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Short report

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Expired Probiotics: What is Really in Your Cabinet?

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Abstract:

Background: The popularity of using probiotics has surged, since they became widely accepted as safe and help improve general health. Inevitably, some of these products are used after expiration when microbial cell viability is below the recommended effective dose. Given that probiotics are live microorganisms administered in adequate amounts, the aim of this study was to measure viability in expired products and assess how packaging and storage conditions impact efficacy, if at all.

Results: Thirty-three expired probiotic products were evaluated, of which 26 were stored in conditions recommended by the manufacturer. The viable microbial counts were enumerated and representative isolates identified by 16S and ITS rRNA gene sequencing. While the products had a mean past expiration time of 11.32 (1 to 22) years, 22 still had viable contents, and 5 were within or above the original product cell count claim. Product formulation, and number of species present did not appear to impact the stability of the products. However, overall packaging type, storage conditions and time since expiry were found to affect viability. All products with viable cells had the strain stipulated on the label.

Conclusion: Despite some selected probiotic products retaining viability long past their expiry date (indicating long term storage is possible), the total counts were mostly well below that required for efficacious use as recommended by the manufacturer. Consuming expired probiotics may not yield the benefits for which they were designed.

Key words: Expired, Probiotic, Microbe, Health benefit

Background:

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1] and are packaged and sold for use, largely via consumption. They can potentially offer various benefits such as preventing antibiotic-associated and traveller's diarrhea [2], reducing the symptoms of irritable bowel syndrome [2], lowering blood cholesterol [3], and impacting a range of other conditions [2]. With increasing recognition of the benefits of probiotics, the global market is expected to surpass \$52 billion U.S. dollars by 2020 [4]. A considerable amount of product has entered the market over the past ten years, with presumably some accumulating in households. A survey indicated that approximately one-fifth of Americans had not tidied their personal medications within the preceding three years [5], so there is potential for consumers to accumulate and use expired probiotic products.

With the accelerating costs of medications, the use of expired healthcare products has become a topical issue. Institutions that hold large stocks of prescription medicines, such as the United States military, have looked at the stability of active ingredients in long-expired prescription medications [6]. Over the past 35 years, hundreds of drugs have been tested and 90% of them were found to be safe and effective after expiration [6-8]. The expiration for medications is often for commercial use rather than being indicative of medical potency [7,8]. Vitamin supplements, for example, lack expiration studies, resulting in active ingredients being underestimated to ensure 100 percent of the supplement remains at expiration [9]. In the case of probiotics, manufacturers often "front load" to ensure a certain number of viable cells are remaining at the expiry date and ideally like them to be above this level for two years at room temperature.

As probiotics comprise live organisms, it is important to monitor handling and storing probiotics so that they do not become contaminated or degrade in a manner that makes their components harmful. While the proportion of the population willing to use probiotics after expiration has not been determined, an estimated 9.34-19.25% of people do not check the expiration date on their medicines before using them and it is probable that some of those who notice their probiotics have expired will still use them regardless [10,11]. A study of university students revealed they presumed that the life of medications can be extended past expiration with refrigeration [12]. Furthermore, as some probiotic products are expensive [13], consumers may be reluctant to discard them.

Probiotic products are available in a variety of forms and packages that include: refrigerated food, metal blister packs, plastic-metal sachets, capsules, and tablets placed in plastic and glass bottles. These formats provide varying degrees of protection from the main causes of microbial cell death under storage, namely high-water activity, oxygen and heat, yet few published scientific studies have compared packaging modalities [3, 14-16]. While there may be a potential benefit from the consumption of dead microorganisms, these are not classified as probiotic. Studies have found health benefits can be conferred

from consumption of dead, heat-treated or killed probiotics, metabolites, cell fractions, culture supernatant and probiotic microbial DNA [17-19]. This is not surprising as anti-inflammatory and immunomodulation effects can be induced by immune system stimulation by cell-free supernatants [20,21], peptidoglycans [21], LPS [22], exopolysaccharides [23], teichoic and lipoteichoic acids [24,25]. This demonstrates that there may be further use for probiotic products beyond expiry [18].

The goal of the present study was to assess the viability of randomly acquired, expired probiotic products and look for correlations with packaging, storage conditions, and the time since expiry. Where possible, the microorganisms present in each probiotic product were identified by DNA sequencing to ensure that the correct labelling of the product had occurred.

