using Bergey's Manual of Determinative Bacteriology [9], and fungal utilization based on established methods [10]. The confirmed colonies were preserved for further studies.

Enumeration of actual probiotic organisms on selective media

The content of a capsule or sachet of each product was dispersed in 9 mL sterile phosphate buffered saline (PBS), pH 7.4, and homogenized thoroughly. Then, ten-fold serial dilutions were prepared in PBS, and appropriate dilutions of PB1-PB4 were plated onto appropriate selective media and incubated under specific conditions. The PB1 dilutions were plated on MRS agar adjusted with 0.05% (w/v) L-cysteine hydrochloride under anerophilic condition; PB1 was plated on nutrient agar and incubated under aerobic condition; PB1 dilutions plated on MRS agar were also incubated under microaerophilic condition. The PB2 and PB3 dilutions were plated on Sabouraud Dextrose Agar (SDA) and incubated aerobically, while, PB4 dilutions were plated on MRS agar incubated under microaerophilic condition. The seeded agar plates were incubated at 37 ºC for 48 h. Colonies were counted at the end of incubation, and the actual number of viable probiotic organisms in each product was expressed in colony-forming units (cfu) per mL. The identified probiotic organisms were compared with those indicated on the product labels.

Studies on pH tolerance of probiotic microorganisms

The pH tolerance was determined by first culturing the isolates on appropriate broth (MRS broth and Nutrient broth) for 20 h at 37 ºC. Then, 1 x 10^6 cfu/mL of each isolate was added to 10 mL of appropriate broth adjusted to pH values of 1.5, 3.0 or 7.0, using 0.1 M HCl. The broth cultures were incubated for 24-48 h at 37 ºC; microbial survival determined on turbidity [11].

Studies on bile salt tolerance of probiotic microorganisms

In the determination of bile salt tolerance, the isolates were first cultured on appropriate liquid media for 20 h at 37 ºC. Specifically, 20 µL of each isolate containing 1 × 10^6 cfu/mL was added to 10 mL of appropriate broth adjusted to bile salt levels of 0.3 and 2 %. The inoculated broths were incubated for 24 - 48 h at 37 ºC, depending on the organism, and percentage survival was determined based on turbidity of the broth culture [11].

Determination of antimicrobial effects of probiotic organisms

The antimicrobial effects of probiotic organisms against some test pathogens (*Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Klebsiella pneumonia*, *Staphylococcus aureus* and *Candida albicans*) were determined with soft agar overlay technique, with some modifications [12]. In this process, MRS agar plates containing confluent growths of the probiotic isolates in spots ranging from 4 - 5 mm were overspread with soft Muller-Hinton agar pre-inoculated with the test pathogens. The overlaid media were allowed to set and subsequently incubated at 37 ºC for 24 – 48 h. The diameter of zone of inhibition (DZI) was recorded as index of antimicrobial effect, while the absence of DZI was considered as lack of antimicrobial effect.

Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.10 program. Test for statistical significance were done using Kruskal-Wallis non-parametric ANOVA, paired *t*-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Each of the probiotic products indicated the contact details, expiration date, dose, batch number, storage instructions and names of the organisms up to species level. Seventy-five percent (75 %) of the products provided information with respect to NAFDAC approval number, product net quantity and microbial count. Fifty percent (50 %) of the investigated probiotic products indicated the excipients used in the preparations. However, only 25 % of the products showed indications, while none of the products showed strain designation. These results are presented in Table 1.

