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Production of Sour-milk Using Mono- and Multi-cultures of Lactic Acid Bacteria Isolated from Nunu

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Abstract
Production of nunu, a nutritious sour cow milk that serve as refreshing drink to many Nigerians, involve spontaneous fermentation by a diverse group of microbial contaminants resulting in products of varying flavor quality. This study investigated the effect of mono and multi-culture of lactic acid bacteria (LAB) as starter in sour-milk production. Organisms were isolated from nunu samples, characterized and identified using molecular techniques. The organisms were used to ferment fresh cow milk individually, and in various combinations. The sensory properties, pH and proximate composition of the products were compared. Among the three LAB isolates, *Lactobacillus helveticus* NBRC15019 had the highest rate of acidification of about 30% pH reduction in 16 hours as against 20% and 15% for *L. fermentum* CIP102980, and *L. helveticus* DSM20075 respectively. There was exponential increase in the microbial load (from a minimum of 4.0 x 10^3 to 6.6 x 10^{13}) within the 24 hours period of fermentation. Consumer ratings favored *L. fermentum* CIP102980 over the others. Fermentation generally improved the protein content from 3.07% in the raw milk sample up to 3.47% in the sample fermented with a combination of the three LAB and compared favorably with that of the commercially sourced nunu sample while the sugar content was reduced from 5.63% to as low as 4.1%. *Lactobacillus helveticus* NBRC15019 has been proved as the most efficient organism in milk acidification. However, combination with the other two LAB strains may help to improve the sensory and nutritional quality of sour-milk.

Keyword: Lactic Acid, bacteria Mono-culture, multi-culture, production, sour-milk.

1. Introduction

Nunu is a nutritious sour cow milk that is most commonly consumed in the northern part of Nigeria. It is a product of spontaneous fermentation of unpasteurized cow milk (Owusu-Kwarteng *et al.*, 2017). Nunu is a watery white to cream colored fluid with a sour yogurt-like taste (Akabanda *et al.*, 2010; 2014). In Nigeria, consumers of nunu usually mix it with a cereal based fermented dough known as fura to make a complete diet. Nunu is highly marketable and constitutes a major source of income to wives of pastorals in northern Nigeria (Egwaikhide *et al*., 2014).

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Production of nunu involves collecting cow milk into a container, usually a calabash and allowing the milk to ferment naturally for 24 hours. Small quantity of the nunu from previous ferment may be added to speed up the fermentation. After fermentation, the milk is churned to separate fat, while the watery fluid, which is very rich in protein is the nunu. Several microorganisms including bacteria and fungi have been isolated from fermenting milk in the production of nunu. Among them, lactic Acid Bacteria (LAB) including *Lactobacillus plantarum*, *L. bulgarius*, *L. helveticus*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *S. lactis* and *S. cremoris* (Okiki *et al.*, 2018; Akabanda *et al.*, 2013) were most common. Others include fecal contaminants such as *Escherichia coli* and *Enterobacter* sp; Gram positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus* sp. and *Bacilus cereus;* and fungi including the yeast *Sacharomyces cerevisae* and molds *Aspergillus flavus* and *Rhizopus nigricans* (Adebesin, *et al.*, 2001).

The fermentation that leads to nunu production is an uncontrolled process involving microorganisms from the previous fermentation or those that contaminate the fresh milk (Owusu-Kwarteng *et al.*, 2017). This kind of fermentation can result in non-standard products with varying flavor and texture quality. Products may also be of poor quality in terms of safety due to the presence of microbial pathogens or their toxic by-products such as mycotoxins, ethyl carbamate and biogenic amides (Capozzi *et al*., 2017). This brings to the fore, the necessity to control the fermentation of milk, particularly regarding the microorganisms involved, for nunu production. In this study therefore, the role of the individual lactic acid bacterial isolates, as well as the effect of their combination was investigated with a view to design a starter culture for nunu production.

2. Materials and Methods

2.1 Study area and sample collection

The study was conducted in Ilorin, North-central Nigeria. Nunu samples from which microorganisms were isolated was collected from retailers at three different locations within Ilorin. Fresh cow milk used as raw material for the fermentation process was sourced from a Fulani settlement in Ilorin. The milk was aseptically collected directly from the cow's udder into a sterile container which was placed in ice bucket and immediately transported to the laboratory for analysis and fermentation.

