Dying in yoghurt: the number of living bacteria in probiotic yoghurt decreases under exposure to room temperature

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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.
Dying in Yoghurt – The number of living bacteria in probiotic yoghurt decreases under exposure to room temperature

Short Title: Probiotic bacteria die at room temperature

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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 on 4°C or put at room temperature (RT) for 6 h or 24 h. An aliquot of each yoghurt was applied on MRS 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 by 6 h and further diminished by 24 h at RT. From the third yoghurt, containing *Lactobacillus acidophilus*, only 53.8% CFU remained after 6 h at RT and after 24 h, just about one fourth 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.

Keywords: Probiotic bacteria, ulcerative colitis, probiotic yoghurts, Lactobacillae
Background

The human colon carries about $10^{10}$ to $10^{12}$ microorganisms per ml gut content. Mostly, these are anaerobic bacteria, but also a small number of aerobe and facultative anaerobe bacteria, such as \textit{Lactobacillae}, are detectable. The normal commensal gut flora represents a complex and well balanced system of microorganisms that plays a crucial role for the digestion of nutrients, the 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].

Since a couple of years, the food industry heavily advertises a new group of products: probiotic foods. These probiotic products, available as yoghurts or yoghurt drinks, contain certain strains of bacteria. \textbf{Though the food industry promotes these products in a way that they would improve general well being, digestion and even the immune system by modulating the commensal gut flora, these possibly beneficial effects have not been proven by scientific data so far.} 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 \textit{lactobacillae} subspecies, what is thought to justify the term “probiotic”.

Specific probiotic products have been established for the treatment of ulcerative colitis (UC), such as \textit{E.coli} Nissle 1917 or VSL#3. The latter contains eight different strains of \textit{Lactobacillae} and \textit{Bifidobacteriae}. For those products the efficacy for the maintenance of remission in UC patients has been proven by a number of clinical trials [3-5]. \textbf{Interestingly, these probiotics exert antimicrobial effects, either via increased production of bactericidal effector proteins, such as human defensins [6; 7] or via stimulation of specific immune responses [8]. Further, they increase epithelial barrier function [9] and modulate pro-inflammatory 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 are packed in an acid-proof capsule. This ensures quality, safety and availability of the probiotic in the human colon. In contrast, probiotic yoghurts contain neither standardized amounts or concentrations of microorganisms nor are they 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 or, and even more important, whether these bacteria are able to survive until they are consumed and whether they are still viable after the passage of the stomach acid. These limitations contribute to the fact that the role for such advertised probiotics with respect to consumer’s health benefit is still controversial.

Here, we wanted to address the point 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*, one of them additionally a strain of *Bifidobacteriae*. The yoghurts were either kept on 4°C (control yoghurts) or put at room temperature (RT) for 6 h 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, since it contains sodium acetate that suppresses the growth of a large number of concurrent bacteria and therefore exclusively favours the growth of lactobacillae. Subsequently, agar plates containing the yoghurt solutions were incubated at 37°C for 48 h under aerobe conditions. Though lactobacillae originally represent anaerobe bacteria, they are aerotolerant, what means that they are also able to live in an oxygen-rich atmosphere. However, lactobacillae do not express the enzyme catalase, what catalyzes the reduction of hydrogen peroxide (H₂O₂) into oxygen and water. Therefore we applied 2 µl of H₂O₂ (Sigma, St. Louis, MO) onto the agar plates to verify that the
bacteria growing on these plates are catalase-negative and therefore most likely \emph{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).

\section*{Results}

The first investigated yoghurt (yoghurt 1) contained \emph{Lactobacillus johnsonii} as a probiotic component. Already 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 even more the ones kept at RT for 24 h, showed a clear separation into two phases: While the lower phase contained the yoghurt part of the product, there was also a clear fluid phase on top of the white phase below. Additionally the yoghurts that had been exposed to RT were of even more liquid and soft consistence compared to the cooled products. 48 h after incubation of the yoghurt dilutions, we then counted the number of CFU on MRS-agar plates. As visible in Figure 1A, the agar plates that contained the yoghurt samples that had been kept at 4°C were completely covered by CFU of \emph{lactobacillae}. While the plates containing the yoghurt samples that were at RT for 6 h already showed a looser assembly of CFUs, the agar plates with the 24 h samples showed only a few CFUs, indicating that only a small number of bacteria was still viable after being exposed to RT for 24 h. Statistical analysis revealed that the 6 h plate still contained about 91% of the CFU that were detectable on the control plates (p<0.05; Figure 1B). However, and in accordance to the visual aspect, microscopic counting of CFUs on the plate that had been at RT for 24 h, revealed that only 44.8% of the CFU were detectable when compared to control plates (p<0.01).

