**Lactobacillus spp. Strains Isolation, Identification, Preservation and Quantitative Determinations from Gut Content of 45-Day-Old Chickens Broilers**

**ABSTRACT**

The objective of this study was to isolate, identify, preserve and determine the quantitative level of the Lactobacillus strains from the gut content of 45-day-old chickens broilers; to test the viability of these strains preserved at 4 °C and room temperature (20 ± 2 °C). Lactobacillus strains were isolated, phenotypically identified and preserved from the gut content of 17 chickens broilers. Identification was performed by morphological, cultural and biochemical characters examination, using apiwebTM and ABIS online software. The quantitative level of Lactobacillus strains in intestinal content (10^5 – 10^9 CFU/g) and the viability of strains preserved at 4 °C and at room temperature (from 8 days to 9 months) was also determined. Twenty-three strains of L. acidophilus, L. brevis, L. plantarum, L. fermentum and L. salivarius from the gut content of chickens broilers were isolated, phenotypically identified, and preserved. Of these, L. plantarum, L. fermentum and L. acidophilus biotype 1 strains were technologically and ecologically suitable to continue the testing of probiotic traits.

**INTRODUCTION**

Recent research on the structure of the normal intestinal microbiota of chickens revealed the presence of Lactobacillus spp. (Lu et al., 2003; Wei et al., 2013; Waite & Tailor, 2014; Duar et al., 2017), known for its beneficial effects on the host’s health. Lactobacilli have a symbiotic role in host fitness, their metabolites contributing to the digestive process and counteracting pathogens (Duar et al., 2017). Zou et al. (2018) showed that Lactobacillus induce a polarizing effect on the chicken cecal microbiome, suggesting a major influential role of this genus in local microbiome, with negative (with Ruminococcaceae, Lachnospiraceae) or positive (with other lactobacilli, Bacteroides, Clostridiales and Christensenellaceae) correlations. At hatching, the poultry microbiota from cecum consists, predominantly, as Enterococcus, cloforms and clostridia (Coates & Fuller, 1977) but, from the 4th day of age, Lactobacillus becomes a significant component of the intestinal microbiota (Zhu et al., 2002). At the 7th day of age, the ileal mucosal microbiota is dominated by Enterococcus, followed by Lachnospiraceae and Enterococcus (Cressman et al., 2010). After the 14th day of age, the small intestine of broilers chicks develop various communities (Pedroso & Lee, 2015) but, from day 21 to 42 of age, Lactobacillus became the most abundant organism in the small intestine. Of this genus, L. salivarius, L. johnsoni, L. reuteri, L. oris and L. crispatus were detected (Nakphaichit et al., 2011). This diversity raises the issue of selecting the best strain for developing bacterial-based feed additives in poultry nutrition.

The objective of our work was to isolate, identify, preserve and assess the quantitative level of the Lactobacillus strains from the gut content...
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MATERIALS AND METHODS

Birds were treated in accordance with Romanian legislation (law no. 305/2006) for handling and protection of animals used for experimental purposes. The birds’ care and use protocol were approved by the Animal Care and Use Committee at the National Research-Development Institute for Biology and Animal Nutrition (INCDBNA-IBNA) Baloteşti, Romania, following the principles of EU Directive 2010/63/EU and Romanian Law on Animal Protection.

Lactic acid bacteria isolation and determination of total bacterial count

The Mountzouris method (2007) completed by Sorescu et al., (2019) was applied. Sample preparation: 1 g intestinal content (ileum and cecum, respectively) per capita from seventeen chicks (Cobb 500, 45-day-old) was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and immediately frozen at – 20 ºC until testing (no more than three months). After defrost, decimal dilutions from every sample were inoculated on Oxoid Man, Rogosa, Sharpe (MRS) agar. Further instead, the procedure presented in other paper (Sorescu et al., 2019) for the isolation and counting of Lactobacillus CFU has been applied.

Identification of bacterial strains

Phenotypic identification of isolated bacterial strains was performed by morphological, cultural and biochemical characters examination, according to Bergey’s Manual of Systematic Bacteriology (Garrity et al., 2009), ABIS on line software (Stoica & Sorescu, 2018) and APIweb™ API50CHL software BioMerieux (France), following the protocol described in (Sorescu et al., 2019). The results obtained by Pelinescu et al. (2009) were also considered.