2.2 Isolation of organisms from nunu samples

Nunu sample, 1 ml was mixed with 9 ml of sterile distilled water and serially diluted to 10^{-5}. Aliquots, 0.1 ml from the 10^{-4} and 10^{-5} diluents were inoculated onto Nutrient and MRS agar plates. The plates were incubated at 37ºC. After growth, distinct colonies were sub-cultured to obtain pure cultures which were transferred onto agar slants and stored at 4ºC.

2.3 Characterization of Isolates and screening for lactic acid bacteria

Organisms were characterized based on colonial, cellular and biochemical characterization. The Gram positive and catalase negative organisms that were able to grow on MRS agar were selected for the fermentation process. They were identified molecularly by sequencing the 16SrRNA using the Big Dye Terminator v3.1 Cycle Sequencing Kit. The sequence was edited and translated into the FASTA format using the Finch tv, Bioedit and Chromas Lite softwares. The FASTA sequences were entered into the NCBI Basic Local Alignment Search Tool (BLAST) centre and three lactic acid bacteria strains were identified.

2.4 Fermentation of fresh milk

The inoculum for the fermentation was prepared by growing the lactic acid bacteria isolates in MRS broth at 37ºC for 24 hours. The inoculums were prepared as single and mixed cultures. The mixed cultures were prepared by combining the organisms equally (v/v). All inoculums were standardized to 0.5 McFarland prior to use for fermentation.

Fresh cow milk, 100 ml each was placed in 250 ml Erlenmeyer flasks and pasteurized at 65ºC for 30 minutes by placing the flasks in water bath. After pasteurization, the flasks were immediately cooled to 28±2ºC. Each flask was inoculated with 5 ml of the inoculum and incubated at 28±2ºC without shaking, for 24 hours. Control was set up by incubating 100 ml of unpasteurized and un-inoculated milk sample.

2.5 Analysis of the organoleptic properties and nutritional composition of samples

Samples of nunu and the laboratory fermented cow milk were assessed for colour, texture, and odour on a five-point hedonic scale by a panel of five individuals who are very familiar with fermented milk. The nutritional composition of the samples were also determined. Samples were assessed for the protein, reducing sugar and total sugar content using the methods of Lowry *et al.* (1951), dinitrosalicylic acid method of Miller (1959) and Anthrone Test (Roe,1955) respectively.

2.6 pH and microbiological analysis

Samples of the fermenting milk were withdrawn at 4 hours intervals for pH and microbiological analysis. The pH was measured using pH meter (Crison basic 20, Barcelona, Spain) calibrated with standard buffers. The microbial load was determined by isolating organisms on MRS agar using the pour plate method. Organisms were enumerated by counting the colonies using colony counter.

3. Result and Discussion

3.1 Organisms

Five bacteria were isolated from the nunu samples. Morphology and biochemical characterization of the bacterial isolates revealed three organisms with features of Lactic Acid Bacteria. The other two include a *Bacillus* sp. and a *Staphylococcus* sp. (Table 1). The three lactic acid bacteria were identified as *Lactobacillus fermentum* strain CIP 102980 (16S ribosomal RNA gene, complete sequence with total score of 1279, query cover of 95%, identity of 95% and accession NR 104927.1), *Lactobacillus helveticus* strain NBRC 15019 (16S ribosomal RNA gene, partial sequence with total score of 1448, query cover 100%, identity 99% and accession NR 113719.1), *Lactobacillus helveticus* strain DSM 20075 (16S ribosomal RNA gene, complete sequence with total score of 1448, query cover 100%, identity 99% and accession NR 042439.1).

Table 1: Morphology and biochemical properties of bacterial isolates.

