biology and life sciences forum

MDPI

Proceeding Paper

Exploration and Insights of Potential Probiotics of Donkey Milk from a Rural Indian Village †

Ankur Kumari [1], Parvati Sharma [1,*] and Anuradha Bhardwaj [2]

[1] Department of Zoology, Chaudhary Bansi Lal University, Bhiwani 127021, India
[2] Biochemistry-Biotechnology Laboratory, National Research Centre on Equine, Hisar 125001, India
* Correspondence: parvati.hsr@gmail.com
† Presented at the 2nd International Electronic Conference on Diversity (IECD 2022)—New Insights into the Biodiversity of Plants, Animals and Microbes, 15–31 March 2022; Available online: https://sciforum.net/event/IECD2022.

Abstract: Non-bovine milk is gaining more international acceptance for research and commercialization due to its usefulness as bovine milk is now reported as hypersensitive to infant's serum due to the high concentration of the casein protein, which may act as potent allergen. The nutritious components of donkey milk are comparable to human milk, i.e., high lysozyme concentration, etc. Many potential probiotics species are identified. In addition to the high lysozymal content, the well-adapted potential probiotics of donkey milk are identified and also categorized on the basis of their relative abundance, and include *Lactobacillus paracasei*, *Lactococcus lactis*, and *Carnobacterium maltaromaticum*. Recently, it was reported that the *Lactobacillus paracasei* and *Lactococcus lactis* speciesare more abundant while the *Leuconostoc*, *Enterococcus*, and *Streptococcus* are the least. These probiotic strains exhibit greater antimicrobial, antioxidant, and anti-proliferative properties in vitro conditions. The present study highlighted the basic composition of donkey milk as well as isolating bacteria and their potential probiotic characteristics against stress conditions like low pH, high bile, etc., and it was found that *Lactobacillus paracasei* and *Enterococcus faecalis* species were predominant in raw donkey milk samples collected from farm sites, while in local field samples, the *Lactococcus lactis* and *Lysinibacillus sphaericus* species were dominant. Thus, in the future, donkey milk probiotics can be an interesting research area and can also provide a novel source for fermented food products with highly efficient probiotics.

Keywords: probiotics; donkey milk; nutritious; antioxidant; anti-allergic

Citation: Kumari, A.; Sharma, P.; Bhardwaj, A. Exploration and Insights of Potential Probiotics of Donkey Milk from a Rural Indian Village. *Biol. Life Sci. Forum* **2022**, *15*, 35. https://doi.org/10.3390/IECD2022-12437

Academic Editor: Ipek Kurtboke

Published: 17 March 2022

1. Introduction

In recent years, donkey milk has captured international attention and experienced an increased market demand due to its human health benefits. Donkey milk is very similar to human milk and exhibits anti-allergic, anti-oxidative, antimicrobial, anti-proliferative, and anti-diabetic activities. Donkey milk production differs greatly from that of conventional dairy species, especially in terms of the milk supply. Recent studies on donkey lactation curves showed that the individual milk yield ranged between 1.54 and 1.73 kg/day on specialized farms [1,2]. The ratio of saturated and unsaturated fatty acids, cholesterol, the triglyceride profile, and the vitamin C content in this milk are also similar to human milk. Donkey milk seems to be a promising alternative food, especially targeted at children's health [2]. Moreover, its high lactose content suggests that the use of donkey milk as probiotic is an ideal substrate for a correct development of intestinal *lactobacilli*, and this makes donkey milk an ideal matrix for the preparation of probiotic drinks with *Lactobacillus rhamnosus* strains [3]. Isolated probiotics from donkey milk (*Lactobacillus paracasei*, *Lactococcus lactis*, and *Carnobacterium maltaromaticum*) modulates gastrointestinal flora and stimulates the immune system [4,5]. Fermented donkey milk enriched with probiotics is one of the most significant advancements in the nutrition sector that can deliver probiotics efficiently

in the host system [6]. Thus, donkey milk (DM) is the one of the most high-demand and healthy non-ruminant dairy products.

In this paper, donkey milk samples from local areas of south Haryana were selected to explore its biochemical properties along with the existence of probiotics in the milk.