Methods:

Test products. Thirty-three products listed in Table 1 were tested, of which 23 are shown in Figure 1. They had been acquired over a number of years and stored at room temperature in sealed containers.

Study outline. The following information was collected for each probiotic product evaluated: genus, and species of bacteria, minimum colony forming units (CFU), packaging type, storage recommendations, expiration date and storage conditions. Products without listed expiration dates were excluded from the study.

One dose (capsule, lozenge powder etc.) of the probiotic product was added to 10 mL of sterile phosphate-buffered saline (PBS). If the dosage format was encapsulated, the powder was added to the PBS; if there was no capsule, the whole contents were added. The mixture was vortexed until homogenous and serially diluted in PBS; five μ L of the first through sixth dilutions was inoculated, drop plate wise, on various types of selective media as described following. When sufficient quantities of the probiotic product were available, six capsules were tested, each with four replicates. Otherwise, four replicates of all available doses were prepared in this manner. All plates were incubated both aerobically and anaerobically in jars at 37 °C for up to 48 hours. All plates were checked at 24 hours and counted if there was growth; however, if no growth was observed, plates were returned to grow for an additional 24 hours. The remaining bacterial content was then calculated based on recoverable CFU compared to guaranteed CFU. Microbial contents of the probiotics were confirmed when possible with selective media based on the manufacture claimed microbial contents. When this was not possible, 16S rRNA gene sequencing, and internally transcribed spacer region (ITS) sequencing for yeast was used to confirm microbial contents.

Selective media for enumeration. Several selective media were used to isolate all possible species from each probiotic. *Streptococcus salivarius* was grown on Mitis Salivarius Agar (Difco, MD)

aerobically at 37°C. SF (*Streptococcus Faecalis*) Medium (Broth) (20.0 g of tryptone, 5.0 g of dextrose, 4.0 g of dipotassium phosphate, 1.5 g of monopotassium phosphate, 5.0 g of sodium chloride, 0.5 g of sodium azide, 32.0 mg of bromocresol purple) with 15 g/L agar was used to isolate *Enterococcus faecalis* (previously, *Streptococcus faecalis* [26]) grown aerobically at 37°C. ST (*Streptococcus thermophilus*) agar (10.0 g of tryptone, 10.0 g of sucrose, 5.0 g of yeast extract, and 2.0 g KzHP04 dissolved in 1000 ml distilled water) [27] was used to isolate *Streptococcus thermophilus*, grown aerobically at 37°C. Standard YPD media (1% (weight/volume) yeast extract, 2% peptone, 2% glucose/dextrose) with 15g/L agar dissolved in distilled water) was used to isolate *Saccharomyces boulardii*, grown aerobically at 37°C.

For differentiation of lactobacilli strains, MRS agar (55 g/L; Difco, MD) and antibiotic MRS agars were used; all grown anaerobically in a jar at 37°C for up to 48 hours. MRS agar with 50 µg mL⁻¹ tetracycline (Alfa Aesar, Ward Hill, MA, USA) and 15 µg mL⁻¹ fusidic acid was used to differentiate *Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1, respectively [28]. Modified deMan-Rogosa Sharpe agar containing bromophenol blue (mMRS-BPB) (55 g MRS, 0.05% L-cysteine (Sigma-Aldrich, St. Louis, MO, USA), pH to 6.5 ± 0.2, 0.002% BPB, and 15 g/L agar dissolved in 1000 ml distilled water) was used to isolate different lactic acid-producing bacterium, from the mixed culture [29].

DNA extraction and PCR amplification. For probiotic products labelled as containing only one strain, a colony was isolated when there was growth. From probiotic products containing multiple species that were differentiable by selective growth media, all differentiable colonies were collected. DNA was extracted from the samples using the InstaGene™ Matrix (Bio-Rad) following the manufacturers' protocol, or the microwave method. For the microwave method, a small portion of a colony is spread on the inside of a PCR tube then microwaved on high for three minutes then PCR mix is added directly to this tube.