Characters	**Isolates**				
	A	**B**	**C**	**D**	**E**
Cell shape	Rods	Rods	Rods	Cocci	Rods
Gram reaction	+	+	+	+	+
Spore	-	-	+	-	-
Motility	-	-	+	-	-
Catalase	-	-	+	+	-
Oxidase	-	-	-	-	-
Lactose	+	+	+	+	+
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Gas	+	+	-	-	+
Starch	+	+	+	+	+
Citrate	+	+	-	-	+
Maltose	+	+	+	+	+
Urease	-	-	-	+	-
Nitrate Red	-	-	+	+	-
Growth at 15ºC	-	-	+	+	-
Growth at 45ºC	+	+	+	+	+
Organism	*Lactobacillus* sp.	*Lactobacillus* sp.	*Bacillus* sp.	*Staphylococcus* sp.	*Lactobacillus* sp.

Key: + (positive); - (negative)

3.2 Organoleptic properties and nutritional composition of the milk samples

The color, odor and appearance of the nunu samples were rated significantly ($p < 0.05$) higher than the laboratory fermented samples. Samples fermented with *Lactobacillus fermentum* strain CIP102980 had the highest ratings among the others. In the combined culture category, fermentations involving *L. fermentum* strain CIP102980 and *L. helveticus* strain NBRC15019 was rated higher than that involving the two strains of *L. helveticus*. The spontaneously fermented milk was rated higher than all that were fermented with combination of isolates (Table 2).

The protein, total sugar, and reducing sugar contents of the samples are presented on Figure 1. The protein content of all the fermented samples except the combination involving the two strains of *L. helveticus* compares favorably with the fresh milk. There was no significant ($p < 0.05$) difference in the total and reducing sugar content of the nunu and samples AB, ABC and X while those of samples A, B, C and BC were reduced significantly ($p < 0.05$).

Table 2: Organoleptic assessment of milk samples.

Samples	**Assessment**			**Acceptance**
	Texture	**Color**	**Odor**	
NUNU	4.20±0.25	4.70±0.15	4.20±0.25	4.37±0.22
A	3.20±0.20	4.60±0.25	4.0±0.00	3.93±
B	3.00±0.00	3.60±0.25	3.40±0.25	3.33
C	2.40±0.25	3.60±0.25	2.8±0.20	2.93
AB	3.40±0.25	3.80±0.20	3.20±0.20	3.47
AC	2.20±0.20	2.60±0.25	2.40±0.25	2.40
BC	2.00±0.00	3.00±0.32	2.00±0.00	2.33
ABC	2.60±0.25	2.60±0.25	2.80±0.37	2.67
X	3.00±0.00	4.00±0.32	3.60±0.40	3.53

Key: NUNU - commercially sourced locally fermented cow milk samples; others are cow milk samples fermented in the laboratory using bacterial isolates singly and in combinations as designated: A - *Lactobacillus fermentum* strain CIP 102980; B - *L. helveticus* strain NBRC15019; C - *L. helveticus* strain DSM20075; X – fresh cow milk fermented spontaneously in the laboratory. Values are mean of assessment by five member panel ± standard deviation.

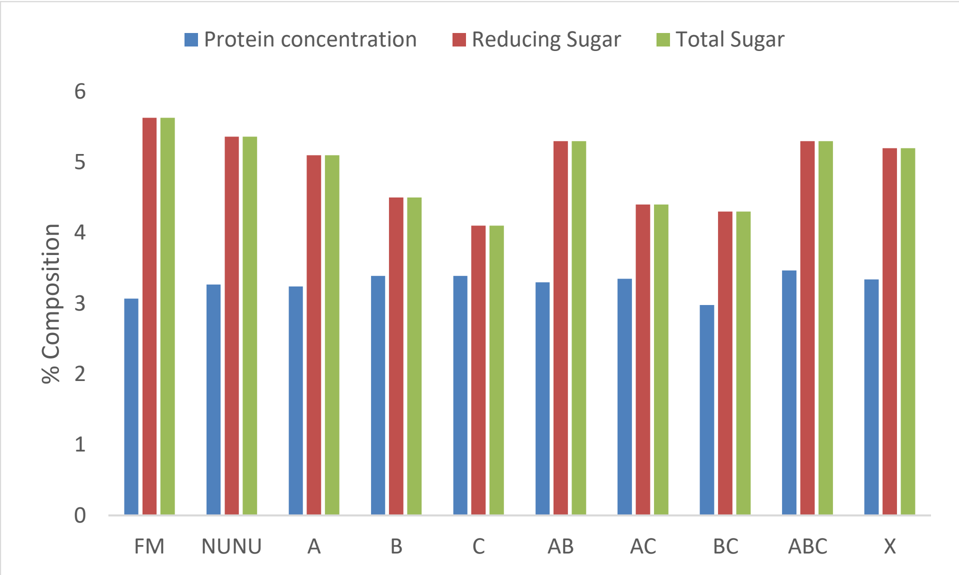

Figure 1: Nutritional composition of milk samples.