2. Material and Methods

2.1. Collection of Samples

A total of 10 milk samples were collected from healthy Rajsthani donkey breeds aged between 4 and 8 years at the mid- and late-lactation stage at donkey farms and from local tribal communities in the villages of Luharijattu (District Bhiwani), Budaak (District Hisar), and Badopal (District Fatehabad), which exist in the Southern Haryana region. In rural Haryana, these donkey breeds are reared along with sheep and goat herds, and they graze in open fields and barren green lawns. Milk samples were collected in sterile sample vials, and, during the sample collection, udder/teats were first wiped with 70% ethanol or a spirit dipped cotton swab whilst wearing sanitized gloves, and a few drops of milk were discarded and then the milk was collected in a sterile vial. The collected samples were stored at very low temperature in an ice basket, and then transported to the laboratory and stored in a freezer at −20 °C for further use.

2.2. Identification of Biochemical Properties of DM Samples

The biochemical properties of the milk samples, i.e., solid non-fat(SNF), fat content, density, Corrected lactometer reading (CLR), proteins, temperature, water content, etc., were all studied by the milk analyzer, Ultra Scan Swift Twinsonic Milk Analyzer, Hindustan Thermostatics, Ambala, India.

2.3. Selective Culture of DM Probiotics

To reduce lysozymal activity and increase the bacterial population in DM, samples were cultured by method Masouras et al. [7].The selective culture of donkey milk probiotics was conducted on MRS agar(Man Rogosa Sharpe, Himedia, Mumbai) andnutrient agar (Himedia, Mumbai), and the total number of bacterial viable counts at the optimum value of the milk was standardized by pouring 200 µL, 300 µL, and 400 µL neat milk. 300 µL milk was standardized for sufficient bacterial culture, and, then, milk samples were prepared by serial dilution (85:15 v/v) in saline solution and peptone, as per the protocol [8,9].Well-defined glossy colonies were obtained and then inoculated in broth for different morphological, biochemical, and confirmatory tests of various isolates. The obtained isolates were also preserved in 30% glycerol stock solution and kept at −80 °C for further analysis.

2.4. Identification of Bacterial Isolates

Microbial isolates were identified by different morphological studies such as gram staining properties, shape and size, motility test, spore staining, etc., and physiological tests such as the bacterial cell wall differentiation test by Potassium hydroxide KOH test, catalase test, and other biochemical characteristics of all tests were reported based on Bergey's Manual [10].The in vitro analysis of probiotic properties such as tolerance to low pH (2.0), tolerance against bile salt (0.3%), and the carbohydrate fermentation test were performed according to the modified protocol [11]. A total of 1 mL of the MRS broth tubes was adjusted at pH 2.0 and 0.3% bile salt concentration by adding 1 M hydrochloric acid (HCl) and 0.3 g bile salt respectively [12]. These tubes were incubated with 200 µL of 48 h grown bacterial culture for 2–3 h at 37 °C, anaerobically. Viable counts were then noted by plating 80 µL of culture on MRS agar plates. All the experiments were technically performed independently in triplicates. After calculating the viable log, cfu/mL survivability was counted as

$$\% \text{ survivability} = (\text{viable log count at time t/viable log count at t} = 0) \times 100$$

3. Result and Discussion

The results of the biochemical analysis of fresh milk samples were recorded as shown in Figures 1 and 2. There are many environmental and genetic factors like lactation stage, health, maternal age, and type of feed, breed, frequency, and milking completeness, etc., which directly or indirectly influence the gross composition of the milk. Donkey milk contains less protein, fats, and inorganic salts but more lactose concentration compared to human milk [13]. In our study, we reported that milk samples collected from local areas contain less SNF and lactose concentration.