Table 1: Content labels of probiotic products

Label content	Compliance (%)
Contact details	100
Expiration dates	100
Dose	100
NAFDAC number	75
Product net quantity	75
Excipients	50
Batch number	100
Storage instructions	100
Indications	25
Microbial count	75
Strain designation	-
Name-species	100
Name-genus	100

The identities of the selectively-isolated organisms and results of their respective Gram staining, catalase test and sugar utilization tests are shown in Table 2. The isolates obtained were labeled as PB1^a, PB1^b, PB1^c, PB2, PB3 and PB4. A careful review of the results showed that PB1^a was a Gram-positive and catalase-negative bacillus, with the utilizable sugars ribose, galactose, maltose, sucrose, D-fructose, D-glucose and D-lactose. Mannitol was the only non-utilizable sugar for PB1^a. Based on the selective medium used in its isolation (0.05% w/v L-cysteine hydrochloride under anerophilic condition) and the results of the identification tests, PB1^a was identified as *Bifidobacterium infantis.*

The probiotic isolates PB1^c and PB4 were Gram-positive and catalase-negative bacilli which utilized ribose, galactose, maltose, sucrose, D-fructose, D-glucose and D-lactose, but did not metabolize mannitol (non-utilizable sugar). These identification results, and the selective medium used for their isolation (MRS agar incubated in a microaerophilic condition) revealed the presence of *Lactobacillus acidophilus.*

Probiotic isolate PB2 was Gram-positive and catalase-positive cocci, with the utilizable sugars maltose, sucrose, D-fructose and D-glucose, and non-utilizable sugars ribose, galactose, mannitol and D-lactose. A positive identification of *Saccharomyces boulardii* was made, based on

the selective medium used for isolating PB2 (SDA incubated in aerobic condition) and the results of the various identification tests.

The probiotic isolate PB3 showed a similar pattern as PB2 with respect to the selective medium used for its isolation, and identification tests, but differed specifically in the galactose utilization test: PB3 was able to utilize galactose, which is consistent with *Saccharomyces cerevisiae.*

It should be noted that *Enteroccocus faecium* which was indicated in the label of PB1[b], was absent in the actual determination.

The quantity indicated on the label of PB1 did not tally with the relative quantity of the probiotic organism obtained. There was no indication of quantity of organisms on label of PB2, while the labels on PB3 and PB4 captured their respective quantities. Apart from PB1[b], the actual quantities of all isolates ranged from 1.6×10^3 to 1.0×10^9 cfu/mL, as shown in Table 3.

The pH and bile salts tolerance are shown in Table 4. *Bifidobacterium infantis*, *S. boulardii, S. cerevisiae, L. acidophilus* (PB1) and *L. acidophilus* (PB4) showed growth in media of pH 3.0 and pH 7.0, as well as in media containing 0.3 and 2.0 % bile salts. However, there were no growths in a medium of pH 1.5 (Table 4).

The antimicrobial effects of the probiotic organisms are shown in Table 5. The antimicrobial effect of *B. infantis* and the respective DZI ranges against the test pathogens were: 6 - 8 mm (*C. albicans*); 9-11mm (*E. coli, K. pneumonia,* and *P. aeruginosa*), and 13 - 15mm (*B. subtilis* and *S. aureus*).

Table 2: Probiotic microorganisms in the probiotic products analyzed

Identification test	Probiotic isolate					
	PB1[a]	***PB1[b]***	***PB1[c]***	***PB2***	***PB3***	***PB4***
Gram staining	GPB	Absent	GPB	GPC	GPC	GPB
Catalase	–	Absent	–	+	+	–
Ribose	+	Absent	+	–	–	+
Galactose	+	Absent	+	–	+	+
Maltose	+	Absent	+	+	+	+
Mannitol	–	Absent	–	–	–	–
Sucrose	+	Absent	+	+	+	+
D-fructose	+	Absent	+	+	+	+
D-Glucose	+	Absent	+	+	+	+
D-Lactose	+	Absent	+	–	–	+
Probiotic organisms	*B. infantis*	–	*L. acidophilus*	*S. boulardii*	*S. cerevisiae*	*L. acidophilus*

GPC = Gram-positive cocci, GPB = Gram-positive bacilli

Table 3: Actual counts of probiotic organisms, in relation to contents shown on label content

