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Dying in Yoghurt: The Number of Living Bacteria in Probiotic Yoghurt Decreases under Exposure to Room Temperature

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Key Words
Probiotic bacteria · Ulcerative colitis · Probiotic yoghurts · *Lactobacillae*

Abstract
Background/Aims: While probiotic bacteria are successfully used in the treatment of ulcerative colitis, the effect of commercially available probiotic products is still controversial. Here, we study whether the number of living probiotic bacteria in yoghurts is altered by an interruption of the cold chain. ***Methods:*** Three commonly available probiotic yoghurts were kept at 4°C or put at room temperature (RT) for 6 h or 24 h. An aliquot of each yoghurt was applied on Man-Rogosa-Sharpe agar and incubated at 37°C for 48 h. Colony forming units (CFU) were counted by microscopy. ***Results:*** The first yoghurt, containing *Lactobacillus johnsonii*, showed a significant decrease in CFU after 6 h of storage at RT, which was further pronounced after 24 h. The number of CFU of the second yoghurt, containing *Lactobacillus GG*, was also decreased after 6 h and further diminished after 24 h at RT. From the third yoghurt, containing *Lactobacillus acidophilus*, only 53.8% of the CFU remained after 6 h at RT; after 24 h, only about one fourth of the CFU were found. ***Conclusions:*** Our data demonstrate that the number of living probiotic bacteria in yoghurt products decreases dramatically after exposure to RT. This represents an important information for consumers of such products.

Background

The human colon carries about 10^{10} to 10^{12} microorganisms per ml gut content. These are mostly anaerobic bacteria, but a small number of aerobic and facultative anaerobic bacteria, such as *Lactobacillae*, are also detectable. The normal commensal gut flora represents a complex and well-balanced system of microorganisms that plays a crucial role in the digestion of nutrients and activation and modulation of immune responses, as well as for intestinal barrier function and host defense [1]. This is supported by observations in patients treated with antibiotics, in whom a disturbed commensal flora can frequently be found, often resulting in flatulence and diarrhea [2].

Over the last couple of years, the food industry has been heavily advertising a new group of products: probiotic foods. These probiotic products, available as yoghurts or yoghurt drinks, contain certain strains of bacteria. Although the food industry promotes these products as being able to improve general well-being, digestion and even the immune system by modulating the commensal gut flora, these claims have not yet been proven by scientific data. In comparison to conventionally available yoghurts or yoghurt drinks, however, these probiotic products are far more expensive. They are comprised of diverse bacteria strains, mostly *Lactobacillae* subspecies, which are thought to justify the term 'probiotic'.

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Specific probiotic products have been established for the treatment of ulcerative colitis, such as *Escherichia coli* Nissle 1917 or VSL#3. The latter contains eight different strains of *Lactobacillae* and *Bifidobacteriae*. For those products, the efficacy for the maintenance of remission in ulcerative colitis patients has been proven by a number of clinical trials [3–5]. Interestingly, these probiotics exert antimicrobial effects, either via increased production of bactericidal effector proteins, such as human defensins [6, 7], or stimulation of specific immune responses [8]. Furthermore, they increase epithelial barrier function [9] and modulate proinflammatory responses of the immune system [10].

However, the clinically used probiotic preparations are produced under controlled conditions and contain a similar amount and a high concentration of living, lyophilized bacteria, which are packed in an acid-proof capsule. This ensures quality, safety and availability of the probiotic in the human colon. In contrast, probiotic yoghurts have no standardized amounts or concentrations of microorganisms, and they are not lyophilized or kept acid-proof. Therefore, it is unclear whether the amount of microorganisms in these products is even enough to cause beneficial effects in the intestine. More importantly, it is unclear whether these bacteria are able to survive until the point when they are consumed and whether they are still viable after the passage of the stomach acid. These limitations contribute to the controversy surrounding the role of such advertised probiotics with respect to the consumer's health benefit.

In this paper, we wanted to address whether appropriate storage of probiotic products might be essential for the quality and potential effectiveness of these preparations in the human colon. Since it is proposed that probiotic yoghurts and yoghurt drinks have to be stored under cool conditions, we investigated whether the number of probiotic bacteria in yoghurts is altered by an interruption of the cold chain.

Methods

Three commonly available probiotic yoghurts from three different manufacturers were obtained commercially. Each of the yoghurts contained a different strain of *Lactobacillae*, with one of them containing additionally a strain of *Bifidobacteriae*. The yoghurts were either kept at 4°C (control yoghurts) or at room temperature (RT) for 6 or 24 h, respectively, before analysis. RT was considered to be continuously 20°C throughout the experiment.