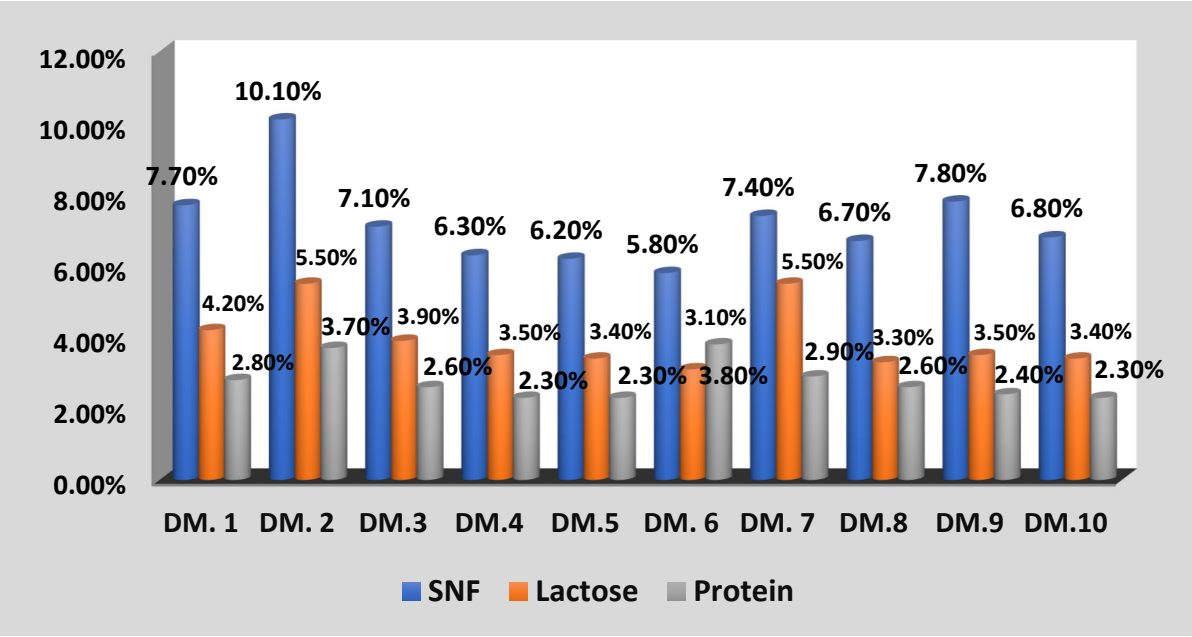

Figure 1. Biochemical profiles of donkey milk samples collected from farms and local areas.

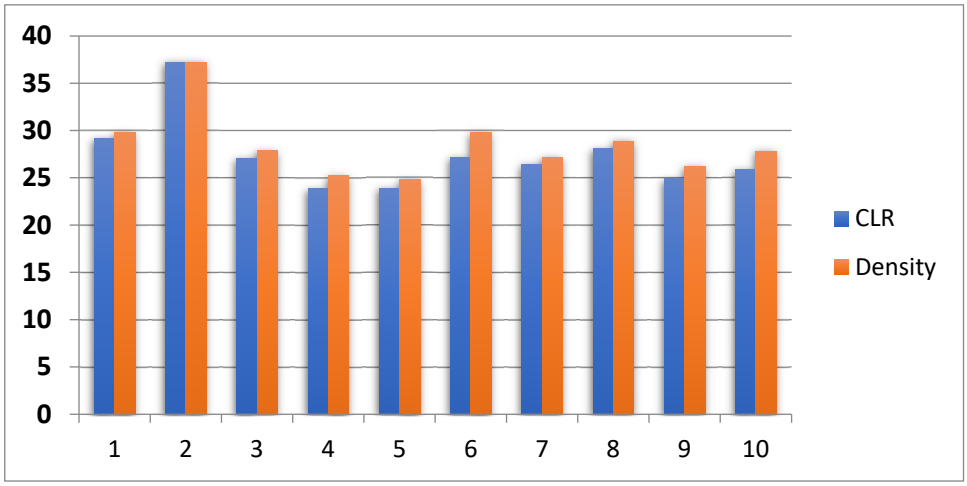

Figure 2. Donkey milk samples' density and CLR values.

The average value of protein, lactose, fat, solid non-fat (SNF) dry matter, and density ranges from 2.30–3.80%, 3.10–5.50%, 0.343–0.438%, 5.80%–10.10%, 8.37–9.50%, and 24.8–29.8%, respectively. The average fat content of donkey milk ranges from 0.5–1.7% or negligible [14,15]. Likewise, we have also reported significant fat concentration in our samples. However, DM 2 which was collected from a farm area containing high (10.10%) SNF & lactose content (5.50%),while DM 5 and DM 6 were collected from a rural area and showed the least lactose content (3.40%) and lactose amount (3.10%). Sample density and

CLR values in both types of the samples did not vary so significantly, and DM 2 possessed high density and high CLR values [16].