Preservation of bacterial strains

The medium-term preservation (months) was done by culture in MRS broth, the viability of bacterial strains being evaluated after 45 days, 3, 7 and 9 months. Long-time preservation (years) was done at – 80 ºC, with addition of glycerol 20%, and bacteria viability is to be assessed every 2 years.

RESULTS AND DISCUSSION

The taxonomic classification of bacterial strains in Lactobacillus spp. was performed through morphologically (Gram positive, non-spore forming rods), culturally (anaerobic growth) and biochemically characters (negative catalase test). Identification of the Lactobacillus spp. was performed based on their biochemical characters. Thus, twenty-three strains of the genus Lactobacillus (L. acidophilus biotype 1 IBNA 64, L. acidophilus biotype 3 IBNA 49, 51, 53, 55, 63, 65, L. plantarum biotype 1 IBNA 45, 46, 48, 61, L. salivarius IBNA 47, 52, 54, 59, 60, 62, 67, 68, L. brevis biotype 2 IBNA 50, and L. fermentum biotype 1 IBNA 56, 57, 69) were isolated, identified and preserved from the intestinal content (ileum and cecum), from seventeen 45 d-old chickens.

The morphological, cultural and biochemical characteristics of the identified strains are presented in Table 1.

Figures 1-4 show smears from L. salivarius IBNA 67, L. plantarum IBNA 46, L. acidophilus biotype 3 IBNA 65 and L. fermentum biotype 1 IBNA 57 cultures in/on MRS broth/agar (Gram staining, × 1000).

Figure 1 – L. salivarius IBNA 67 culture in MRS broth (Gram staining × 1000).

Figure 2 – L. plantarum IBNA 47 culture on MRS agar (Gram staining × 1000).
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**Figure 3** – *L. acidophilus* biotype 3 IBNA 65 culture in MRS broth (Gram staining × 1000).

**Figure 4** – *L. fermentum* biotype 1 IBNA 57 culture in MRS broth (Gram staining × 1000).

**Table 1** – Morphological, cultural and biochemical characteristics of the *Lactobacillus* strains isolated from intestinal contents of broiler chickens at 45 days.

| Tests | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|---|---|---|---|---|---|
| Morphological characters | a | a | b | a | a | b, c |
| Cultural characters | x | x | x, y, z | y | x | y |
| Catalase test | 0(1)* | 0(6) | 0(4) | 0(1) | 0(3) | 0(8) |
| Fermentation (API50CHL) | | | | | | |
| glycerol | 0(1) | 0(6) | 1(4) | 0(1) | 0(3) | 0(8) |
| L-arabinose | 0(1) | 0(6) | 3(4) | 1(1) | 0(3) | 0(8) |
| D-ribose | 0(1) | 0(6) | 4(4) | 1(1) | 0(3) | 0(8) |
| D-xylene | 0(1) | 0(6) | 0(4) | 1(1) | 0(3) | 0(8) |
| D-galactose | 1(1) | 5(6) | 4(4) | 1(1) | 3(3) | 8(8) |
| D-mannose | 1(1) | 3(6) | 4(4) | 0(1) | 2(3) | 8(8) |
| L-rhamnose | 0(1) | 0(6) | 0(4) | 0(1) | 0(3) | 0(8) |
| inositol | 0(1) | 0(6) | 0(4) | 0(1) | 0(3) | 7(8) |
| D-mannitol | 1(1) | 0(6) | 4(4) | 0(1) | 7(3) | 8(8) |
| D-sorbitol | 0(1) | 0(6) | 2(4) | 0(1) | 0(3) | 0(8) |
| Methyl-α-D-mannopyr. | 0(1) | 0(6) | 1(4) | 0(1) | 0(3) | 0(8) |
| N-acetylgulosamine | 1(1) | 6(6) | 4(4) | 0(1) | 0(3) | 8(8) |
| amygdalin | 1(1) | 0(6) | 3(4) | 0(1) | 0(3) | 8(8) |
| arbutin | 1(1) | 0(6) | 4(4) | 0(1) | 0(3) | 0(8) |
| esculin | 1(1) | 6(6) | 4(4) | 0(1) | 0(3) | 0(8) |
| salicin | 1(1) | 0(6) | 4(4) | 0(1) | 0(3) | 0(8) |
| D-cellobiose | 1(1) | 1(6) | 4(4) | 0(1) | 0(3) | 0(8) |
| D-maltose | 1(1) | 3(6) | 4(4) | 1(1) | 3(3) | 8(8) |
| D-lactose | 1(1) | 0(6) | 4(4) | 1(1) | 3(3) | 8(8) |
| D-melibiose | 1(1) | 7(6) | 4(4) | 1(1) | 3(3) | 8(8) |
| D-trehalose | 1(1) | 0(6) | 4(4) | 0(1) | 0(3) | 8(8) |
| D-melezitose | 0(1) | 0(6) | 1(4) | 0(1) | 0(3) | 0(8) |
| aramid (starch) | 1(1) | 2(7) | 4(4) | 0(1) | 0(3) | 0(8) |
| gentiobiose | 1(1) | 2(7) | 4(4) | 0(1) | 0(3) | 0(8) |
| D-tagatose | 0(1) | 0(6) | 1(4) | 0(1) | 0(3) | 0(8) |
| D-arabitol | 0(1) | 0(6) | 0(4) | 1(1) | 0(3) | 0(8) |
| potassium gluconate | 0(1) | 0(6) | 3(4) | 1(1) | 0(3) | 0(8) |