From each brand, we tested four different yoghurts at each time point. 5 mg of each of the yoghurts were dissolved in 10 ml phosphate buffered saline (PBS) and thoroughly mixed. The yoghurt solutions were further diluted (1:10) and 100 μl of each of the solutions was applied on Man-Rogosa-Sharpe (MRS) agar plates (Merck, Darmstadt, Germany). MRS agar was chosen as it contains sodium actetate, which suppresses the growth of a large number of concurrent bacteria and, therefore, exclusively favors the growth of *Lactobacillae*. Subsequently, agar plates containing the yoghurt solutions were incubated at 37°C for 48 h under aerobic conditions. Though *Lactobacillae* originally represent anaerobic bacteria, they are aerotolerant, i.e. they are also able to live in an oxygen-rich atmosphere. However, *Lactobacillae* do not express the enzyme catalase, which catalyzes the reduction of hydrogen peroxide (H_2O_2) into oxygen and water. Therefore we applied 2 μl of H_2O_2 (Sigma, St. Louis, Mo., USA) onto the agar plates to verify that the bacteria growing on these plates are catalase-negative and, therefore, most likely *Lactobacillae*. To quantify the amount of surviving bacteria after exposure to RT and incubation, colony forming units (CFU) were counted using a conventional light microscope (Zeiss, Jena, Germany).

Results

The first investigated yoghurt (yoghurt 1) contained *Lactobacillus johnsonii* as a probiotic component. The visual aspect revealed a clear difference between the yoghurts kept at 4°C and the ones that had been exposed to RT. Initially, we evaluated the consistence of the product samples. The yoghurts that had been at RT for 6 h, and especially the ones kept at RT for 24 h, showed a clear separation into two phases: the lower phase contained the yoghurt part of the product, while a clear fluid phase was found on top of the white phase. Additionally the yoghurts that had been exposed to RT were of even more liquid and had a soft consistency compared to the cooled products. We then counted the number of CFU on MRS agar plates 48 h after incubation of the yoghurt dilutions. As visible in figure 1a, the agar plates containing the yoghurt samples that had been kept at 4°C were completely covered by CFU of *Lactobacillae*. While the plates containing the yoghurt samples that were at RT for 6 h already showed a looser assembly of CFU, the agar plates with the 24 h samples showed only a few CFU. This indicated that only a small number of bacteria were still viable after being exposed to RT for 24 h.

Statistical analysis revealed that the 6-hour plate still contained about 91% of the CFU that were detectable on the control plates ($p < 0.05$; fig. 1b). However, and in accordance with the visual aspect, microscopic counting of CFU on the plate that had been at RT for 24 h revealed that only 44.8% of the CFU were detectable when compared to the control plates ($p < 0.01$).

The second analyzed yoghurt product (yoghurt 2) contained the probiotic component *Lactobacillus GG*.