Legend: FM – Fresh unfermented cow milk samples; NUNU – commercially sourced locally fermented cow milk samples; others are cow milk samples fermented in the laboratory using bacterial isolates singly and in combinations as designated: A - *Lactobacillus fermentum strain CIP102980* [*Lf*]; B - *L. helveticus* strain NBRC15019 [*Lh* NBRC15019]; C - *L. helveticus* strain DSM20075 [*Lh* DSM20075]; X – fresh cow milk fermented spontaneously in the laboratory. Values are means of three independent replicates.

3.3 The pH and microbial load of fermenting milk samples

The pH of the fermenting milk samples decreased from 6.9 for the fresh milk sample to as low as 4.2 for the unpasteurized and un-inoculated sample (Figure 3) while the microbial load of all the samples increased significantly ($p < 0.05$) as fermentation progressed (Table 3).

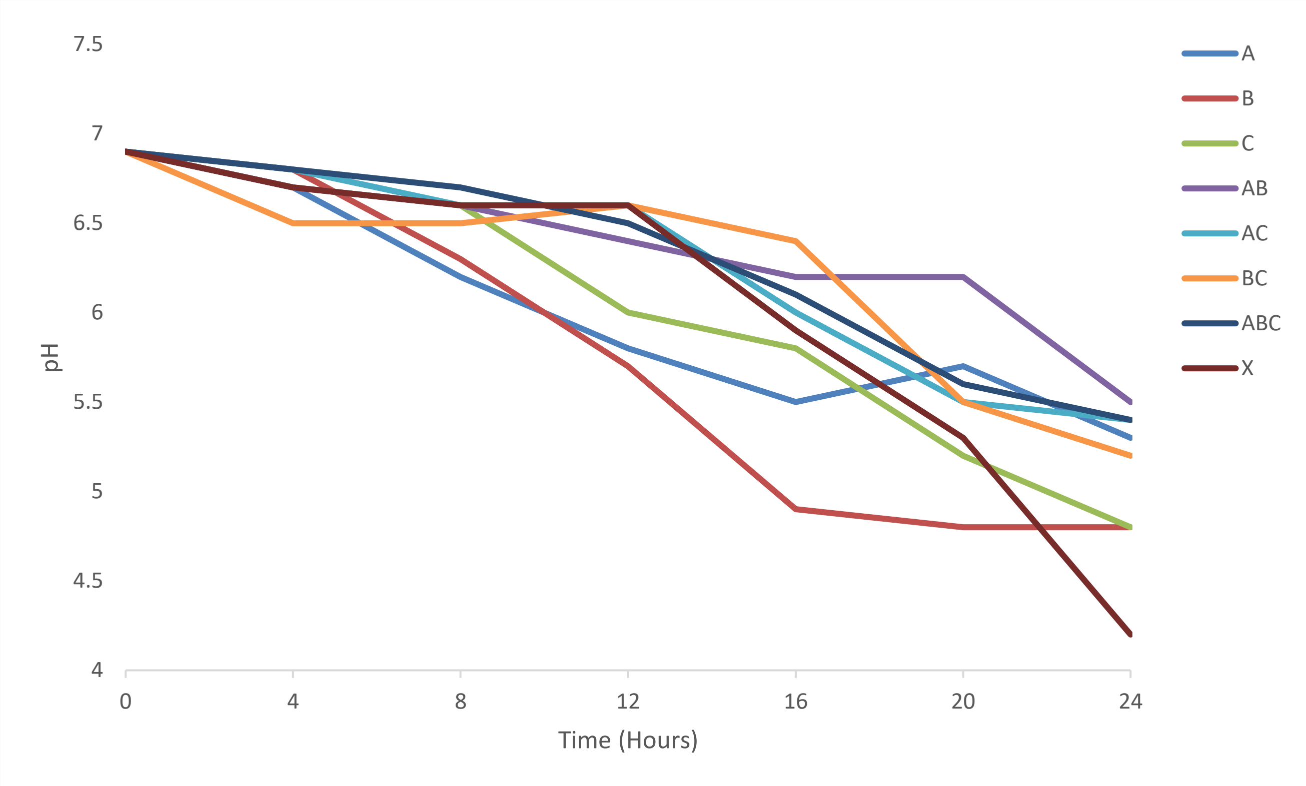

Figure 3: Changes in the pH of milk samples during fermentation.