Bacterial DNA PCR protocol: 5 µl 10x PCR Buffer, 3 µl MgCl₂ (50mM), 4 µl BSA (10mg/ µl), 2 µl pA (100µM), 2 µl pH (100µM), 1 µl Taq, 2 µl dNTP (10µM), 2 µl template DNA then topped off to 50 µl with NFH₂O. Run at 95°C for 2 minutes, then 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute followed by a 10-minute hold at 72°C. PCR of bacterial sequences was conducted with 16S rRNA primers pA (AGAGTTTGATCCTGGCTCAG) and pH (AAGGAGGTGATCCAGCCGCA) [30].

Fungal DNA PCR protocol was taken from [31]. PCR of fungal sequences was conducted using ITS primers, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [31]. Samples were amplified using Taq DNA polymerase. Reactions were primed at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min; and a final extension at 72°C for 10 min. Nucleotide Basic Local Alignment Search Tool (BLAST) was then used to determine the identity of the bacterial and yeast species in the probiotic.

Statistical analysis. The proportion of cells that remained viable at the time of testing was used as a measure of viability. Data were log-transformed to increase validity, additivity, and linearity. All graphs were plotted and statistically analyzed using GraphPad Prism 8 (La Jolla, CA). Significantly higher viability in package type and packaging materials was identified using One-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc test with Dunnett's multiple comparison test. An unpaired t-test determined significantly higher viability based on storage conditions. One-way ANOVA with Tukeys was used to analyze all other violin plots. Spearman correlation was used to analyze years vs. viability.

Results and Discussion:

For all probiotics, the proportion of cells that remained viable at the time of testing was compared to the time since expiry (Figure 1b). There was a negative relationship between the time since expiration and viability, suggesting that after expiration, viability decreases over time. However, there is no specific point at which viability ceases.

Pill type (encapsulated, compressed powder or loose powder) had no impact on the viability; most were encapsulated. Evaluation of the manufacturers' probiotic labels was confirmed with either sequencing or selective media (Table 1). For viable probiotics, all consistently matched up to the manufacturers' labelling. All products with more than 6 unit dosages are shown in Figure 1a. These results indicate that labelling of genus and species in the products tested was accurate. This finding is interesting since past studies have shown inaccuracy in scientific labelling of probiotic genera and species [32,33]. One evaluation of *Lactobacillus* probiotics found that three of the ten human probiotics products had misidentified the species present [32]. A second study showed incorrect labeling of 9 out of 21 products, particularly in veterinary probiotic products [33].

The impact on viability of the numbers of species or strains in a product was assessed, with between one and twelve present in a given product. There was no difference in viability between one or more species when looking at total cell counts of the products or individual species cell counts where tested (Figure 2a). In this study, the number of species in a probiotic product had no observable impact on viability after expiration; however, only limited investigations of individual strain's viability were conducted here.

When recommended storage conditions were not followed, there was a significant decrease in strain viability past expiration (Figure 2b ($p < 0.05$ ($p = 0.0191$) unpaired t-test, $n = 33$)). One probiotic was stored in an "Ultra protective casing" and it had 108% label claim viability at 7 years past expiration (Figure 2c). In general, manufacturers recommend that probiotics are stored in a cold, dark, dry place or the refrigerator. Most of our expired probiotics were stored in a dark, dry box or a refrigerator for the time

in our possession. This result is consistent with the literature, which indicates that refrigeration leads to higher viability than storage in ambient conditions [40–43]. Overall, only two of the seven probiotics stored not according to the manufacturers' instructions had viability. Probiotics A and B had much higher viability than the other incorrectly stored probiotics. This could be because both expired relatively recently (four and seven years ago, respectively) and were kept in metal packaging (Table 1).

Packaging material had a significant impact on bacterial viability, while capsule composition did not. The protective abilities of various packaging materials were evaluated (Figure 1d, 1e). Capsule composition was categorized based on three main ingredients found in most of the tested probiotics (Figure 1f). Probiotics formulated in capsules in a metal container were found to have significantly higher viability compared to a similar product in a plastic bottle ($p < 0.05$ ($p = 0.0476$) One-way ANOVA with Tukeys, $n = 33$). Based on packaging material alone, metal was also found to have significantly higher recoverable viability than plastic ($p < 0.05$ (0.0106) One-way ANOVA with Tukeys, $n = 33$). Although there is no significant difference between plastic and glass overall, smaller-scale analyses revealed that there may be a difference in some instances. Probiotics T and U (Table 1) expired 13 years ago, were stored correctly and presumably contained the same organism (*S. boulardii* 17). Probiotic T, packaged in a plastic sachet, had no viability while probiotic U, packaged in a glass bottle, had 98.3% viability. All other comparisons between packaging material and dosage composition were found to be non-significant. The type of seal used on the packing lids was not evaluated here and may have also been a factor.