1 = *L. acidophilus* biotype 1 IBNA 64; 2 = *L. acidophilus* biotype 3 IBNA 49, 51, 53, 55, 63, 65; 3 = *L. plantarum* biotype 1 IBNA 45, 46, 48, 61; 4 = *L. brevis* biotype 2 IBNA 50; 5 = *L. fermentum* biotype 1 IBNA 56, 57, 69; 6 = *L. salivarius* IBNA 47, 52, 54, 59, 60, 62, 67, 68.

a = Gram positive, non-spore forming rods, grouped in pairs, chains, filaments and, rare, in palisade; b = Gram positive short rods, with rounded end, non-spore forming, arranged in pairs, short chains or irregular clumps; c = Gram positive short and thick rods or coccoid cells, non-spore forming, arranged in short chains and irregular clumps.

x = small colonies, 0.5-1.5 mm in diameter, rarely larger, up to 2.0 mm, smooth type, round, opaque, semi-transparent or transparent, and whitish, grey or colourless on MRS agar; y = large colonies, 2.0-4.0 mm in diameter, rarely smaller, smooth type, round, opaque, white or whitish; z = small colonies, 1.0-1.5 mm in diameter, rough type, transparent, flattened, round, colourless.

* = number of positive strains from number of tested strains; ? = dubious, weekly positive.

All strains were negative for the fermentation of erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, dulcitol, methyl-α-D-glucopyranoside, inulin, glycogen, xyloitol, D-luranose, D-lyxose, D-fucose, L-fucose, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. All strains were positive for the fermentation of D-glucose, D-fructose, D-saccharose and D-rafinose.
Table 2 presents the origin (ileum or cecum content) and the quantitative level of the isolates presence in ecological niche.

Table 3 presents the results of strains identification by apiweb™ soft, API50CHL V.5.1, BioMerieux (France), and ABIS online software.

Table 2 – The origin and the level of Lactobacillus spp. strains presence in the ecological niche (45-day-old chickens’ broiler intestinal content).