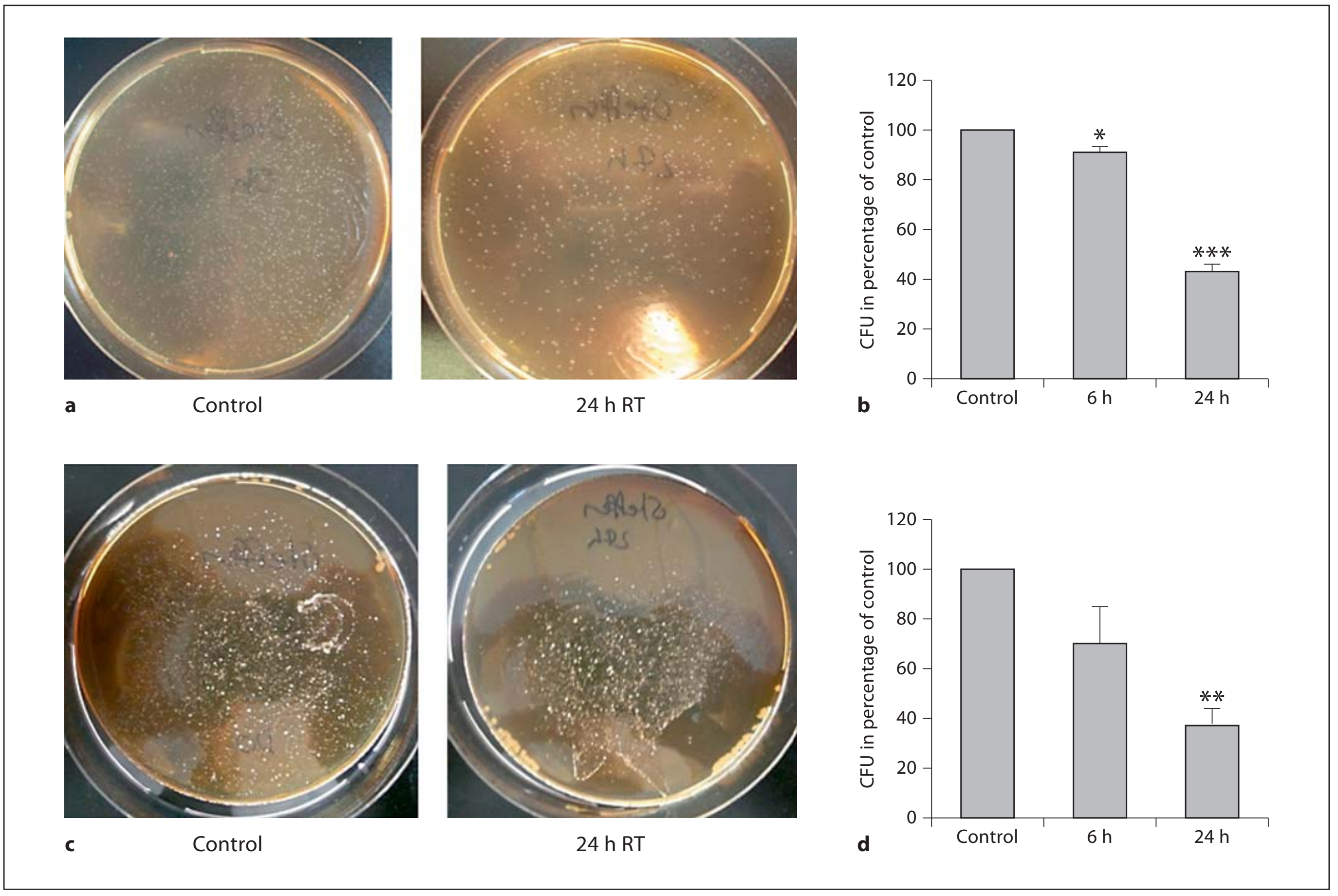

Fig. 1. Survival of probiotic bacteria on MRS agar plates. Representative images of CFU on MRS agar plates (control and 24 h at RT) of yoghurt 1 solutions after incubation for 48 h at 37°C (**a**) and statistical analysis of four similar experiments (**b**). Representative images of CFU on MRS agar plates (control and 24 h at RT) of yoghurt 2 solutions after incubation for 48 h at 37°C (**c**) and statistical analysis of four similar experiments (**d**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control.

Similar to the previous observations, there was a clear separation into a fluid and a solid phase in the yoghurts that had been exposed to RT. After incubation, the control plates were full of *Lactobacillae* CFU. However, even after 6 h, there was a clear difference in the number of visible CFU (fig. 1c). Statistical analysis revealed that after 6 h of RT exposure, the number of CFU decreased by 30% ($p < 0.01$). After 24 h, this decrease was even further pronounced and only 36.8% of the CFU that were detectable on control plates could be counted by microscopy ($p < 0.01$; fig. 1d).

The third yoghurt (yoghurt 3) contained *Lactobacillus acidophilus* and a *Bifidobacteriae* subspecies which was not further described. In these products, a clear separation between a fluid and a solid phase was also detectable. In addition, the yoghurt mass featured small clumps. Here, as visible in representative images in figure 2a, in the yoghurt samples that had been at RT for 6 h, only 53.8% of the CFU remained ($p < 0.001$) compared to the number of CFU on control plates. Interestingly, in the 24 h of RT, only about one fourth of the CFU (as compared to the control samples) could be counted by microscopy (27.1%; $p < 0.001$; fig. 2b).

Conclusions

Our data demonstrate that the number of living probiotic bacteria in yoghurt products decreases dramatically when they are exposed to RT. Additionally, the de-