The microbial population in fresh raw milk is comparatively less than other non-bovine milk due to presence of more natural antimicrobials such as lysozyme or lactoferrin [2]. However, in this study, we have identified a total of 25 colonies after combining biochemical tests of probiotic potential. The total viable bacterial count ranged between 1.8 to 2.8 log cfu/mL. Donkey milk exhibited differential microbial composition, and we reported less than 4 log cfu/mL whilst [17] reported a high bacteria count 5 log cfu/mL. Among all bacterial isolates, a total of eight species, as mentioned in Table 1, DM.1(a), DM.2(a), DM.3(a), DM.4(a), DM.5(a), DM.5(c), DM.6(b), DM.8(a), are *Lactococcus lactis*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Lysinibacillus sphaericus*, *Enterococcus faecalis*, *Lysinibacillus fusiformis*, *Brevibacillus choshinensis*, and *Enterococcus durans*, respectively.

Table 1. Morphological and biochemical characteristics of bacterial Isolates of donkey milk on MRS agar.

Bacterial Isolates	Characteristics on MRS Agar Plates	Microscopic Characteristics (Gram Staining)	Log cfu/mL	KOH Test	Catalase Test	Growth at (6%NaCl)
DM.1(a)	Small, smooth,	Gram (+)ve, single	2	(−)ve	(−)ve	(−)ve
DM.2(a)	Small, flat, creamy colour	Gram (+)ve, cocci, short chains	2.3	(−)ve	(−)ve	(+)ve
DM.3(a)	Small, white	Gram (+)ve, cocci	2.5	(−)ve	(−)ve	(+)ve
DM.4(a)	Medium, rounded, creamy	Bacilli, gram (+)ve, non-spore	1.8	(−)ve	(−)ve	(−)ve
DM.5(a)	Very small, glossy	Cocci, gram (+)ve,	2.9	(−)ve	(−)ve	(+)ve
DM.5(c)	Circular, medium, off− white	Gram (+)ve, cocci, straight, chain	2.6	(−)ve	(−)ve	(+)ve
DM.6(b)	Large, pale yellow	Gram (+)ve, bacilli	1.9	(−)ve	(−)ve	(−)ve
DM.8(a)	Medium, glossy white	Gram (+)ve, bacilli, tapering ends	2.8	(−)ve	(−)ve	(+)ve

We have also reported more prevalence of coccus shaped bacteria than bacilli [18], demonstrating that coccus-shaped lactic acid bacteria are more lysozyme resistant than lactobacilli. For the first time, *Lactococcus lactis* were isolated, and were also reported as the second most prevalent bacterial species in the DM. *Lysinibacillus sphaericus*, *Brevibacillus choshinensis*, and *Lysinibacillus fusiformis* species are Gram-positive, rod-shaped endospore forming bacteria, exhibiting high chemical resistant potential. These species were also isolated from raw cow's milk. The presence and its potential in commercial probiotic formulations have also been reported [19]. *Lactobacillus paracasei* species are bacillus-shaped, mesophilic, and lysozyme resistant bacteria.

Among all of the species, only *Enterococcus faecalis* can reduce all the sugar bases used in the experiment, while *Leuconostoc mesenteroides* can reduce all of the sugars except cellobiose, as shown in Table 2.

All species showed good survivability at low pH and bile salt concentrations. Among all bacterial species, *Enterococcus faecalis* exhibited a very high survivability, *Lactobacillus paracasei* showed the least tolerance against low pH, and *Brevibacillus choshinensis* showed a low survivability rate at bile salt concentration, as shown in Figure 3. The physiological conditions of the human gastrointestinal tract (GI tract) vary with age and gender, and, normally, stomach pH is 1.5–2.5 while the bile concentration lies in the range of 0.3–0.5% [19]. GI tract probiotics must resist pH changes and bile salt concentration, and these species showed good resistance against these changes.

Table 2. Results of carbohydrate fermentation tests of bacterial isolates of DM grown in MRS broth.