The second analyzed yoghurt product (yoghurt 2) contained the probiotic component \emph{Lactobacillus GG}. 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 \emph{lactobacillae} CFUs. However, even after 6 h was a clear difference in the number of CFUs visible (Figure 1C). Statistical analysis revealed that after 6 h RT exposure, the number of CFU was
decreased by 30 % (p<0.01). After 24 h, this decrease was even further pronounced and only 36.8 % of CFUs that were detectable on control plates could be counted by microscopy (p<0.01; Figure 1D).

The third yoghurt (yoghurt 3) contained *Lactobacillus acidophilus* and a *bifidobacteria* subspecies which was not further described. In these products there was also a clear separation between a fluid and a solid phase 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 % CFU remained (p<0.001) compared to the number of CFU on control plates. Interestingly, in the 24 h RT only just about one fourth CFU as compared to the control samples could be counted by microscopy (27.1 %; p<0.001; Figure 2B).

**Conclusions**

Our data demonstrate that the number of living probiotic bacteria in yoghurt products decreases dramatically when they are exposed to room temperature. Additionally, the decrease in living bacteria is dependent on the duration of the 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 too less bacteria remaining in the product to be useful. As we have shown, even after 6 h, a significant amount of bacteria already had died (in yoghurt three about 50 % of bacteria). Given the fact that the cold chain is interrupted for a couple of minutes up to a few hours at any conventional shopping, the question arises, whether these probiotic products can show any health benefit for their consumers. According to current hypothesis, a certain number of bacteria is needed to cause any effects in the GI 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 high likely that an additional significant number of probiotics does not survive the stomach passage and only a slight number of bacteria can arrive finally in the colon. Unfortunately, the producers of those probiotic products do not indicate the concentration or amount of bacteria within their products. However, Schillinger showed that the majority of probiotic yoghurts contain
about $10^5$ viable *Lactobacillae* per gram yoghurt, when stored continuously in the refrigerator [11]. Nevertheless, a direct comparison to clinically used probiotics is impossible. Additionally, each bacteria strain induces different effects *in vitro* as well as *in vivo*. In contrast to clinically used probiotic strains, for most of the commercially advertised bacteria no clinical or basic research data are available of the nature of their effects in the human GI tract, neither whether they are beneficial or detrimental nor whether they have any effect at all. This clarifies that one must question whether these commercial probiotic products will have even any (healthy) effects at all in the human GI tract.

Taken together, we show that the survival of probiotic bacteria in commercially available products is critically dependent on the conditions 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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**Abbreviations**

CFU, colony forming units; MRS agar, Man-Rogosa-Sharpe Agar; PBS, phosphate-buffered saline; RT, room temperature; UC, ulcerative colitis.
Disclosures

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Reference List


**Figure legends**

**Figure 1.** Survival of probiotic bacteria on MRS-agar plates. (A) Representative images of CFU on MRS-agar plates (control and 24 h RT) of yoghurt 1 solutions after incubation for 48 h at 37°C and (B) statistical analysis of four similar experiments. (C) Representative images of CFU on MRS-agar plates (control and 24 h RT) of yoghurt 2 solutions after incubation for 48 h at 37°C and (D) statistical analysis of four similar experiments. * indicates p < 0.05, ** = p < 0.01, *** = p < 0.001 vs. control.
Figure 2. Survival of probiotic bacteria on MRS-agar plates. (A) Representative images of CFU on MRS-agar plates (control and 24 h RT) of yoghurt 3 solutions after incubation for 48 h at 37°C and (B) statistical analysis of four similar experiments. *** indicates p < 0.001 vs. control.