| Strains           | Origin, sample number             | CFU/g intestinal content (log10) |
|-------------------|-----------------------------------|----------------------------------|
| L. acidophilus    |                                   |                                  |
| biotype 1 IBNA    | ileum content, 91                 | 8.301                            |
| 64                | ileum content, 63                 | 8.698                            |
| 51                | ileum content, 68                 | 7.301                            |
| 53                | cecum content, 72                 | 8.662                            |
| 55, 63, 65        | ileum content, 76, 90, 91         | 7.602; 8.176; 7.698              |
| L. plantarum      |                                   |                                  |
| biotype 1 IBNA    | cecum content, 59 (large colonies)| 8.518                            |
| 45                | 59 (small colonies)               | 9.431                            |
| 48, 61            | ileum content 63, 88              | 6.301; 7.301                     |
| L. brevis         |                                   |                                  |
| biotype 2 IBNA    | ileum content, 66                 | 5.477                            |
| 50                |                                   |                                  |
| L. fermentum      |                                   |                                  |
| biotype 1 IBNA    | cecum content, 78                 | 9.079                            |
| 56                | ileum content, 81                 | 7.954                            |
| 69                | cecum content 97                  | 9.672                            |
| L. salivarius     |                                   |                                  |
| IBNA              | ileum content, 63, 72             | 7.531; 6                         |
| 47, 52            | ileum content, 73                 | 8                                 |
| 54                | ileum content, 82                 | 7.176                            |
| 60                | cecum content, 87                 | 8                                 |
| 62, 67, 68        | ileum content, 90, 93, 95         | 8.301; 7.113; 8.397              |

L. salivarius and L. acidophilus are included in the vertebrate adapted lifestyle lactobacilli group and were isolated from human, pigs, hamsters, horses, chickens (Duar et al., 2017) and turkeys (Sorescu et al., 2019). L. fermentum and L. plantarum belongs to the nomadic species group of lactobacilli (Duar et al., 2017). L. fermentum was isolated from fermenting plant material, sewage, milk products, mouth and faeces of humans, and intestines of pig, rat, cattle, mouse and birds (Garrity et al., 2009), including turkeys and 26-day-old chicks (Sorescu et al., 2019). L. plantarum was isolated from fruit flies, vertebrate digestive tract, plants, dairy products, environments, silage (Duar et al., 2017). L. brevis has a free-living lifestyle (Duar et al., 2017) and was isolated from silage, beer, milk, cheese, sauerkraut, sourdough, cow manure, mouth, feces and intestinal tract of human, cattle, rats, pigs and birds (Garrity et al., 2009), including 26-day-old chicks (Sorescu et al., 2020).

The strains described in this paper, isolated from the intestinal content, can be important for developing probiotic compounds for the same bird species because they are host-adapted and have a high ecological fitness. Moreover, this higher fitness is relevant in the process of outcompeting the pathogens.

Differentiation of Lactobacillus strains was performed as described before by Sorescu et al. (2019) (on turkeys) and Sorescu et al. (2020) (on 26-day-old chickens), mainly on the basis of some morphological characters (aspect of bacilli and grouping of them), some cultural characters (colony size, smooth or rough type, colour and degree of transparency/opacity) and especially, biochemical characters (fermentation of glycerol, L-arabinose, D-ribose, D-xyllose, D-galactose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, Methyl-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-trehalose, D-melezitose, starch, gentibiose, D-tagatose, D-arabitol and potassium gluconate). It can be noticed that Lactobacillus strains isolated from chickens aged 45d, generally fermented more carbohydrates (31) than those from 26-day-old chickens (21) and turkeys (15), which may interfere...
with the absorption and metabolism of these carbohydrates in the host gut, if these strains are used in poultry nutrition. L. delbrueckii subsp. delbrueckii was identified only from 26-day-old chickens and L. plantarum only from 45-day-old chickens.

As in turkeys (Sorescu et al., 2019) and 26-day-old chickens (Sorescu et al., 2020; Ciurescu et al., 2020) cases, in the intestinal cecum content of 45-day-old chickens, the numbers of CFU lactobacilli/g were higher (10^8 - 10^9) than in the ileum area (10^5 - 10^8), obviously especially in the case of isolation of the same species from both intestinal segments (L. acidophilus biotype 3, L. plantarum, L. fermentum, L. salivarius). Unlike the results on turkeys (probably due to the age difference – 73d versus 26d, and 45d and the species) and similar to the results on 26-day-old chickens, L. fermentum and L. plantarum strains had relative higher presence (up to 10^9 CFU/g) than other lactobacilli (up to 10^8 CFU/g) in the intestinal content of the 45-day-old chickens, which suggests a possible ecologic and, therefore, probiotic advantage for them. This fact is interesting, because the L. fermentum and L. plantarum are considered to be nomadic species, while the L. acidophilus and L. salivarius strains are adapted to vertebrate species.