Bacterial Isolates	Te	Ce	Ga	Mb	Su	Xy	Ma	Mo	Rf
DM.1(a)	P	N	P	N	P	N	N	N	P
DM.2(a)	N	P	N	P	P	P	P	P	P
DM.3(a)	P	N	P	P	P	P	P	N	N
DM.4(a)	N	N	N	P	N	N	N	N	P
DM.5(a)	P	P	N	N	P	P	P	P	N
DM.5(c)	P	P	P	P	P	P	P	P	P
DM.6(b)	P	P	N	P	N	P	P	P	P
DM.8(a)	P	N	P	N	P	N	N	N	P

Keys: P = Positive, N = Negative: Te = Trehalose, Ce = Cellobiose, Ga = Galactose, Mb = Melibiose, Su = Sucrose, Xy = Xylose, Ma = Maltose, Mo = Mannose, Rf = Raffinose.

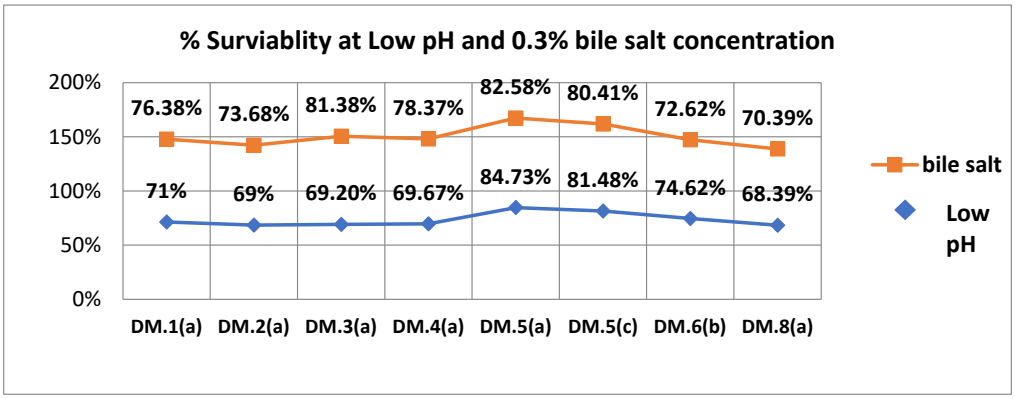

Figure 3. % Survivability rate of bacterial isolates of donkey milk at 0.3% bile salt concentration and at low pH.

4. Conclusions

The comparison of biochemical characteristics, i.e., lactose, SNF, proteins, and the fat content of donkey milk from local areas and farms of southern Haryanan, found no significant difference in fat content, but lactose content was less in the field samples than in the farm milk samples. However, lactose content was enough to support the lactic acid bacteria growth as reported by their selective growth on MRS agar. *Lactobacillus paracasei* and *Enterococcus faecalis* species were predominantly present in the donkey milk samples collected from farm sites, while in local field samples, *Lactococcus lactis* and *Lysinibacillus sphaericus* species were dominant. The probiotic potential of all the bacterial isolates was reported by observing their good survivability rate against low pH and bile salt concentration.