Probiotics B, D, X, Z2 (Table 1) contained more than the manufacturers' labelled microbial amount; at the point of testing, they had over 100 percent viability. Probiotic B and D were both contained in metal, while probiotic X and Z2 were in glass bottles with light protection. There was no correlation between the probiotics; therefore, this difference may be due to packaging materials. Overall, glass and metal were found to be better packaging material to ensure the long-term viability of probiotics, though further studies with greater numbers are required to confirm this.

These results suggest that packaging type impacts the long-term viability of probiotics after expiration. This is consistent with several other studies looking at the effect of packaging on probiotic viability prior to expiration [38–40]. This is attributed to the various levels of protection against exposure to oxygen, light, and moisture, which varied in each format [41]. In particular, plastic has a very high oxygen permeability compared to other materials [41]; so, the observed difference between metal and plastic packaging could be ascribed to their permeability to oxygen. Glass has been found to have a protective effect on probiotic viability [42,43]; however, this was not observed in this study, although the products were expired and had been exposed to stresses for longer than those considered in past studies. We also need to consider that not all products in glass containers were stored according to the manufacturers' instructions. The lack of a significant difference between glass and plastic might also be

because glass, despite providing better protection against oxygen, allows more light to reach the probiotic. Over time, all packages lose integrity and allow contaminants that led to microbial death, regardless of composition or format. These results show that none of the packaging tested consistently provided adequate protection over long periods of time.

The correlation between years expired and viability seen in this study is similar to several other studies of probiotics [34-37,41,44]. This result is expected because older probiotics have been exposed to external stresses (such as oxygen, light, and moisture) known to cause microbial death [43] for more extended periods, resulting in greater microbial death.

This study has some limitations to be considered when interpreting the results. First, we used the proportion of cells remaining to quantify viability. This proportion was computed under the assumption that the number of live cells present at the time of expiry was exactly as guaranteed by the manufacturer. Though this was acceptable for the study, it is likely not accurate across all products because manufacturers add excess bacteria or yeast to ensure their products contain the guaranteed count at end of shelf-life. Since the true count at the time of expiry may have been greater or less than estimated, this could have increased or decreased the proportions used in our analysis. In future experiments, it may be better to quantify the number of dead cells and use this to calculate a proportion that more accurately represents the change in viability. Second, the groups considered in each analysis varied in terms of characteristics other than the ones used to define the collection (e.g., the products in the "stored correctly" group varied in terms of time since expiry, packaging, and the number of species). Ideally, the groups would differ only in terms of the variable being investigated, so that any conclusion can be clearly linked to that factor.

Conclusion:

This study found that many probiotic products retained their viability long after expiration, albeit lower than the recommended threshold for efficacy. Currently, expired probiotics are not safe to consume past expiration despite containing viable cells due to the lack of safety studies. Companies often calculate expiration date based on accelerated studies alone. Ideally, they should re-evaluate their viable count to determine expiration in real time to more accurately reflect when the product no longer meets its efficacy threshold.

Declarations:

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: Data sharing not applicable to this article as no datasets were

generated or analysed during the current study.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Study idea and design were put together by JB and HW. Probiotic product CFU collection was done primarily by HW and CC with contribution from SS. DNA extraction was performed by HW and CC. Data analysis and manuscript writing was done by HW and CC with edits made by JB and GR. All Authors read and approved the final manuscripts.

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Figure and Table Legend:

Table 1. Comparison of all probiotics.