As identification systems, both software (apiwebTM and ABIS) proved to be appropriate, especially for L. plantarum, L. salivarius and L. brevis biotype 2, where the same taxonomic classification was obtained, but with different percentage results, the way of calculating them being totally different. Instead, for L. acidophilus biotype 1, L. acidophilus biotype 3, which

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### Table 3 – The results of parallel identification of strains by apiweb™ soft, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

| Strains             | API, %ID          | ABIS, %SIM |
|---------------------|-------------------|------------|
| L. acidophilus biotype 1 IBNA 64 | IBNA 64, L. acidophilus 1, 74.4 | L. kalivensis, 94 |
|                     |                   | L. crispatus, 19.0 | L. manihotivorans, 92 |
|                     |                   | L. acidophilus, 88 |
| L. acidophilus biotype 3 IBNA 49 | IBNA 49, L. acidophilus 3, 99.5 | L. kefitranofaciens ssp. kefitranofaciens, 88 |
|                     |                   | L. acidophilus, 85 |
|                     |                   | L. curvatus ssp. melibiosus, 90 |
|                     | IBNA 51, L. acidophilus 3, 97.9 | L. acidophilus, 82 |
|                     |                   | L. acidophilus, 88 |
|                     | IBNA 51, L. acidophilus 3, 97.9 | L. acidophilus, 84 |
|                     | IBNA 53, L. acidophilus 3, 99.8 | L. acidophilus, 82 |
|                     | IBNA 55, L. acidophilus 3, 97.7 | L. gigerorum, 85 |
|                     | IBNA 55, L. acidophilus 3, 97.7 | L. acidophilus, 84 |
|                     | IBNA 63, L. acidophilus 3, 99.8 | L. delbrueckii ssp. delbrueckii, 88 |
|                     | IBNA 65, L. acidophilus 3, 95.6 | L. acidophilus, 77 |
| L. plantarum biotype 1: IBNA 45 | IBNA 45, L. plantarum 1, 35.7 | L. plantarum, 89 |
|                     | IBNA 46, L. plantarum 1, 70.4 | L. plantarum, 88 |
|                     | IBNA 48, L. plantarum 1, 99.7 | L. plantarum, 86 |
|                     | IBNA 61, L. plantarum 1, 47.8 | L. plantarum, 91 |
| L. brevis biotype 2 IBNA 50 | IBNA 50, L. brevis 2, 98.5 | L. fermentum (possibility of L. reuten), 95 |
|                     |                   | L. brevis (possibility of L. buchneni), 94 |
| L. fermentum biotype 1: IBNA 56 | IBNA 56, L. fermentum 1, 73.2 | L. kefitranofaciens ssp. kefitranofaciens, 97 |
|                     |                   | L. fermentum (possibility of L. reuten), 91 |
|                     |                   | L. kefitranofaciens ssp. kefitranofaciens, 91 |
|                     |                   | L. fermentum (possibility of L. reuten), 88 |
|                     | IBNA 57, L. fermentum 1, 97.5 | L. kefitranofaciens ssp. kefitranofaciens, 96 |
|                     | IBNA 69, L. fermentum 1, 92.4 | L. salivarius: IBNA 47, L. salivarius, 23.3 | L. salivarius, 91 |
|                     |                   | L. salivarius, 23.3 | L. salivarius, 94 |
|                     | IBNA 52, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 54, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 59, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 60, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 62, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 67, L. salivarius, 23.3 | L. salivarius, 93 |
|                     | IBNA 68, L. salivarius, 23.3 | L. salivarius, 93 |

For apiweb identification is presented the %ID (percentage of identification), and %SIM for ABIS (percentage of similarity with respectively specie).
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are biochemically close to *L. fermentum*, and for *L. fermentum* biotype 1, ABIS software is not yet refined enough for an exact identification.