Author Contributions: A.K., P.S. and A.B. have equal contribution for experimental work as well as to write the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting this paper was generated by Department of Zoology, Chaudhary Bansi Lal University and ICAR-NRCE, Hisar, and are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bordonaro, S.; Dimauro, C.; Criscione, A.; Marletta, D.; Macciotta, N.P. The mathematical modelling of the lactation curve for dairy traits of the donkey (Equusasinus). *J. Dairy Sci.* **2013**, *96*, 4005–4014. [CrossRef] [PubMed]
2. Coppola, R.; Salimei, E.; Succi, M.; Sorrentino, E.; Nanni, M.; Ranieri, P.; Belliblanes, R.; Grazia, L. Behaviour of Lactobacillus rhamnosus strains in ass's milk. *Ann. Microbiol.* **2002**, *52*, 55–60.
3. Francavilla, R.; Lionetti, E.; Castellaneta, S.; Ciruzzi, F.; Indrio, F.; Masciale, A.; Fontana, C.; La Rosa, M.M.; Cavallo, L.; Francavilla, A. Randomised clinical trial: *Lactobacillus reuteri* DSM 17938 vs. placebo in children with acute diarrhoea—A double-blind study. *Aliment. Pharm.* **2012**, *36*, 363–369. [CrossRef] [PubMed]
4. Zhu, Y.; Michelle, L.T.; Jobin, C.; Young, H.A. Gut microbiota and probiotics in colon tumorigenesis. *Cancer Lett.* **2011**, *309*, 119–127. [CrossRef] [PubMed]
5. Ojetti, V.; Gigante, G.; Gabrielli, M.; Ainora, M.E.; Mannocci, A.; Lauritano, E.C.; Gasbarrini, G.; Gasbarrini, A. The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients: Randomized trial. *Eur. Rev. Med. Pharmacol. Sci.* **2010**, *14*, 163–170. [PubMed]
6. Massouras, T.; Bitsi, N.; Paramithiotis, S.; Manolopoulou, E.; Drosinos, E.H.; Triantaphyllopoulos, K.A. Microbial Profile Antibacterial Properties and Chemical Composition of Raw Donkey Milk. *Animals* **2020**, *10*, 2001. [CrossRef] [PubMed]
7. Zhang, X.; Zhao, L.; Jiang, L.; Dong, M.; Ren, F. The antimicrobial activity of donkey milk and its microflora changes during storage. *Food Control* **2008**, *19*, 1191–1195. [CrossRef]
8. Andrighetto, C.; Dalmasso, A.; Lombardi, A.; Civera, T.; Bottero, M.T. Isolation and characterisation of lactic acid bacteria from donkey milk. *J. Dairy Res.* **2016**, *83*, 383–386.
9. Kleerebezem, M.; Hugenholtz, J. Metabolic pathway engineering in lactic acid bacteria. *Curr. Opin. Biotechnol.* **2003**, *14*, 232–237. [CrossRef] [PubMed]
10. Kaushik, J.K.; Kumar, A.; Duary, R.K.; Mohanty, A.K.; Grover, S.; Batish, V.K. Functional and probiotic attributes of an indigenous isolate of Lactobacillus plantarum. *PLoS ONE* **2009**, *4*, e8099. [CrossRef] [PubMed]
11. Rastogi, S.; Mittal, V.; Singh, A. In vitro evaluation of probiotic potential and safety assessment of *Lactobacillus mucosae* strains isolated from donkey's lactation. *Probiotics Antimicrob. Proteins* **2019**, *12*, 1045–1056. [CrossRef] [PubMed]
12. Saarela, T.; Kokkonen, J.; Koivisto, M. Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatr.* **2005**, *94*, 1176–1181. [CrossRef]
13. Martini, M.; Altomonte, I.; Salari, F. Amiata donkeys: Fat globule characteristics, milk gross composition and fatty acids. *Ital. J. Anim. Sci.* **2014**, *13*, 123–126. [CrossRef]
14. Bhardwaj, A.; Pal, Y.; Legha, R.A.; Sharma, P.; Nayan, V.; Kumar, S.; Tripathi, H.; Tripathi, B.N. Donkey milk composition and its therapeutic applications. *Indian J. Anim. Sci.* **2020**, *90*, 837–841. [CrossRef]
15. Cavallarin, L.; Giribaldi, M.; Rio, M.D.L.D.S.-D.; Valle, E.; Barbarino, G.; Gennero, M.S.; Civera, T. A survey on the milk chemical and microbiological quality in dairy donkey farms located in North Western Italy. *Food Control* **2015**, *50*, 230–235. [CrossRef]
16. Neviani, E.; Carminati, D.; Veaux, M.; Hermier, J.; Giraffa, G. Characterization of *Lactobacillus helveticus* strains resistant to lysozyme. *Le Lait* **1991**, *71*, 65–73. [CrossRef]
17. Celandroni, F.; Vecchione, A.; Cara, A.; Mazzantini, D.; Lupetti, A.; Ghelardi, E. Identification of *Bacillus* species: Implication on the quality of probiotic formulations. *PLoS ONE* **2019**, *14*, e0217021. [CrossRef] [PubMed]
18. Wang, J.; Jonkers, H.M.; Boon, N.; De Belie, N. *Bacillus sphaericus* LMG 22257 is physiologically suitable for self-healing concrete. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 5101–5114. [CrossRef] [PubMed]
19. Dunne, C.; O'Mahony, L.; Murphy, L.; Thornton, G.; Morrissey, D.; O'Halloran, S.; Feeney, M.; Flynn, S.; Fitzgerald, G.; Daly, C.; et al. In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. *Am. J. Clin. Nutr.* **2001**, *73* (Suppl. 2), 386S–392S. [CrossRef] [PubMed]