Legend: Series are cow milk samples fermented in the laboratory using bacterial isolates singly and in combinations as designated: A - *Lactobacillus fermentum strain CIP102980* [*Lf*]; B - *L. helveticus* strain NBRC15019 [*Lh* NBRC15019]; C - *L. helveticus* strain DSM20075 [*Lh* DSM20075]; X – fresh cow milk fermented spontaneously in the laboratory. Values are mean of three independent replicates

Table 3: Bacterial load of milk samples during fermentation.

Samples	**Bacterial load (log cfu/ml)**					
	4 hours	**8 hours**	**12 hours**	**16 hours**	**20 hours**	**24 hours**
A	7.2×10^{3}	9.8×10^{5}	1.42×10^{8}	1.4×10^{10}	1.14×10^{12}	6.0×10^{13}
B	4.0×10^{3}	8.6×10^{5}	6.4×10^{7}	1.2×10^{10}	9.4×10^{11}	4.6×10^{13}
C	5.0×10^{3}	1.08×10^{6}	1.14×10^{8}	9.8×10^{9}	6.2×10^{11}	4.2×10^{13}
AB	7.0×10^{3}	6.4×10^{5}	8.6×10^{7}	7.8×10^{9}	1.02×10^{12}	5.0×10^{13}
AC	1.0×10^{4}	1.18×10^{6}	1.2×10^{8}	8.4×10^{9}	1.12×10^{12}	6.6×10^{13}
BC	4.0×10^{3}	9.8×10^{5}	8.2×10^{7}	7.4×10^{9}	5.6×10^{11}	3.8×10^{13}
ABC	9.8×10^{3}	7.4×10^{5}	8.6×10^{7}	6.2×10^{9}	4.4×10^{11}	3.0×10^{13}
X	1.3×10^{4}	8.0×10^{5}	1.02×10^{8}	6.0×10^{9}	4.8×10^{11}	5.2×10^{13}

Legend: Samples are cow milk samples fermented in the laboratory using bacterial isolates singly and in combinations as designated: A - *Lactobacillus fermentum strain CIP102980* [*Lf*]; B - *L. helveticus* strain NBRC15019 [*Lh* NBRC15019]; C - *L. helveticus* strain DSM20075 [*Lh* DSM20075]; X – fresh cow milk fermented spontaneously in the laboratory.

4. Conclusion

The lactic acid bacteria isolated from nunu samples in this study are among those that are commonly found in milk products where they play important roles in its fermentation for improved flavor and shelf quality (Akabanda *et al.,* 2010; Okiki *et al.,* 2018). *Lactobacillus fermentum* is a heterofermentative organism that produce lactic acid along with other products such as carbondioxide and ethanol thereby impacting preservative benefits. Most strains have probiotic properties with ability to produce bacteriocins and inhibit the growth of many pathogens including *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Enterrococcus feacalis*, *Proteus mirabilis* and *Listeria monocytogenes* (Owusu-Kwarteng et al., 2015; Fuochi *et al.,* 2017; Subramanyam and Mekala, 2020). In addition, they inhibit the growth and urease activity of *Helicobacter pylori* (Garcia et al., 2017) and have been applied in the treatment of infections such as urogenital (Gardiner *et al.,* 2002). *L. helveticus* is used in cheese production (Moser *et al.,* 2017) where it plays a primary role of preventing bitterness and producing nutty flavors in cheeses.