A - Dr. Milk Probiotic Milk, Blis Technologies Ltd., Otago, New Zealand; **B** - DS, Innēov France, France; **C** – Bio-Tura, Pharma Nord, Marlton, New Jersey, United States; **D** - Yakult RI, Yakult Honsha Co., Tokyo, Japan; **E** - Culturelle, CAG Functional Foods, Omaha, Nebraska, United States; **F** - GY-NA-TREN FLORA, Natren, Westlake Village, California, United States; **G** - Ultimate Multi Probiotic,

Natural Factors, Canada; **H** - Milk-Free Acidophilus, Schiff Vitamins, Salt Lake City, Utah, United States; **I** – Florgynal vaginal capsule, Laboratories IPRAD, Paris, France; **J** - Ultimate Flora, Renew Life, Oakville, Ontario, Canada, **K** - Plantadophilus, Transformation Enzyme Corp., Houston, Texas, USA, **L** - Yogurt, Instuit Rosell, Montreal, Quebec, Canada; **M** - Womens’ Multi Probiotic, Natural Factors, Canada; **N** - Reuterin, BioGaia AB, Stockholm, Sweden; **O** - Theralac, Therabiotics, INC., Victoria, Minnesota, United States; **P** - Trevis, Chr. Hansen Biosystems A/S, Hørsholm, Tanska, Denmark; **Q** - Entero-Dophilus Globetrotter, Instuit Rosell, Montreal, Quebec, Canada; **R** – Investigational drug, Chr. Hansen Biosystems A/S, Hørsholm, Tanska, Denmark; **S** - Florastor, MFI Pharma, Richmond Hill, Ontario, Canada; **T** - Lactipan, EMS Sigma Pharma, Hortolândia, São Paulo, Brazil; **U** – Floratil 200, Merck, Kenilworth, New Jersey, United States; **V1-5** – UREX-cap-5, manufactured by Chr. Hansen, Hoersholm, Denmark, received for clinical studies; **W** – RepHresh Pro-B, manufactured by Chr. Hansen, Hoersholm, Denmark, distributed by Lil’ Drug Store Products, Inc., Cedar Rapids, IA; **X** – BiO-LiFE PRO-UTix, manufactured by FA Herbs Sdn Bhd, Kuala Selangor, distributed by BiO-LiFE Marketing Sdn Bhd, Kuala Lumpur, Malaysia; **Y1-2** – femdophilus, manufactured by Chr. Hansen, Hoersholm, Denmark, distributed by Jarrow Formulas, Los Angeles, CA; **Z1-2** – Ecoflora, manufactured by Tablets (India) Ltd., Pondicherry, India; **AA** – LaciBios femina, manufactured by Exeltis, Poland.

^a Average CFU calculated from 6 pills. NA — Not available

All bottles previously opened unless otherwise stated

Figure 1. Probiotic viability past expiration. **A)** Expired probiotics with more than six remaining doses. **B)** Graph of remaining viability (%) of expired probiotics versus years since expiration.

Figure 2. Expired probiotic packaging and material. **A)** Number of species per probiotic relation to viability post expiration (One-way ANOVA with Tukeys). There is no statistically significant relationship between the number of initial species and viability of probiotics after expiration. **B)** Storage conditions (either in compliance with manufacturer’s suggestions or not) significantly impacted probiotic viability (P=0.0191, unpaired t-test). **C)** The probiotic with the greatest percentage (108%) of the initial CFU that remained was packaged in a special ultra-protective packaging. **D)** Overall packaging type including packaging and pill casing (P=0.0476, n=33, One-way ANOVA with Tukeys), **E)** the packaging material only (P=0.0106, One-way ANOVA with Tukeys), and the **F)** composition of the pill (One-way ANOVA with Tukeys). N=33

Figures

Figure 1

Probiotic viability past expiration. A) Expired probiotics with more than six remaining doses. B) Graph of remaining viability (%) of expired probiotics versus years since expiration.

Figure 2

Expired probiotic packaging and material. A) Number of species per probiotic relation to viability post expiration (One-way ANOVA with Tukeys). There is no statistically significant relationship between the number of initial species and viability of probiotics after expiration. B) Storage conditions (either in compliance with manufacturer's suggestions or not) significantly impacted probiotic viability ($P=0.0191$, unpaired t-test). C) The probiotic with the greatest percentage (108%) of the initial CFU that remained was packaged in a special ultra-protective packaging. D) Overall packaging type including packaging and pill casing ($P=0.0476$, $n=33$, One-way ANOVA with Tukeys), E) the packaging material only ($P=0.0106$, One-way ANOVA with Tukeys), and the F) composition of the pill (One-way ANOVA with Tukeys). $N=33$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ProbioticComparisonTable.pdf](#)