Probiotic sample	Probiotic organism		Quantity (cfu/mL)	
	Label	***Actual***	***Label***	***Actual***
PB1[a]	*B. infantis*	*B. infantis*		5.0×10^3
PB1 PB1[b]	*E. faecium*	Absent	1.2×10^7*	–
PB1[c]	*L. acidophilus*	*L. acidophilus*		1.0×10^6
PB2	*S. boulardii*	*S. boulardii*	–	1.0×10^9
PB3	*S. boulardii*	*S. cerevisiae*	5.0×10^9	1.6×10^3
PB4	*L. acidophilus*	*L. acidophilus*	1.0×10^8	1.0×10^5

Bifidobacterium infantis = B.infantis, Saccharomyces cerevisiae = S. cerevisiae, Lactobacillus acidophilus = L. acidophilus, Saccharomyces boulardii = S. boulardii, Enteroccocus faecium = E. faecium, *Uncategorized

Table 4: Effect of pH and bile salts on growth of probiotic organisms

Probiotic organism	pH			Bile salt (%)		
	1.5	3.0	7.0	0	0.3	2.0
B. infantis	–	+	+	+	+	+
L. acidophilus (PB1)	–	+	+	+	+	+
S. boulardii	–	+	+	+	+	+
S. cerevisiae	–	+	+	+	+	+
L. acidophilus (PB4)	–	+	+	+	+	+

Note: + = growth, - = no growth

Table 5: Antimicrobial effects of the probiotic organisms

Probiotic organism	Pathogenic organism					
	B. subtilis	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*S. aureus*	*C. albicans*
B. infantis	+++	++	++	++	+++	+
L. acidophilus (PB1)	+++	++	+	+	++	+
S. boulardii	++	++	++	+	++	+
S. cerevisiae	+	+	+	+	++	+
L. acidophilus (PB4)	++	+	+	+	++	+

B. subtilis =Bacillus subtilis, E. coli =Escherichia coli, K. pneumonia =Klebsiella pneumonia, P. aeruginosa =Pseudomonas aeruginosa, S. aureus =Staphylococcus aureus, = C. albicans =Candida albicans. (+ = 6 – 8 mm; ++ = 9-11 mm; +++ = 13 – 15 mm)

The antimicrobial effect of *L. acidophilus* (obtained from probiotic product PB1), and the respective DZI ranges against the test pathogens were: 6 - 8 mm (*K. pneumonia, P. aeruginosa,* and *C. albicans*); 9 - 11mm (*E. coli* and *S. aureus*), and 13 – 15 mm (*B. subtilis*).

For *S. boulardii* the antimicrobial effect and ranges of DZI against the pathogens tested were: 6 - 8 mm (*P. aeruginosa* and *C. albicans*) and 9 - 11mm (*B. subtilis, E. coli, K. pneumonia,* and *S. aureus*). The antimicrobial effect of *S. cerevisiae* and ranges of DZI against the various pathogens tested were: 6 - 8mm (*B. subtilis, E. coli, K. pneumonia, P. aeruginosa* and *C. albicans*); and 9 - 11mm (*S. aureus*). For *L. acidophilus* (obtained from probiotic product PB4) the antimicrobial effect and ranges of DZI against test pathogens were: 6 - 8mm (*E. coli, K. pneumonia, P. aeruginosa* and *C. albicans*); and 9 -11mm (*B. subtilis* and *S. aureus*).

DISCUSSION

There was label compliance of 100 % in the names of probiotic organisms which were shown in terms of genus and species, and also in storage condition, dose, expiration date, contact details and batch number, as expected for a probiotic drug. Results from label content study did not differ significantly from label indications. However, product net quantity, NAFDAC number, microbial count, excipient and indications were not fully stated. None of the probiotic products showed strain designation. The partial compliance and absence of strain designation seen in this study are consistent with results obtained in a similar study [13]. The incomplete labeling suggests that these products were not subjected to scrutiny by the relevant regulatory agencies in Nigeria. Since probiotic organisms are strain-specific, strain designation is necessary because it enables prescribers to match the probiotic organisms used in the products with their origins and health benefits. There is a need for absolute compliance with the label contents of the probiotic formulations for the benefit of both the prescriber and the end users.