It provides numerous health benefits to humans including lowering of blood pressure. It also has beneficial psychological effects such as lowering of anxiety, depression, and anger among others (Messaoudi *et al.,* 2011). As a probiotic organism, *L. helveticus* possesses the ability to survive gastrointestinal transit, adhere to epithelial cells, antagonize pathogens, modulate host immune responses, and affect the composition of the intestinal microbiota (Taverniti and Guglielmetti, 2012). *L. fermentum* and *L. helveticus* are among the lactic acid bacteria proposed as starter culture for the production of nunu because of their probiotic and antimicrobial properties, as well as the amino acid profile of the product (Akabanda *et al.*, 2014).

The decrease in the pH of the milk sample during fermentation attests to the production of acid and further confirms the activities of the lactic acid bacteria in the milk. Rate of acidification of the milk samples, which was a measure of the pH decrease was fastest with *L. helveticus* NBRC15019 and not as fast with the other strain and the *L. fermentum*. This indicates that acidification could be strain dependent and may not be generalized for any species of *Lactobacillus*. Akabanda *et al*. (2014) also reported a fast acidification rate for *L. helveticus* compared to the other lactic acid bacteria in their study. Acidification rate was relatively lower in all the combined culture fermentation despite inclusion of *L. helveticus* NBRC15019, showing that this kind of combination may not be beneficial for efficiency, and where products requiring coagulation is desired. In terms of acidification therefore, *L. helveticus* NBRC15019 was the best candidate to ferment milk efficiently among the isolated organisms. Combination with the other strains may however be necessary for improved flavor and aroma quality. The spontaneously fermented milk samples had the lowest pH of all after 24 hours of fermentation and this may be due to the involvement of many acid producing microbial contaminants in the fermentation. High rate of acidification is desired in dairy industry for flavor and to help eliminate acid intolerant organism which may cause spoilage or be pathogenic to consumers of the products. A general decrease in pH during the fermentation of milk to produce nunu have also been reported in previous studies (Akabanda *et al.,* 2010; Adesokan *et al.*, 2011).

In line with Akabanda *et al.* (2013), the decrease in the pH of fermenting milk in this study, corresponds with exponential increase in the microbial load. Among the LAB, *L. helveticus* strain NBRC15019 which produced the highest acidity grew fastest thereby indicating that the population of organism was directly proportional to the rate of acidity. Organoleptic properties of nunu play an important role in its acceptability as consumers are more inclined to opt for products with good sensory qualities. In this study, after the 24 hours of fermentation, the sensory changes of the products revealed that milk fermented with only *L. helveticus* strain NBRC15019 was the most acceptable in terms of colour and odour while product of a combination involving *L. fermentum* strain CIP102980 and *L. helveticus* strain NBRC15019 was rated highest in texture. However, a general improvement in consumers' acceptance was obtained for all the inoculated milk compared to the spontaneously fermented products. This is similar to the report of an earlier study (Akabanda *et al*., 2014) and showed that a precise control of milk processing condition can positively influence the sensory qualities of the fermented product as stated by Wolf *et al*. (2013).

The drop in the reducing and total sugar contents of the milk samples during fermentation indicates usage for growth and other metabolic activities by the fermenting organisms in addition to its conversion to lactic acid and other products. This is evident as *L. helveticus* strain DSM20075 utilized sugar the most and its product did not have the highest acidity. It therefore follows that utilization of sugar may not necessarily measure the acidity rate since the sugar can be converted to other products such as ethanol, acetic acids etc. Normally, during fermentation, the milk sugar, lactose, is metabolized by the fermenting organism into lactic acid. Thus, nunu contains less lactose compared to raw milk and it is better tolerated by people who are lactose intolerant (Panesar, 2011). Although, there was a general increase in the protein content of the final products, fermentation using a combination of *L. fermentum* CIP102980, *L. helveticus* NBRC15019, and *L. helveticus* DSM20075 yielded nunu product with the highest protein content. This underscored the importance of combined LAB fermentation.

In controlling the fermentation for the production of sourmilk, *L. helveticus* strain NBRC15019 was shown to be the best in terms of acidification and consumer acceptability as its product compares favorably with the spontaneously fermented milk. Its combination with *L. fermentum* strain CIP102980 and *L. helveticus* strain DSM20075 however yielded highest protein content. This study therefore recommends the combined culture fermentation for overall product quality.

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