The resistance at 4 ºC and room temperature are relevant technologically characters of the strains. A longer resistance is a positive trait of the strains during their selection. *L. plantarum*, *L. fermentum* biotype 1, *L. brevis* biotype 2 and *L. acidophilus* biotype 1 isolates resisted for the longest period of time, 66 days to 9 months at 4 ºC and 45 days to 3 months at room temperature. These results are useful in screening the phenotypic characters of the candidate strains in order to prepare a probiotic product, involving resistance at least two months at 4 ºC. Considering the presence of just 10⁸ CFU/g for *L. brevis*, the *L. fermentum* biotype 1, *L. plantarum* and *L. acidophilus* biotype 1 strains only were selected for further testing of the probiotic characteristics. Same reasons led to the selection of strains in previous studies: *L. fermentum* - from turkey's gut for same reasons and other strains of *L. fermentum* and *L. brevis* - from 26-day-old chicks (Sorescu et al., 2020). Also, viability, the cell wall condition and the growth stage of the probiotic have an important influence on its performance (Papadimitriou et al., 2015). Therefore, the commercially successful probiotics were based on their technological robustness, they retaining viability during product shelf-life (O’Toole et al., 2017).

**CONCLUSION**

In this study 23 strains of the *Lactobacillus* genus (*L. acidophilus* biotype 1-one strain, *L. acidophilus* biotype 3 - six strains, *L. plantarum* - four strains, *L. brevis* biotype 2 - one strain, *L. fermentum* biotype 1 - three strains and *L. salivarius* - eight strains) have been isolated from the gut content (ileum and cecum) of 17 broiler chickens.

The total bacterial cell was counted as 10⁵-10⁶ in cecum and 10⁵-10⁷ in ileum. *L. plantarum*, *L. fermentum* biotype 1, *L. brevis* biotype 2 and *L. acidophilus* biotype 1 isolates resisted for the longest period of time. From all isolated strains, the *L. fermentum* biotype 1, *L. plantarum* and *L. acidophilus* biotype 1 are technically and ecologically suitable as potential

| Table 4 – Testing the viability of *Lactobacillus* spp. strains preserved at 4ºC and room temperature. |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Strains                                          | Viability at 4 ºC | Viability at room temperature |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| *L. acidophilus* biotype 1                        | IBNA 64           | 66 days                     | 45 days                                         |
| *L. acidophilus* biotype 3:                       | IBNA 49           | 8 days                      | 8 days                                          |
| IBNA 51                                           | 45 days           | 45 days                     |                                                  |
| IBNA 53                                           | 45 days, a single passage | < 45 days                  |                                                  |
| IBNA 55                                           | 45 days           | 50 days, a single passage    |                                                  |
| IBNA 63                                           | 68 days           | < 45 days                   |                                                  |
| IBNA 65                                           | 45 days           | < 45 days                   |                                                  |
| *L. plantarum* biotype 1:                         | IBNA 45           | 7 months                    | < 3 months                                      |
| IBNA 46                                           | 7 months          | 3 months                    |                                                  |
| IBNA 48                                           | 9 months          | 3 months, a single passage   |                                                  |
| IBNA 61                                           | 3.5 months        | < 3 months                  |                                                  |
| *L. brevis* biotype 2 IBNA 50                     | IBNA 56           | 3 months                    | 45 days                                         |
| *L. fermentum* biotype 1:                         | IBNA 57           | 4 months                    | 45 days                                         |
| IBNA 69                                           | 3 months          | < 45 days                   |                                                  |
| *L. salivarius:* IBNA 47                         | IBNA 47           | 45 days                     | < 45 days                                      |
| IBNA 52                                           | 45 days           | < 45 days                   |                                                  |
| IBNA 54                                           | 45 days           | 45 days, a single passage    |                                                  |
| IBNA 59                                           | < 45 days         | < 45 days                   |                                                  |
| IBNA 60                                           | < 45 days         | < 45 days                   |                                                  |
| IBNA 62                                           | 45 days, a single passage | < 45 days                  |                                                  |
| IBNA 67                                           | < 45 days         | < 45 days                   |                                                  |
| IBNA 68                                           | < 45 days         | < 45 days                   |                                                  |
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probiotics and worth continuing the testing of their probiotic properties.

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