In the isolation and identification of probiotic organisms, the indicated label contents of single-species products PB2 and PB4 i.e. *S. boulardii* and *L. acidophilus,* respectively, were consistent with their actual contents. The multiple-species product (PB1) ought to contain three probiotic organisms labelled $PB1^a$, $PB1^b$ and $PB1^c$, based on the label. The labels of $PB1^a$ and $PB1^c$ probiotic organisms indicated contents of *B infantis* and *L.acidophilus* which were also consistent with the actual contents, while *E. faecium* which was stated on the label of $PB1^b$ was absent in the actual determination. Another single-species product PB3 actually contained *S. cerevisiae* instead of *S. boulardii* indicated on the label. The discrepancies between label claims and actual contents seen in this study are similar to findings in a related work [14]. These negative features may have resulted from omissions or losses during technological processing of the microorganisms, and they are likely to affect the overall synergistic and species-specific effects of the probiotic organisms. The absence of a particular strain from PB1 and the mislabeling in PB3 are not in conformity with the Food and Agriculture Organization (FAO) and World Health Organization (WHO) recommendations on products containing probiotics. Comprehensive review and strict adherence to standard operating procedures (SOPs) should be used to minimize the observed errors.

In the enumeration test, the label content for PB1 did not categorize the different probiotic organisms. This product actually contained low concentration of $PB1^a$ (*B. infantis*) and adequate concentration of $PB1^c$ (*L. acidophilus)*. The quantity of probiotic organisms (*S. boulardii*) was not shown in label contents of PB2 with respect to adequate/actual concentration, while PB3 and PB4 actually contained lower concentrations than were stated in their respective labels, although

the differences were not statistically significant. Several studies which evaluated the quality of probiotics have shown similar enumeration patterns [15]. Poor storage conditions of probiotic products in community pharmacies are likely to contribute to the low concentrations of organisms observed among the probiotic products analyzed.

All the probiotic organisms isolated from products PB1, PB2, PB3 and PB4 exhibited tolerance to pH 3.0 and pH 7.0, but they did not tolerate pH 1.5. A good source of probiotics should survive in a medium of pH 3.0, which is consistent with what was observed in all the probiotic organisms isolated from the probiotic products [16]. This implies that all the probiotic organisms can withstand the denaturing effect of gastric acid during transition from the stomach to the duodenum. Moreover, the probiotic isolates showed tolerance to bile salts at levels of 0.3 and 2 %, which is within and above the 0.3 % bile tolerance recommended for the selection or identification of tolerance of probiotic organisms for human use [17]. This demonstrates the ability of the probiotic organisms to resist the cell membrane-solubilizing effect of bile as they move through the duodenum to the distal ileum and colon. The tolerance of the organisms to pH and varying concentrations of bile salts were within acceptable ranges for the gastrointestinal viability of each of the studied probiotic products.

The individual probiotic species isolated from the pharmaceutical products demonstrated growth-inhibitory properties as shown by their significant antimicrobial effects against *P. aeruginosa, E. coli, B. subtilis, K. pneumonia*, *S. aureus* and *C. albicans.* The observed antimicrobial properties of the isolates are consistent with results obtained in a similar investigation [18]. The lower DZI seen for *C. albicans* may have resulted from the morphological differences (*C. albicans* is a fungus), when compared with other test organisms (bacteria) used in this determination. The low DZI values are consistent with the beneficial antimicrobial effects of probiotic organisms in the formulations.

CONCLUSION

Four products PB1, PB2, PB3 and PB4 have been analyzed in this study. The results reveal some degree of non-compliance of the products with regulatory requirements. There is an urgent need for compliance with extant regulatory specifications for probiotic products. The findings in this study point to the need for stricter regulation of the quality of probiotic products by the appropriate regulatory agencies.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contributions of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Oloton E. and Obaseki E. contributed to the study conceptualization and design, data acquisition, manuscript preparation, and critical revision of the manuscript. All the authors contributed significantly to the study, and they all agree with the contents of the manuscript.

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