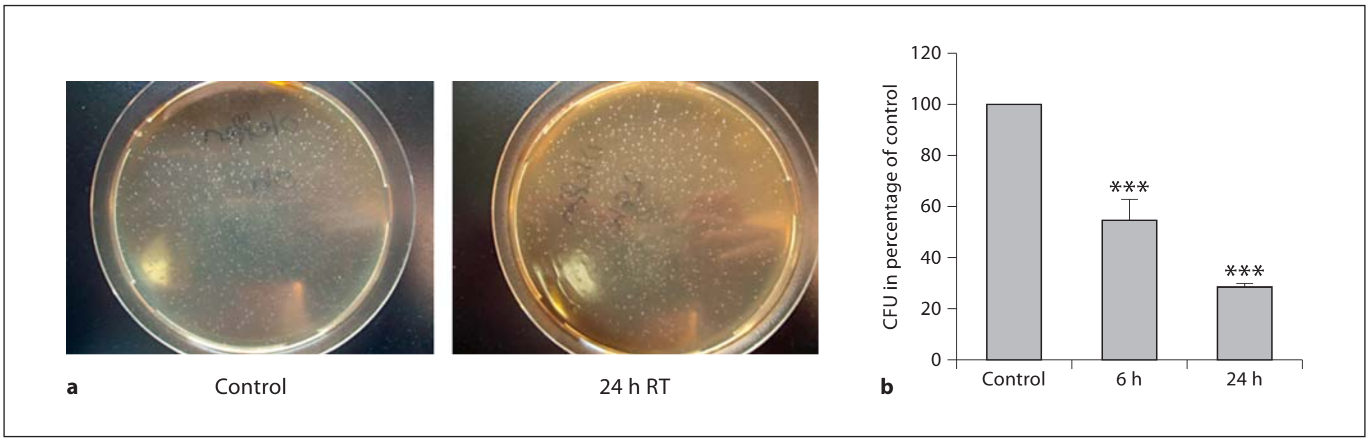

Fig. 2. Survival of probiotic bacteria on MRS agar plates. Representative images of CFU on MRS agar plates (control and 24 h at RT) of yoghurt 3 solutions after incubation for 48 h at 37°C (**a**) and statistical analysis of four similar experiments (**b**). *** $p < 0.001$ vs. control.

crease in living bacteria is dependent on the duration of RT exposure. This leads to the question whether, already by conventional shopping, the number of probiotic bacteria declines to such an extent that there are not enough bacteria remaining in the product to be useful. As we have shown, even after 6 h, a significant amount of bacteria had already died (in yoghurt 3 about 50% of the bacteria).

Given the fact that the cold chain is interrupted for a couple of minutes up to a few hours during any conventional shopping, the question arises whether these probiotic products can show any health benefit for their consumers. According to the current hypothesis, a certain number of bacteria is needed to cause any effect in the gastrointestinal tract. However, in contrast to clinically used probiotic compositions, such as *E. coli* Nissle 1917 or VSL#3, the probiotics in the yoghurt are not protected by an acid-proof capsule. Therefore, it is very likely that an additional significant number of probiotics does not survive the stomach passage and only a slight number of bacteria actually arrive in the colon.

Unfortunately, the producers of those probiotic products do not indicate the concentration or amount of bacteria within their products. However, Schillinger [11] showed that the majority of probiotic yoghurts contain about 10^5 viable *Lactobacillae* per gram of yoghurt, when stored continuously in the refrigerator. Nevertheless, a direct comparison to clinically used probiotics is impossible. Additionally, each bacteria strain induces different effects in vitro as well as in vivo. For most of the commercially advertised bacteria (in contrast to clinically used probiotic strains), no clinical or basic research data on the nature of their effects in the human gastrointestinal tract are available: neither whether they are beneficial or detrimental, nor whether they have any effect at all. For this reason, one must question whether these commercial probiotic products will have even any (health) effects at all in the human gastrointestinal tract.

Taken together, we show that the survival of probiotic bacteria in commercially available products is critically dependent on the conditions of how the products are stored. This might also have a strong implication for a putative beneficial effect of the probiotic compositions and, therefore, represents important information for the consumers of such probiotic yoghurts.

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References

1 Guarner F, Malagelada JR: Gut flora in health and disease. Lancet 2003;361:512–519.

2 Beaugerie L, Flahault A, Barbut F, Atlan P, Lalande V, Cousin P, Cadilhac M, Petit JC, Study Group: Antibiotic-associated diarrhoea and *Clostridium difficile* in the community. Aliment Pharmacol Ther 2003;17: 905–912.

3 Kruis W, Schütz E, Fric P, Fixa B, Judmaier G, Stolte M: Double-blind comparison of an oral *Eschericia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 1997;11: 853–858.

4 Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT: Non-pathogenic *Eschericia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999;354:635–639.

5 Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB: VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol 2005;100:1539–1546.

6 Möndel M, Schroeder BO, Zimmermann K, Huber H, Nuding S, Beisner J, Fellermann K, Stange EF, Wehkamp J: Probiotic *E. coli* treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol 2009;2:166–172.

7 Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, Schröder JM, Stange EF: NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Eschericia coli* Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 2004;72:5750–5758.

8 Mastrangeli G, Corinti S, Butteroni C, Afferni C, Bonura A, Boirivant M, Colombo P, Di Felice G: Effects of live and inactivated VSL#3 probiotic preparations in the modulation of in vitro and in vivo allergen-induced Th2 responses. Int Arch Allergy Immunol 2009;150:133–143.

9 Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, Bleich A, Bruder D, Franzke A, Rogler G, Suerbaum S, Buer J, Gunzer F, Westendorf AM: Probiotic *Eschericia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One 2007;2:e1308.

10 Rasche C, Wolfram C, Wahls M, Worm M: Differential immunomodulating effects of inactivated probiotic bacteria on the allergic immune response. Acta Derm Venereol 2007;87:305–311.

11 Schillinger U: Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. Int J Food Microbiol 1999; 47:79–87.